REVIEW ARTICLE

Advanced 3D-Printing Bioinks for Articular Cartilage Repair

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Abstract: Chondral lesions caused by stressors, such as injury or inflammation, lead to osteoarthritis (OA). OA is a degenerative joint disease that has become a challenge worldwide. As the articular cartilage is incapable of self-regeneration due to the absence of vessels and nerves, novel cartilage repair techniques are urgently needed. Three-dimensional (3D) bioprinting, which allows the precise control of internal architecture and geometry of printed scaffolds, has stepped up to be a promising strategy in cartilage restoration. With regards to 3D bioprinting, bioinks with proper chemical and mechanical properties play one of the most critical roles in designing successful cartilage tissue constructs. In particular, hydrogels as 3D hydrophilic cross-linked polymer networks are highly recommended as bioinks because of their fine biocompatibility, easy fabrication, and tunable mechanical strength. Herein, we highlight the widely used polymers for hydrogel preparation and further provide a non-exhaustive overview of various functional modified additives (such as cells, drugs, bioactive factors and ceramic) to exploit the unique properties suitable for bioprinted cartilage. Finally, a prospective on future development for 3D-bioprinting in cartilage repair is elucidated in this review.

Keywords: Osteoarthritis; Hydrogels; Silk fibroin; Collagen; Polyethylene glycol

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1. Introduction

Articular cartilage is an avascular connective tissue that works to lubricate the friction between the joint surfaces[1]. The only cell constituting this hyaline tissue is the chondrocyte. It is usually embedded in the extracellular matrix (ECM) mainly consisting of type II collagen (COL II) network and aggrecan proteoglycan (**Table 4**)[2]. The damage of cartilage may lead to osteoarthritis (OA) (**Table 4**), which is the most common degenerative joint disease that affects over 303 million people worldwide[3]. OA is characterized by severe joint pain, swelling, and sound or sensation of grating in the synovial joints caused by the progressive loss of articular cartilage[4]. Briefly, stressors such as cartilage injury or inflammation may cause the hypersecretion of pro-inflammatory cytokines. It promotes the expression of metalloproteinases (MMPs) and a disintegrin and an MMPs with thrombospondin motifs (ADAMTS) (**Table 4**). MMP and ADAMTS enzymes then contribute to the decomposition of aggrecan proteoglycan, which is regarded as a significant early event contributing to the deconstruction of cartilage tissue[2]. In addition, collagenases of the MMP family, such as MMP-13, lead to the degradation of ECM COL. In turn, the defect

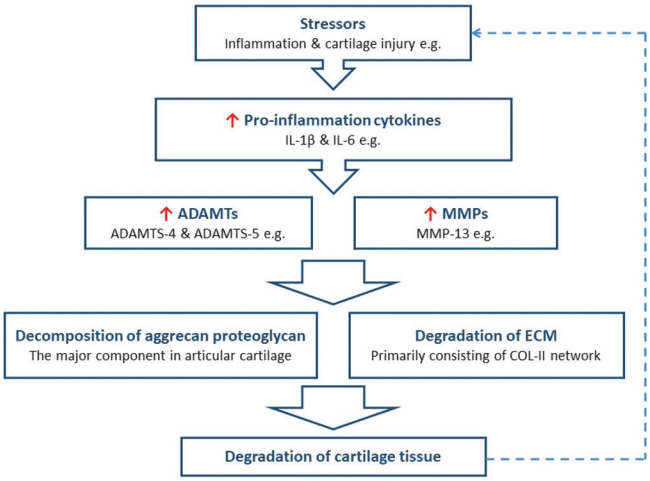

Figure 1. The general pathological process underlying OA. Cartilage damage caused by injury or inflammation promotes the hypersecretion of pro-inflammatory cytokines such as IL-1β and IL-6, which then enhance the expression of MMPs including MMP-13 and ADAMTS such as ADAMTS-4 and ADAMTS-5[6]. As a result, aggrecan proteoglycan and ECM COL are degraded, leading to further deconstruction of damaged cartilage tissue.

in cartilage aggravates the inflammation of the joint, thereby enhancing proteolytic enzymes hypersecretion and promoting the progression of OA (**Figure 1**)[5].

As fully differentiated joint cartilage is incapable of self-regeneration due to its lack of vessels and nerves, there is an urgent need for techniques of cartilage repair. Three-dimensional (3D) bioprinting has now been regarded as a promising cartilage tissue engineering technique that can replace the battered or lost cartilage with 3D-printed biological materials. An ideal 3D printing process mainly includes small processing time, high printing resolution, and compatibility with cells if the material is cell-laden. With topographically and morphologically correct structures, the printed scaffold should be able to guide cell differentiation and migration, thereby influencing ECM deposition and ultimately displaying properties that are similar to the native tissue[7]. In addition, the 3D printing technique allows the porosity, internal architecture, mechanical, and structural properties of the printouts to be tuned via controlling their manufacturing process. It is also capable of printing materials carrying different concentrations of bioactive factors and cells[8].

Bioprinting inks are one of the key elements for 3D-printing cartilage repair. Hydrogels, composed of 3D cross-linked networks made of water-soluble polymers, are one of the main sources of developing bioinks[9]. Their fine biocompatibility enables hydrogels to serve as temporary ECM-like microenvironment, which is efficient for the survival, proliferation, and differentiation of encapsulated cells[10]. Currently, the hydrogel materials applied as inks in the field of 3D printing involve natural materials including hyaluronic acid (HA), alginate, collagen (COL), silk fibroin (SF), and synthetic polymers such as gelatin methacryloyl (GelMA) and polyethylene glycol (PEG) (**Table 4**) [11]. This review focus on the properties of the above five most commonly used hydrogels. We also discuss the development and applications of such hydrogel-based bioinks modified with functional additives. Finally, challenges and future directions of hydrogels in the field of cartilage regeneration are stated.

2. Overview of bioinks for 3D printed cartilage engineering

The 3D-bioprinting technique applied in cartilage tissue engineering usually contains three important elements, i.e., cells, growth factors, and printed scaffolds, which are composed of various bioinks. Repair mechanisms of bioinks mainly involve two ways: (i) The printouts serve as a temporary ECM environment to promote chondrogenesis and angiogenesis, leading to the generation of new cartilage tissue; (ii) the engineered biomaterials replace the battered or lost cartilage to restore the functions of defected joint. Three key standards for selecting a suitable bioink involving a mechanical strength that is close to the native cartilage, superior biocompatibility that avoids cytotoxicity, and high degradation speed according to the speed of cartilage regeneration for scaffolds working as temporary ECM. Inks made from natural resources usually possess good biocompatibility, but most of them lack mechanical strength, while most bioinks consisting of synthesized polymers are the opposite **(Figure 2**). With the use of the right bioinks, printouts should be able to provide sufficient mechanical and structural support and adequate nutrition supply[7].

To generate functional and high-quality neocartilage, native progenitor cells and stem cells are widely used along with cartilage scaffolds to improve the repair of cartilage defects. For example, mesenchymal stem cells including adipose-derived stem cells and bone marrow-derived mesenchymal stem cells (BMSCs) (**Table 4**), which are multipotent stem cells that are capable of rapid proliferation and are promising for cartilage regeneration[8]. In addition, chondrocytes are also popular cell additives for their application in scaffold-based cartilage repair. Cell density needs to be carefully designed when developing a cell-laden bioink, because various studies have shown that it may significantly influence the properties of both the bioink and the printout[12]. For instance, as the density of primary chondrocytes increases, the gelation rate and storage modulus of COL bioinks by extrusion printing decrease. However, cell densities of up to 100×10^6 cell/mL do not impair the resolution and printability of these bioinks[13]. The viability of the cell is not affected by either the cell density or the printing process. For GelMA bioinks, a cell density of up to 40×10^6 cell/mL has been shown to have no effect on the resolution under a printing condition with

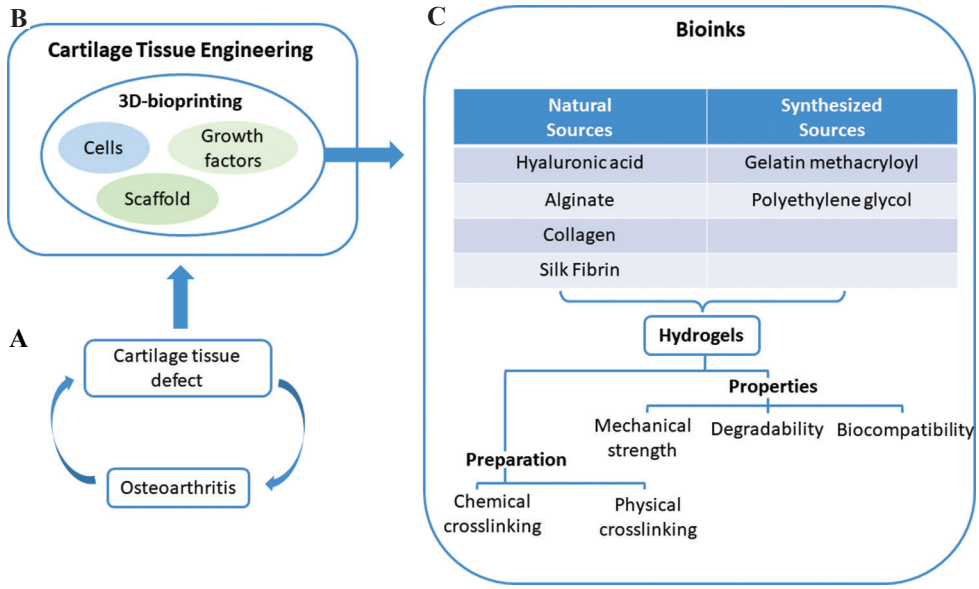

Figure 2. (A-C) A basic summary of 3D-bioprinting repair of cartilage tissue.

speed ratios from 0.07 to 2.24 mm^2 by the Integrated Tissue-Organ Printer system. In addition, both storage modulus and loss modulus increase as the cell density increase, with no change in the shear viscosity observed[12]. Nevertheless, encapsulated human glioblastoma cells have been demonstrated to impair the printing resolution of gelatin bioinks using extrusion 3D bioprinting[14]. Furthermore, higher cell densities may enhance the steady shear viscosity while reducing the threshold of extrusion pressure, which contributes to the bioink compressibility and the friction between cells and the hydrogel during the printing process.

Common bioinks additives include (i) additives that improve biocompatibility and repair efficacy; (ii) additives that enhance hydrogel crosslinking and mechanical properties; and (iii) additives that refine printing resolutions. Growth factors are important additives of bioinks. They are important for inducing cellular response, thereby stimulating cell differentiation and tissue regeneration. In addition, they are essential in enhancing chondrogenesis and inhibiting chondrocyte hypertrophy[15]. Basically, the most widely applied growth factors include transforming growth factor-β (TGF-β) (**Table 4**) which promotes cell proliferation and chondrogenesis; bone morphogenetic proteins that improve the production of ECM; insulin-like growth factors which promote the differentiation of mesenchymal stem cells, fibroblast growth factors that maintain ECM homeostasis, and platelet-derived growth factors which enhance the formation of heterotopic cartilage[16]. In addition to the growth factors, additives are also used for the crosslinking of hydrogels and the enhancement of mechanical properties in bioink development. Methacrylate anhydride is one of the most popular chemicals for generating methacrylate functional groups in the bioink polymers[17]. Compared with the natural sources which are usually crosslinked physically, the methacrylate functionalized polymers are photocrosslinked covalently, thereby improving the mechanical strength of the hydrogel. Moreover, nanomaterials, including graphene, nanoclay, and ceramics nanoparticles, are also applied to reinforce hydrogel-based bioinks[18]. In addition, additives to improve the printing resolution of printouts are also used. For example, the click reaction between thiols and alkene groups added to the bioink polymers can solidify the material immediately during 3D printing, thereby enabling the fabrication of complex yet high-quality constructs[19]. Furthermore, photoabsorbers, such as tartrazine, are also popular additives for the crosslinking and resolution improvement of photocrosslinkable hydrogels[20].

Different bioinks correspond to different 3D printing techniques. One of the most commonly used technology is extrusion-based printing (EBP) (**Table 4**), which requires the bioink to be loaded into plastic or stainless steel cartridges and then extruded through a printing nozzle onto a platform (**Figure 3A**). It supports 3D printing with cell-laden bioink and its printouts are of moderate resolution[7,21]. Moreover, as mechanical extrusion printing allows both bioink deposition and withdrawal, it enables a clean cut of the bioink strand and the correction of printing errors, thereby achieving an improved shape fidelity of the printouts[22]. Nevertheless, the viscosity of the bioinks applied must be high enough to avoid shape collapse[23]. As for cell-encapsulated bioinks, shear-thinning characteristics are required for the hydrogels to prevent cells from damage caused by shear stress when existing the nozzle[21]. Gelation methods, including temperature/pH change and photocrosslinking, can be

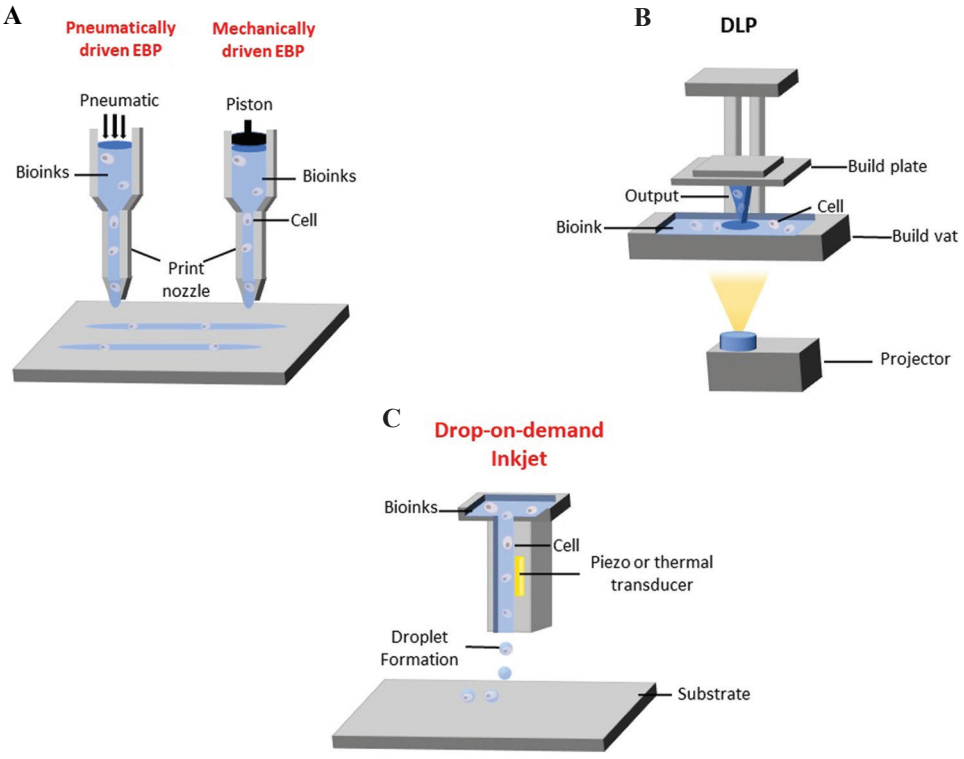

Figure 3. A brief introduction to three popular 3D-bioprinting techniques with cell-laden bioinks. (A) Schematic diagram of extrusion-based 3D printing. Cell-laden bioinks contained in the micro-syringe are extruded onto the substrate on the collection plate through the print nozzle by pneumatic pressure or piston. The dimensions of a structure are translated into X, Y, and Z coordinates during printing by a computer, which controls the nozzle and substrate. (B) Schematic diagram of digital light processing 3D printing. The construct is printed with the increased height from the photocrosslinkable liquid bioink in the build vat as the build plate moves up vertically. (C) Schematic diagram of the 3D-printing process using cell-laden bioinks by drop-on-demand inkjet.

applied depending on the materials during deposition[7]. Another widely applied printing technique is light-based 3D printing using digital light processing (DLP) technology (**Table 4**) (**Figure 3B**). Different from EBP, the printout of DLP is generated from a reservoir filling with liquid bioink and is attached to the platform above. As the platform moving up, the height of the 3D construct is then increased[24]. Compared with other 3D-printing techniques, DLP has superior vertical structure fidelity and high printing resolution[21]. However, it usually requires bioinks to be photocrosslinkable. In addition, the viscosity of bioinks should be maintained in a specific range so that the printout can withstand dissociation from the bottom of the build vat while attaching to the build plate or the layer above[25]. With the exception of these two methods mentioned above, drop-on-demand 3D printing has also been applied in cartilage tissue engineering (**Table 1**).

2.1. HA

HA is a polymeric glycosaminoglycan (GAG) (**Table 4**) consisting of duplicated β−1,4-d-glucuronic acid-β−1,3-N-acetyl-Dglucosamine residues (**Figure 4A**). As one of the major components in ECM, HA promotes chondrogenesis significantly[28]. It was first considered as a potential material for tissue engineering in 1997[29] and was first applied clinically in 1999[30]. HA can interact with cell surface hyaladherins such as Receptor for Hyaluronan Mediated Motility, which is important for cell migration under the conditions of inflammation and tissue repair[31]. Thus, it exhibits superior biocompatibility and the ability to promote chondrogenesis. However, implants composed of fragments or low-molecular-weight HA lack biological interaction with encapsulated cells and surrounding tissue, leading to inflammation or degradation of scaffolds. As a result, increasing chemical-modified HA derivatives have been developed (**Table 2**)[32].

HA and its derivatives are widely applied in 3D printing, especially EBP[38]. In 2015, Kesti *et al*. designed a novel bioink by blending polymer poly(N-isopropylacrylamide) grafted hyaluronan (pNIPAAM) with methacrylated hyaluronan (MeHA) (**Table 4**). The high-resolution scaffold generated showed immediate termination of flow and rapid gelation, but it required the elution of HA-pNIPAAM after printing to prevent high death of embedded cells[39]. Later in 2017, the ultraviolet (UV)-crosslinkable MeHA hydrogel was applied to print a porous and rigid scaffold. The printout showed improved storage moduli and elastic moduli. However, precise control

Table 1. Summary of different 3D-printing technologies in cartilage tissue repair and their bioinks.

3D-printing technique	Advantages	Disadvantages	Bioink requirements	Bioink examples	Reference
Extrusion-based	Support cell-laden bioinks Moderate-resolution Improved shape fidelity	Slow printing speed Inferior cell viability (40-80%) Expensive cost	High viscosity Shear-thinning characteristics for cell-laden bioinks	GelMA HA Collagen	[21,22,25,26]
Digital-light processing	Superior vertical structure fidelity Hight resolution Fast printing speed Mild condition for cells (viability: 85-95%)	Separating force between the platform and the printed surface May need the addition of cytotoxic photoinitiator	Photocrosslinkable Viscosity maintained within a specific range	GelMA PEGDA Silk-fibrin	[21,25,27]
Drop-on-demand inkjet	Medium printing speed High cell viability (>85%)	Poor structure fidelity Low cell density (<106 cells mL − 1)	Thermoplastic Viscosity maintained within a specific range	Alginate PEGDA Collagen	[21,25]

Table 2. HA derivatives, their fabrication and gelation methods.

HA derivatives	Fabrication	Gelation method	Reference
Thiol-modified HA	Modifying the carboxylate groups of GAGs and polypeptides with hydrazide reagents	Difunctional electrophiles	[33,34]
Haloacetate-modified HA	Using excessive bromoacetic anhydride to synthesize HA bromoacetic with a substitution of 18%	Crosslinker-free when combined with thiol-modified HA	[35]
Dihydrazide-modified HA	Addition of adipic dihydrazide and other hydrazides	Ketones and aldehydes; can also acylhydrazide with acylating agents	[36]
Tyramine-modified HA	Coupling tyramine to a small percentage of HA carboxylates	Addition of horseradish peroxidase and hydrogenperoxide	[37]

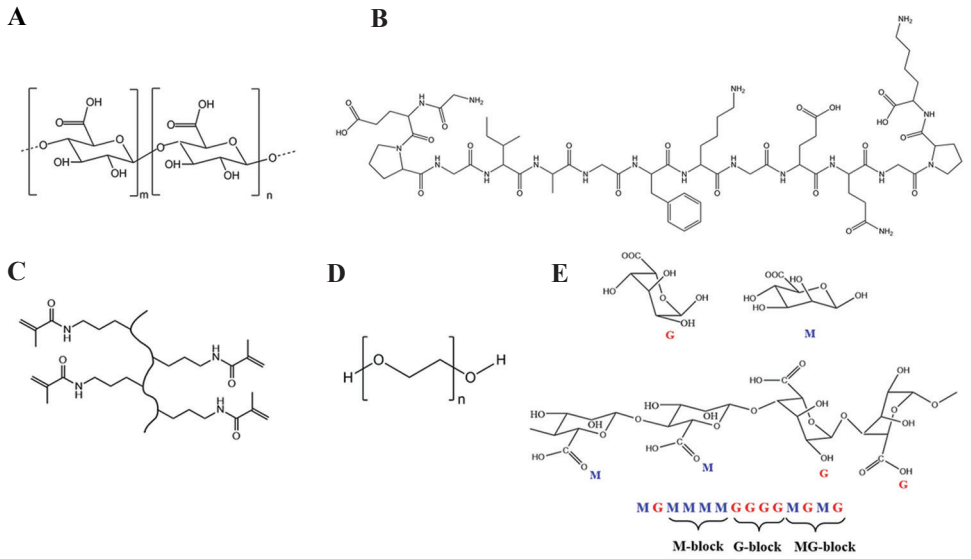

Figure 4. The structure of (A) hyaluronic acid, (B) type II collagen, (C) gelatin methacryloyl, (D) polyethylene glycol, and (E) alginate.

of MeHA concentration was needed for this photosensitive bioink[40]. Currently, MeHA has been combined with GelMA to build a scaffold carrying human mesenchymal stem cells (hMSCs) (**Table 4**)[41]. The GelMA-MeHA hydrogel exhibited benign cell viability through 8 weeks of culture and it also improved regeneration of both cartilage

and subchondral bone in rabbits with osteochondral defects. In addition, instead of using UV light, the GelMA-MeHA scaffold could gelate via the illumination of visible light. On the other hand, acellular HA scaffold has been used to deliver growth factors, including human leukocyte-platelet-rich plasma (PRP) and leukocyte-platelet-rich fibrin, to cartilage defects. The following *in vivo* results confirmed that the newly generated cartilage tissue had improved biomechanical strength by these growth factor-loaded HA scaffold[42].

2.2. Alginate

Alginate is a biocompatible, biodegradable gelling agent that can be obtained from the cell wall of brown seaweed[43]. It is composed of 1,4-β-D-mannuronic acid (M) and 1,4-α-L-guluronic acid (G). Longer M or G blocks divided by MG alternating regions organized into this long anionic linear copolymer, which has suitable flexibility and shear-thinning capability required by 3D printing (**Figure 4E**)[44]. Alginate hydrogels are usually crosslinked rapidly by dropping an alginate solution into a calcium ion liquor, such as calcium chloride ($CaCl_2$) solution[45]. The compressive modulus of alginate inks is about 30 kPa, which is higher than that of native human articular cartilage, which is about 10.60 ± 3.62 MPa[46,47]. Owing to fast degradation rates, they are also considered to be lacking biological stability, which is important for cell viability[48]. Currently, scientists have found that oxidation of alginate was able to improve the *in vivo* biodegradability of alginate-based inks by creating more reactive sites[49]. Yang *et al.* also demonstrated that alginate bioink combined with COL I or agarose showed improved mechanical strength, with the compressive modulus increased by over 1.87- and 2.38-fold, respectively[47]. Coating 3D-printed alginate scaffold with homogeneous nano apatite has also been shown to significantly improve Young's modulus of the construct and the differentiation rate of encapsulated rat bone marrow cells[50].

Alginate-based bioinks have been applied widely in cartilage tissue engineering during the recent decade. Back in 2012, scientists used alginate hydrogel to demonstrate that the 3D printing techniques could be applied in osteochondral tissue engineering for the 1st time[51]. In 2014, researchers developed a novel ink using alginate/acrylamide solution with an epoxy-based UV-curable adhesive. It was shown to improve the mechanical strength of the printed scaffold compared with that composed of pure alginate[52]. In 2015, Kundu *et al*. built a 3D scaffold consisting of alginate hydrogel with chondrocytes and layer-by-layer deposition of polycaprolactone (PCL) (**Table 4**)[53]. Cells encapsulated showed up to 85% viability immediately after printing, but the long-term effects were not examined. In addition, by using nude mouse model, the authors demonstrated that mice implanted with PCL/alginate/chondrocyte/TGF-β scaffold showed significantly more COL fiber and better cartilaginous tissue formation. However, the long degradation period of PCL could prevent tissue ingrowth[53]. Later, Kosik-Kozioł *et al.* formulated an alginate/short submicron polylactide ink in 2017[54]. This material was able to increase Young's modulus of printouts threefold compared with that of pure alginate scaffold. Neocartilage ECM deposition was also observed during *in vitro* experiments. More recently, Olate-Moya *et al*. designed a new ink by conjugating photocrosslinkable alginate with gelatin, chondroitin sulfate, and graphene oxide, which further enhanced the printability of this material[55]. The 3D printed scaffold via the microextrusion process exhibited optimized resolution and increased cell proliferation when carrying human adipose tissue-derived mesenchymal stem cells. In the same year, Schwarz *et al.* used an oxidized alginate-gelatin hydrogel together with human nasoseptal chondrocytes to create 3D printed grid-like scaffolds, which showed high shape fidelity and improved resolution[56].

2.3. COL

COL is the most common protein in human and is a major component in ECM. It supports the regeneration of several connective tissue, including bone, cartilage, and skin[57]. In general, polypeptide chains constituting COLs are composed of a sequence of different peptides $(glycine\text{-}X\text{-}Y)_3$, in which X and Y are usually proline and hydroxyproline (HYP) (**Table 4**) (**Figure 4B**)[11]. For many years, COL-bioink has been regarded as a biocompatible material, as it can provide anchor sites for cell adhesion[58]. However, the traditional procedure for the cross-linking of COL hydrogel usually includes the addition of toxic chemical agents, such as glutaraldehyde and 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide[59]. In addition, the degradation rate of the 3D-printed COL scaffold is impacted by various factors, including the penetration of cells and the presence of non-specific proteinases. It means that the extent and rate of degradability of COL-based scaffolds is difficult to control[11]. For its printability, it is difficult for printouts by COL-bioink to maintain porous structure due to its inferior viscosity and weak mechanical strength[60]. Thus, applying COL directly as a source for bioink is tough.

The properties of COL can be tuned by blending with other materials or tailoring its fibrillogenesis, including the enzymatic process. In 2016, Shim *et al*. built a 3D-printed scaffold with hMSCs and hydrogel composed of HA and pepsin-treated COL (atelocollagen) [61]. The crosslinking process of this bioink did not involve any cytotoxic reagent. Results showed that the knee joint defect of rabbits with this construct implanted was covered by thick neocartilage tissue at the center space at week 8. But the expression of COL X, which is usually restricted to the lower part of articular cartilage, was observed in

the superficial layer of the neo-tissue[61]. Stratesteffen *et al.* developed a GelMA-COL ink via drop-on-demand 3D printing method in 2017[62]. Using the co-culture of human endothelial cells and hMSCs, the addition of COL increased cell spreading, storage modulus, and viscosity of this material[62]. In 2018, Yang *et al*. assessed the mechanical properties and biocompatibility of alginate bioink, alginate/agarose bioink, and alginate/COL I bioink. Among these three materials, alginate/COL I hydrogel had higher cell viability and cartilage gene marker expression levels than the other two kinds of inks, but with more inferior compressive modulus and tensile strength compared with alginate/agarose one[47]. Simultaneously, another work constructed a 3D-printed porous scaffold via COL crosslinked by tannic acid (TA) (**Table 4**), a non-toxic plant polyphenol[63]. This bioink could gelate at a temperature around 37 °C, suggesting that it is applicable in the human body. For its printability, TA-crosslinked preosteoblast-laden COL bioink was able to be printed into a construct with a pore size of 512 ± 46 μm and strut size of 315 ± 10 μm. The findings from *in vitro* experiments also indicated that this COL -based construct with an optimal TA concentration of 0.5 wt% could maintain cell viability of 95% throughout the 14-day preosteoclast culture. A recent work conducted by Wang *et al*. developed a bi-phasic scaffold with gradient mechanical strength via cryogenic 3D printing[64]. TGFβ1-loaded COL I hydrogel was filled in the printed frame to form the cartilage zone. At 37°C, the compressive strength of the cartilage layer was 0.12 Mpa and the elastic modulus was 1.05 Mpa, which are similar to those of natural human cartilage tissue. Additionally, the shear strength between the cartilage zone and the subchondral zone was 0.4 Mpa. The interface also had a peel strength of 470 N/m. These two results indicated that the ink was capable for cryogenic 3D printing of two-layer osteochondral scaffold. It has also been demonstrated that the expression of cartilage gene markers, such as SOX9 and COL II, was significantly upregulated in the cartilage layer with TGF-β1 through *in vitro* experiments using rat BMSCs[64].

2.4. SF

SF, mainly produced by *Bombyx mori* silkworms, is composed of 43% glycine, 30% alanine, and 12% serine[65]. Sericin, which is a UV-resistant protein that glues the silk fibers, needs to be removed by the degumming process to produce soluble SF[66]. The processed SF is then dissolved in solvents such as lithium bromide, formic acid, ionic liquid, and CaCl2/ethanol/water solvent system[67]. Aqueous silk solution can be turned into different forms and structures, including films/membrane, powder, hydrogel, porous sponges, and nanofibers (**Figure 5**) [68]. SF hydrogel is usually crosslinked by the addition of crosslinkers such as glutaraldehyde and genipin[69]. It is also characterized by a low adverse immune reaction, compatible degradation rates, and superior elasticity[66]. *B. mori* silk without sericin has an initial modulus of 15 – 17 Gpa, which is stronger than most other resources. In addition, it can be incorporated with other biopolymers, such as gelatin, to develop particular bioinks, enabling scaffolds fabricated with tunable mechanical properties and controlled pore sizes[70].

The utilization of SF as a natural source of 3D-printing bioinks has advanced rapidly in recent 3 – 4 years. In 2017, Shi *et al.* developed a BMSC-laden SF/gelatin bioink for articular cartilage repair[71]. Scientists observed a significant increase in HYP and GAG accumulation during a 21-day *in vitro* culture with the addition of BMSC affinity peptide E7, indicating a superior chondrogenesis ability. However, the mechanical properties of this biomaterial are not shown. In 2019, bioink consisting of SF and gelatin was further improved to be crosslinker-free, as most of the chemical agents added for SF polymerization are toxic[69]. In addition, a study by Kim *et al*. developed an advanced SF-based bioink (Sil-MA) by methacrylating SF via glycidyl methacrylate and built a scaffold for cartilage repair using DLP 3D-printing (**Table 4**)[72]. Their results showed that scaffold by bioink composed of 30% Sil-MA exhibited a compressive modulus of 910 kPa, which was able to hold a kettlebell weighing 7kg and recovered without any deformation after removing the bell. As for its printability, 30% Sil-MA scaffold with interconnected pores of sized up to 700 μm was successfully printed and the inner structure was visible to the naked eye. Using 3D-printed 30% Sil-MA cartilaginous trachea, significant cartilage matrix formation and the presence of chondrocytes were observed after 4 weeks of culture *in vitro*, suggesting superior ability to promote cartilage formation and biocompatibility of Sil-MA as a novel bioink[72].

2.5. GelMA

GelMA is a gelatin derivative that mainly contains methacrylamide groups with a minority of methacrylate groups (**Figure 4C**). It is usually crosslinked via UV light illumination with the addition of a photoinitiators, such as Irgacure 2925 and lithium acylphosphinate (LAP) salt (**Table 4**). The photocrosslinking of GelMA can produce

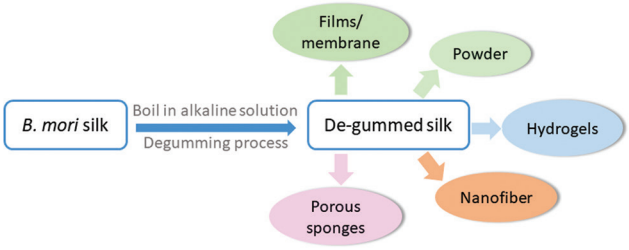

Figure 5. Schema of the *Bombyx mori silk* processing.

mild crosslinked hydrogel with low cytotoxicity[73]. Since GelMA was introduced by Van Den Bulcke *et al*. in 2000, several studies have shown that the physical properties and cell response parameters of GelMA could be tuned by manipulating its synthesis and processing. For example, a study in 2012 demonstrated that the compressive modulus of GelMA was directly correlated with the degree of methacryloyl substitution[74]. Cryogenic treatments, including freeze-drying, can also help control the pore sizes of the GelMA hydrogel[75]. In addition, the stiffness of this material can be modified by the degree of crosslinking[76]. As for its biocompatibility, the arginine-glycine-aspartic acid sequence contained in GelMA is significant for promoting cell attachment[77], indicating a potential capability for promoting chondrogenesis. High cell viability is also observed in cell-laden GelMA hydrogel[73]. As for its disadvantages, studies have revealed that GelMA-pure hydrogel is poor in mechanical strength compared to the initial cartilage tissue. It also exhibits a high swelling rate, which increases wound pressure and results in the lack of stability required for the maintenance of space for cartilage regeneration[78].

The application of GelMA in 3D printing is advancing in recent years. In 2019, Chen *et al*. built a 3D-printed cartilage ECM/GelMA/exosome scaffold to deliver mesenchymal stem cell exosomes, which is significant for the disorder of intercellular mitochondria communication in OA[79]. The construct was printed successfully by a stereolithography-based 3D printer with a resolution of 0.05 mm and suitable pore size (100 – 500 μm). By using the rabbit model, the authors also showed that the ECM/GelMA/exosome scaffold was able to restore the functions of chondrocyte mitochondrial to enhance chondrocyte migration and cartilage regeneration[80]. In the same year, Lam *et al.* also developed a bioink consisting of GelMA. The scaffold was then embedded with porcine chondrocytes of different concentrations to investigate its biocompatibility and repair efficacy[81]. Significant chondrogenic differentiation and enhanced cartilage ECM formation was observed after 14 days of *in vitro* culture. The shapes and distribution of cells were also maintained throughout the 2 weeks. In addition, GelMA scaffold with high chondrocyte density promoted cartilage-specific COL type II formation compared to the MeHA-based constructs[81]. In 2020, Luo *et al*. developed a BMSCs-containing bioink with 5% of GelMA[82]. This cell-laden GelMA hydrogel was capable for the construction of scaffolds with accurate and complex shapes. In addition, BMSC differentiation and generation of cartilage fiber tissue were observed after 4 weeks since the GelMA scaffold was implanted intramuscularly in nude mice. In a more recent study by Irmak and Gümüsderelioglu, a photocrosslinkable hydrogel consisting of GelMA and PRP, which contains various growth factors, was 3D printed into tissue-specific structures[83]. The GelMA/PRP scaffold could significantly promote the proliferation and differentiation of ATDC5 cells as suggested by *in vitro* cell culture study. However, the authors did not provide information about the efficacy of cartilage repair *in vivo* by this novel material.

2.6. PEG

PEG hydrogel is composed of synthetic liquid-swollen polymer networks (**Figure 4D**) that have been approved by the Food and Drug Administration for medical applications in human and have become one of the most popular resources to design hydrogels for cartilage repair[84]. It can be synthesized by photopolymerizing PEG precursors with the addition of photoinitiators[85].

Table 3. Conclusion of the gelation methods, biocompatibility, advantages and disadvantages of hydrogels mentioned in this article. The score goes from "+" to "+++", suggesting relatively low, medium and high biocompatibility.

Materials	Gelation methods	Biocompatibility	Highlight	Reference
HA	1. Chemical agents 2. Photocrosslinking 3. Electropolymerization	++	Bioactive properties but poor of mechanical strength, which can be improved by crosslinking with materials such as PEG.	[32,66,97]
Alginate	Cation adding	+++	Suitable flexibility and shear-thinning capability, but poor of biomechanical properties and stability.	[44,45,66,97]
Collagen	1. Chemical agents 2. Physical methods (heating, drying, irradiation e.g.)	+++	Good biocompatibility with inferior viscosity and mechanical properties; crosslinking via chemicals may involve toxic agents.	[11,59,66,73]
Silk Fibroin	1. Chemical agents 2. Cryogelation	++	Low adverse immune reaction, tunable degradation rate and elasticity; crosslinking via chemicals may involve toxic agents	[66,69]
GelMA	Photocrosslinking	+++	Fast gelation and tunable properties; low cytotoxicity but lacks mechanical strength.	[73,76,77]

Table 4. Acronyms applied in this review and their full name

Acronyms	Full name	Acronyms	Full name	Acronyms	Full name	Acronyms	Full name
OA	Osteoarthritis	PEG	Polyethylene glycol	LAP	Lithium acylphosphinate salt		
ECM	Extracellular matrix	pNIPAAM	Polymer poly (N-isopropylacrylamide) grafted hyaluronan				
MMP	Metalloproteinase	MeHA	Methacrylated hyaluronic acid	BMSC	Bone marrow-derived mesenchymal stem cells	EBP	Extrusion-based printing
ADAMTS	A disintegrin and a metalloproteinase with thrombospondin motifs			hMSC	Human mesenchymal stem cells	DLP	Digital light processing
HA	Hyaluronic acid	PCL	Polycaprolactone	PRP	Platelet-rich plasma	IPN	Interpenetrating network
COL	Collagen	Sil-MA	Methacrylated SF	GAG	Glycosaminoglycan		KLF3 Antisense RNA 1: KLF3-AS1
SF	Silk fibroin	PEGDA	PEG diacrylate	TGFβ	Transforming growth factor β		G-protein-coupled receptor kinase interacting protein 1: GIT-1
GelMA	Gelatin methacryloyl	TA	Tannic acid	HYP	Hydroxyproline		

PEG has been shown to facilitate chondrogenic ECM regeneration back in 2002[86]. It is capable for maintaining the viability of non-adhesive cells such as chondrocytes, while discouraging the adhesion and spreading of adhesive cells including osteoblast and fibroblast[87]. Studies have found that this biologically inert property of PEG could be improved by the inclusion of hydroxyapatite and Laponite, a kind of synthetic smectite clay[88,89]. As for its mechanical properties, the compressive modulus of PEG hydrogel is about 0.75 MPa, which is relatively stronger than other hydrogels but still low compared with that of native human articular cartilage[90].

Scientists have been working on the development of PEG hydrogel in 3D printing for the past decade. In 2014, Zhang *et al*. developed a 3D-printed PEG scaffold with β-tricalcium phosphate ceramic, which is a special form of tricalcium phosphate used as bone graft substitute[91]. The scaffold was of 50%-65% porosity and was fully interconnected. Formation of new tissue with smooth but raised surface was observed 24 weeks after implantation using rabbit model with trochlea defects[90]. Gao and other scientists fabricated a PEG-GelMA scaffold encapsulating hMSCs by inkjet printing in 2015[92]. The contained cells were shown to maintain in their initially deposited position during printing and the cell viability was over 80%. 63% of embedded hMSCs underwent chondrogenic differentiation after 21 days of *in vitro* chondrogenesis, but cell hypertrophy was also observed. The compressive modulus of either cell-laden or non-cell-laden PEG-GelMA scaffold was also lower than that of their corresponding PEG scaffold[92]. In 2018, Wang *et al*. developed a UV-crosslinkable ink containing GelMA and PEG diacrylate (PEGDA) (**Table 4**)[78]. Their results showed that the compressive stress of hydrogels with both GelMA and PEGDA was significantly higher than that of GelMA alone, but the novel ink became fragile as the concentration of GelMA increased due to enhanced crosslinking density. For its cytocompatibility, the viability of MC3T3-E1 cells during a 7-day culture was maintained above 99%. Recently, Qiao *et al*. combined triblock polymer networks of PCL-b-PEG-b-PCL with GelMA, BMSCs and growth factors to construct a native-like tri-layered 3D scaffold via melt electrowriting, a high-resolution additive manufacturing process[93,94]. The three layers, superficial cartilage (S), deep cartilage (D), and subchondral bone (B), were fabricated with PCL-b-PEG-b-PCL filaments of different diameters, spacing, and orientations based on the native osteochondral COL fiber architecture. The compressive moduli of the structure were benign (S: 283.6 ± 22.3 kPa, D: 964.2 ± 56.8 kPa, B: 55.8 ± 5.4 MPa), but the mechanical strength of cartilage parts was still inferior to that of natural tissue. Significant accumulation of osteochondral tissue-related zonal marker proteins and the presence of spatially

orientated cells were also observed, indicating a process of chondrogenesis and osteogenesis[93].

3. Current clinical trials based on hydrogel scaffold against cartilage damage

Until now, articular cartilage scaffolds for commercial use or clinical settings can be divided into three types: cell-laden constructs such as BioSeed®-C and CaReS® with seeded chondrocytes, cell-free constructs such as MaioRegen and TruFit with MSC derivates, and scaffold-free constructs with degradability, such as Chondrosphere®[95]. In addition, scientists have performed a clinical trial comparing patients receiving microfracture treatment with those implanted with BST-CarGel, an acellular scaffold containing polysaccharide chitosan. It was shown that BST-CarGel with a debrided cartilage lesion could develop a more stable, voluminous, and adherent blood clot compared with the traditional surgical strategy for full-thickness cartilage defects[96]. Moreover, current ongoing clinical trials include a study to investigate the efficacy of decalcification bone scaffold when combined with microfracture in the clinical repairment of articular cartilage defects and another project comparing microfracture with COL scaffold laden with adipose-derived stem cells.

However, challenges still lie in the way of hydrogel scaffolds' application from bench to bedside. Firstly, none of the above products are 3D-bioprinted. The printing process of one tissue-based human scale scaffold may take several hours, leading to an extremely long fabrication process and high costs if 3D-printed scaffolds are put into large scale production[23]; Second, even if a rapid and automatic printing process is developed, it may still be difficult to find a material with biomimetic components and structure. In addition, underlying molecular mechanisms of cartilage regeneration are still unclear, causing difficulties to navigate the regulatory pathways[95]. Therefore, the clinical application of 3D-printed cartilage scaffolds still has a long way to go unless more advance is made in pathological studies, bioink development, and customized 3D printing technologies.

4. Conclusion and future direction

As a technology that initially appeared at the end of the 20th century, 3D printing can manipulate the structure of engineered tissue scaffolds with high resolution and accuracy[10]. In the recent decade, the 3D printing technique has been increasingly applied in the repair of articular cartilage, which is usually unable to self-regenerate as it lacks vessels and nerves. Hydrogels have become the most used resources for bioinks due to their elastic property and ECM-mimetic crosslinked network structure. The mechanical and structural properties of printed constructs can be tuned by manipulating their printing process or crosslinking with other materials. In addition, cells, drugs, and other bioactive factors such as cytokines, can also be combined with 3D-printed tissue scaffolds to enhance the repair and regeneration of cartilage (**Table 3**)[11].

Generally, hydrogels lack mechanical strength and are incapable to bear long-term repetitive loading *in vivo*[23]. Thus, future directions of 3D-bioprinted cartilage tissue include developing tougher bioinks that can withstand the long-term compression and shear in joint environment[98]. For example, interpenetrating network (IPN) hydrogels, which are fabricated by combining multiple independent but interdigitating polymer networks at molecular level, has been shown to be an efficient way to enhance the mechanical properties of the biomaterial (**Table 4**)[99]. A recent study by Shojarazavi *et al*. developed an injectable IPN hydrogel composed of ionic crosslinked alginate, enzymatically crosslinked phenolized ECM and silk fibrin nanofibers[100]. The results show that with optimized concentration of alginate and silk fibrin nanofibers, the compression modulus and the mechanical stiffness of the hydrogels could be both improved.

Developing functional scaffold is a new tendency for 3D-printing cartilage repair. A general idea of functionalizing cartilage scaffolds is delivering drugs which target enzymes or cytokines that hinder cartilage regeneration. For example, MMP-13 has been found to be significant for the hypertrophy of BMSCs, thereby inhibiting the therapeutic effects of BMSCs for cartilage repair[101]. Thus, hydrogel carriers of MMP-13 inhibitors can be developed to reduce the hypertrophy of mesenchymal stem cells during chondrogenesis[102]. Additionally, functional scaffolds with novel physical properties can be fabricated to enhance the efficacy of other existing treatments of osteochondral defects as well. Pulsed electromagnetic fields, a therapy for bone repair of low-risk and low-cost, has been found to improve the growth and healing of engineered cartilage[103]. Thus, inks that are conductive and able to build electro-microenvironment can be developed and applied in the field of 3D-printing cartilage tissue repair. Functionalizing cartilage scaffolds with cell derivates to avoid the side effects of cell-based therapies are also catching increasing attention. Previously, hydrogel scaffolds encapsulated with chondrocytes developed rapidly. However, the limited number of chondrocytes from donor sites and undesired effects, such as chondrocyte dedifferentiation, hinder their clinical efficacy[104]. Thus, MSC-laden scaffolds began to appear. Nevertheless, current clinical studies using engineered articular cartilage with MSCs demonstrated problems such as undesired MSC dedifferentiation, tumorigenicity and disease transmission[105]. To overcome these

limitations, MSC-derived exosomes can be combined with bioinks instead. In 2018, Liu *et al*. demonstrated that the therapeutic ability of MSC-derived exosomes specifically relied on lncRNA KLF3 Antisense RNA 1 (KLF3-AS1). It works by separating miRNA206 from its target G-protein-coupled receptor kinase interacting protein 1 (GIT-1), which worked to inhibit chondrocytes apoptosis and promoted chondrogenesis[106]. Thus, developing 3D-printed hydrogel scaffolds carrying exosomes from lncRNA KLF3-AS1-overexpressing human MSCs can be an interesting direction for cartilage regeneration of osteoarthrosis patients. Moreover, single functional research about the regulatory role of miRNAs is far from sufficient because miRNAs are multitargeting and contribute to a complex regulatory network in chondrocytes about their proliferation, migration, autophagy and apoptosis[107]. As a result, future studies also need to comprehensively investigate the roles of miRNAs and their targets in the regulation of chondrocyte fate.

In all, 3D-bioprinting is an advancing and efficient technology for cartilage tissue repair. With the development of printing methods, novel bioinks and the understanding of molecular mechanisms regulating cartilage regeneration, 3D printed scaffold may become a promising therapy for clinical use in the near future.

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Conflict of interest

The authors declare no conflict of interest

Author contributions

Y.M. and Q.L. conceived the idea. Q.L. drafted the manuscript. Y.M. directly contribute to the manuscript by adjusting its content and structure, as well as modifying the grammar. X.Y. and W.W. further revised the paper by giving advice on its structure, content, figures and tables.

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