

# Hybrid and Composite Scaffolds Based on Extracellular Matrices for Cartilage Tissue Engineering

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REVIEW ARTICLE

# Hybrid and Composite Scaffolds Based on Extracellular Matrices for Cartilage Tissue Engineering

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Cartilage consists of chondrocytes and a special extracellular matrix (ECM) having unique biochemical, biophysical, and biomechanical properties that play a critical role in the proliferation and differentiation of cells inherent to cartilage functions. Cartilage tissue engineering (CTE) requires recreating these microenvironmental physicochemical conditions to lead to chondrocyte differentiation from stem cells. ECM-derived hybrid scaffolds based on chondroitin sulfate, hyaluronic acid, collagen, and cartilage ECM analogs provide environments conducive to stem cell proliferation. In this review, we describe hybrid scaffolds based on these four cartilage ECM derivatives; we also categorize these scaffolds based on the methods used for their preparation. The use of hybrid scaffolds is increasing in CTE to address the complexity of cartilage tissue. Thus, a comprehensive review on the topic should be a useful guide for future research.

**Keywords:** cartilage, tissue engineering, hybrid scaffolds, extracellular matrix (ECM), ECM derivatives

## Impact Statement

Scaffolds fabricated from extracellular matrix (ECM) derivatives are composed of conducive structures for cell attachment, proliferation, and differentiation, but generally do not have proper mechanical properties and load-bearing capacity. In contrast, scaffolds based on synthetic biomaterials demonstrate appropriate mechanical strength, but the absence of desirable biological properties is one of their main disadvantages. To integrate mechanical strength and biological cues, these ECM derivatives can be conjugated with synthetic biomaterials. Hence, hybrid scaffolds comprising both advantages of synthetic polymers and ECM derivatives can be considered a robust vehicle for tissue engineering applications.

## Introduction

**A**RTICULAR CARTILAGE (AC) is a connective tissue comprising also the main component of joints' surface, which covers and protects bones. Cartilage is composed of specialized cells and extracellular matrix (ECM).<sup>1</sup> Three major types of cartilaginous tissues are distinguished: hyaline cartilage, fibrocartilage, and elastic cartilage. The

chemistry and the supramolecular structure of the matrices determine the biomechanical and functional properties of these three cartilage types. Hyaline cartilage is the most abundant and well characterized, found as AC on the surfaces of bones.<sup>2,3</sup> AC can bear loads of up to around 20 MPa during normal joint movements.<sup>1</sup> The major macromolecular components of hyaline cartilage include collagen (COL) type II and X, cartilage oligomeric matrix protein, and proteoglycans,

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among which aggrecan (ACAN) is the major component. Collagens and proteoglycans play critical roles in maintaining cartilage ECM structure. The mechanical strength of the different zones of AC is defined by changes in chemistry and biomechanical cues of the macromolecular components.<sup>3-5</sup> Mechanical characteristics and load-bearing ability of AC are related to and optimized by the arrangement of COL fiber organization, proteoglycan content, and chondrocyte shape.<sup>6</sup> The ECM also forms a niche for stem cells (SCs) and provides biochemical and physical signals to control their proliferation.<sup>7</sup> Recent studies demonstrated that SCs necessitate a particular tissue niche for proliferation and differentiation.<sup>8</sup> In addition to the biochemical environment, mechanical cues of the ECM, such as stiffness, also impact SC lineage differentiation.<sup>9</sup> Soft, medium, and hard matrices can induce mesenchymal stromal cells (MSCs) to differentiate into neurons, myocytes, and osteoblasts, respectively.<sup>10</sup> MSCs are site regulated; they secrete different factors to modulate tissue protection and regeneration by interacting with their niche microenvironment mediated by ECM components.<sup>11</sup>

Recent studies demonstrate that chondrocytes sense physical cues such as receptor/ligand density, rigidity/softness, and shape (dimensionality) of the ECM surface by mechanoreceptors. In response to these cues, the cells modulate the organization of the cytoskeleton, extracellular adhesion sites, and cell shape.<sup>12,13</sup> Studies performed on artificial scaffolds, such as hydrogels, porous/fibrous scaffolds, and ECM-based constructs, demonstrated that SCs expand and differentiate, faster on the softer materials such as COL than stiffer substrates such as chitosan (CHSN).<sup>14,15</sup> One approach to create three-dimensional (3D) structures that mimic the cartilage natural niche is to utilize scaffolds based on cartilage ECM, but generally these scaffolds have lower mechanical strength and suffer from lot-to-lot variations.<sup>16</sup> Synthetic and polymeric materials, including poly(DL-lactic-co-glycolic acid) (PLGA), poly( $\epsilon$ -caprolactone) (PCL), poly(D,L-lactic acid) (PLA) and their copolymers, poly vinyl alcohol (PVA) and also methacrylamide (MA) modification, have been used to fabricate scaffolds for AC replacement and to serve as cell transplantation vehicle for tissue engineering (TE).<sup>17-20</sup> Synthetic polymeric scaffolds allow for good control of mechanical properties, but suffer from a lack of biological properties. These biomaterials are hydrophobic, lack functional adhesion sites and cell recognition signals, and therefore must be modified before cell seeding.<sup>21</sup> In contrast, scaffolds manufactured from ECM components contain surface structures conducive to cell growth.<sup>22</sup> Hence, hybrid scaffolds comprising both advantages of synthetic polymers and ECM derivatives can be considered a strong vehicle for TE applications.<sup>23</sup>

Although other reviews related to cartilage ECM derivatives have been published already, this review focuses on cartilage ECM-derived hybrid and composite scaffolds, prepared and used specifically for cartilage tissue engineering (CTE). Four sections describe (1) COL and COL-based scaffolds, (2) hyaluronic acid (HA) and HA-based scaffolds, (3) chondroitin sulfate (CS) and CS-based scaffolds, and (4) cartilage ECM analogs (cECMa) and cECMa-based scaffolds. The fifth section reviews the different hybrid scaffold processing approaches, and in the sixth section, results from ECM-based scaffolds are discussed in detail.

## Hybrid and Composite Scaffolds for CTE

### *COL-based scaffolds*

Collagens can be considered the most important proteins of connective tissue in mammals and also the most abundant proteins in their body.<sup>24</sup> About 30 different forms of COL have been defined according to the amino acid sequences and the structures that they are arranged into. The three most common types of collagens are type II, type I, and type X, in hyaline cartilage, fibrocartilage, and hypertrophic cartilage, respectively.<sup>25</sup> As abundant and biocompatible natural products, collagens have been thoroughly investigated and are widely used as TE scaffolds.<sup>26</sup> Important drawbacks of collagens are, however, inferior mechanical strength and quick degradation, compromising their use for CTE applications. These disadvantages can be partially rectified by crosslinking<sup>27</sup> collagens with natural and synthetic polymers to afford blends and composite materials of superior properties.<sup>28</sup> For instance, blend scaffolds composed of COL crosslinked with CHSN have improved mechanical strength than unmodified COL.<sup>29</sup> A mixture of COL and CHSN exhibits higher mechanical strength because of their miscibility, hydrogen bonding, and electrostatic interactions that reinforce the composite.<sup>30</sup> Partial hydrolysis of COL leads to gelatin (GEL) production. Despite its similarity to chemical COL composition, it lacks antigenicity and immunogenicity. GEL has been used for cartilage, bone, and nerve TE.<sup>31</sup> Hybrid scaffolds composed of COL and synthetic materials, such as PLA, PLGA, PVA, PCL, MA, poly(ethylene glycol) (PEG), or natural biomaterials, including CHSN, agarose (AGR), alginate (ALG), elastin, silk fibroin (SF), and others that have been used for CTE, are listed in Table 1.

### *HA-based scaffolds*

Hyaluronic acid (HA) is a key component in the ECM of cartilage. It is an anionic glycosaminoglycan (GAG) of up to 4000 kDa with hydrogel-like properties.<sup>63,77-80</sup> Elasticity and viscosity of the synovial fluid and shock absorbance capacity in articular joints are due to HA. HA is the primary ligand of the CD44 receptor, and several functions of the CD44 receptor are mediated through interaction with HA.<sup>81,82</sup> Crosslinking is the most common modification of HA, to form hydrogels for TE applications. The methacrylation is one of the frequently used technique for modification of HA hydrogels. Changing the methacrylation percentage directly effects on the crosslinking density and stiffness of the hydrogel matrix.<sup>83</sup> HA has also been chemically functionalized for the attachment of reporter groups for drug delivery systems. Composite materials of HA with natural and synthetic polymers were developed into biomimetic scaffolds with ability of enhancing wound healing and angiogenesis.<sup>84</sup> Hybrid and composite scaffolds composed of HA and synthetic materials (PLGA, PVA, PEG, and MA) and with natural biomaterials (CHSN, fibrin, AGR, ALG, and dextran) have been used for CTE (Table 2).

### *CS-based scaffolds*

CS is a GAG, a long unbranched and polar polysaccharide consisting of amino sugars and sugar acids. CS plays a critical role as a main component of the ECM in cartilage functions.<sup>125</sup> The ECM regulates metabolism as well as

TABLE 1. COLLAGEN-BASED HYBRID SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING

Author	Scaffold composition	Biological assessment	Fabrication process	Advantages	Ref.
Zhu <i>et al.</i>	GEL-MA/PEG diacrylate/(TGF- $\beta$ 1)-embedded nanospheres	hBMSCs <i>in vitro</i>	Tabletop stereolithography-based 3D bioprinter	Cell-laden bioprinted construct, a promising strategy for CTE	32
Cheng <i>et al.</i>	COL-CHSN/GO-np	Hydrogels transplanted in the rats	Composite 3D printing	The hydrogel/GO-np protected the CT by the signal pathway of Rank/Rankl/OPG	33
He <i>et al.</i>	PLCL/COL I	Rabbit articular chondrocytes <i>in vitro</i>	3D printing technology, LDM	PLCL scaffolds coated with COL I show a great potential application in TE	34
Kaczmarek <i>et al.</i>	CHSN/COL/GAGs crosslinked by sodium ALG	Chondrocytes <i>in vitro</i>	FD	Modified biomechanical properties, open-pore structures after 3 days in a perfusion bioreactor	35
Yang <i>et al.</i>	COL I/ALG bioink	Rat articular chondrocytes <i>in vitro</i>	3D cell printing	Bioprinted ALG/COL with favorable mechanical strength and biological functionality	36
Gao <i>et al.</i>	COL I/activated CS (with NHS)	Rabbit articular chondrocytes <i>in vitro</i>	Hydrogels	Injectable and self-crosslinkable hydrogels	37
Chen <i>et al.</i>	PEG/COL	MC3T3-E1 cells	Double network hydrogel	Potential as a load-bearing tissue repair material	38
Mekhileri <i>et al.</i>	Thermoplastic (PEGT/PBT) cell-laden GEL-based hydrogel microspheres	Human chondrocytes <i>in vitro</i>	3D plotting, high-throughput fabrication techniques	Automated and scalable pathway for bioassembly of both simple and complex 3D tissue constructs	39
Bas <i>et al.</i>	MA-GEL/PCL fiber	—	MEW	A strategy based on a numerical model was applied to accelerate the design of specific scaffolds for TE	40
Saghebasl <i>et al.</i>	(PNIPAAm-PCL-PEG-PCL-PNIPAAm)/GEL	Human chondrocytes <i>in vitro</i>	Thermosensitive hydrogel	Useful hydrophilic properties for growth and cell embedding and secretion of ECM	41
Song <i>et al.</i>	Blends of duck's feet COL/PLGA	Rabbit costal chondrocytes <i>in vitro</i>	SC/SL	Scaffolds with pore size 250 to 425 $\mu$ m, highly suitable constructs for enhanced cartilage repair	42
Clearfield <i>et al.</i>	COL-HA/COL-hydroxyapatite	—	Unidirectional freeze casting/lyophilization bonding process	Combination of biomimetic compositional and architectural multidirectional scaffolds	43
Huang <i>et al.</i>	Sodium cellulose sulfate/GEL	hMSCs <i>in vitro</i>	Electrospinning	Use as a scaffolding material for CTE	44
D'Amora <i>et al.</i>	PCL/COL	—	3D printing	Mimicking tissue gradients for interface TE	45
Jeon <i>et al.</i>	O A/GEL-MA IPN-structured hybrid hydrogels	hMSCs <i>in vitro</i>	Hydrogel	Biocompatible, biodegradable, and tough elastomeric hydrogels	46
Agheb <i>et al.</i>	GEL-tyrosine	Chondrocyte	Electrospinning	Excellent matrices in cell adhesion and proliferation	47
Kalaithong <i>et al.</i>	PLCL/GEL	L929 mouse fibroblast cells	Electrospinning and wet spinning	Wet-spun scaffold gave the best combination of properties	48
Shi <i>et al.</i>	SF/GEL	BMSCs <i>in vivo</i>	3D printing	Promising biomaterial for knee cartilage repair	49
Almeida <i>et al.</i>	ALG functionalized with COL I or II	Human IFP-derived stem cells	FD	Shape-memory ALG scaffold	50
Levato <i>et al.</i>	GEL methacryloyl bioinks	BMSCs and chondrocytes	Bioprinting	Biofabrication of 3D constructs with multiple cell types	51

(continued)

TABLE 1. (CONTINUED)

Author	Scaffold composition	Biological assessment	Fabrication process	Advantages	Ref.
Wang <i>et al.</i>	COL/SF/PLGA	BMSCs <i>in vitro</i> and fully thick AC defects in rabbits	COL/SF incorporated with PLGA microspheres	Enhance AC regeneration, good integration between the scaffold and the surrounding tissue	52
Naseri <i>et al.</i>	Cellulose nanofibers/a genipin-crosslinked GEL/CHSN ALG/COL	Chondrocytes	FD	Good mechanical properties	53
Studer <i>et al.</i>	PVA/heparin/GEL	ECPs <i>in vitro</i> and <i>in vivo</i>	—	Introducing a highly chondrogenic and off-the-shelf cell type	54
Bas <i>et al.</i>	GEL/PCL membranes	—	UV photopolymerized hydrogels	Promoting cellular adhesion and sequestering growth factors	55
He <i>et al.</i>	(PLA-co-PCL)/COL I	BMSC/chondrocyte cocultures, subcutaneously in nude mice	Electrospinning	May provide a potentially clinically feasible approach for cartilage repairs	56
Liu <i>et al.</i>	TGF- $\beta$ 1-loaded GEL microspheres into PLGA framework	hMSCs in rabbit model	Dynamic liquid electrospinning	Improved repair scores and compressive modulus	57
Yin <i>et al.</i>	COL/CHSN/PLA	ADMSCs <i>in vitro</i>	—	Enhances quality of tissue-engineered cartilage	58
Haaparanta <i>et al.</i>	PHBHHx/COL	Adult bovine chondrocytes	FD	COL/PLA hybrids promising scaffolds for CTE	59
Lomas <i>et al.</i>	COL/PLGA	hESCs and hMSCs	SL	Successfully used to culture hMSCs and hESCs	60
Dai <i>et al.</i>	PCL fiber/fibrin-COL hydrogel	Bovine chondrocytes subcutaneous in nude mice	PLGA mesh	New approach consists of designed shapes for regeneration of CT	61
Xu <i>et al.</i>	HA/COL	Chondrocytes	Fiber-hydrogel	Printed hybrid scaffolds with good mechanical properties	62
Kim <i>et al.</i>	CHSN-AGR-GEL	<i>In vitro</i> cell adhesion, proliferation/chondrocyte implantation in rabbit ears	FD	Easily processed	63
Bhat <i>et al.</i>	Genipin-crosslinked COL II scaffolds	Goat chondrocytes	Cryogels	Designed 3D scaffold for CTE	64
Chen <i>et al.</i>	PEG-based hydrogels/type II COL, or HA	MSCs for cartilage repair in osteochondral defect	—	New cartilage formation with natural cartilage structure	65
Hwang <i>et al.</i>	COL/PVA	MSCs <i>in vitro</i>	Hydrogel	Differentiation of MSCs to chondrocytes marginally enhanced in hydrogel constructs	23
Abedi <i>et al.</i>	Genipin-crosslinked CHSN/COL	Seeded with autologous MSCs for osteochondral defect in rabbit joints	Nanofibers	Controlling cell differentiation to chondrocytes	66
Bi <i>et al.</i>	PVA/GEL/nanohydroxyapatite/polyamide-6	(BDCs) and (ADCs) used to observe biocompatibility of scaffolds	FD	Optimized scaffold in genipin concentration of 1.0% and temperature of 20°C exhibited good biological properties	67
Qu <i>et al.</i>	HA/COL/CS	Culturing neonatal rabbit MSCs	FD	High mechanical strength composite with promotion of cell adhesion and proliferation ability	68
Zhang <i>et al.</i>	HA/COL/CS	In rabbit models	FD	Crosslinking in mild conditions	69

(continued)

TABLE 1. (CONTINUED)

Author	Scaffold composition	Biological assessment	Fabrication process	Advantages	Ref.
Ho <i>et al.</i>	PCL-COL	MSCs	Electrospun meshes	Inhibition of hypertrophic response from cells	70
Dai <i>et al.</i>	PLGA/COL	Bovine chondrocytes, subcutaneously in nude mice	COL microsponge formed in PLGA mesh	PLGA mesh aided as a mechanically strong frame while COL microsponges enhanced cell seeding and tissue formation	71
He <i>et al.</i>	PLA-COL	Chondrocytes	Hybrid sponge	New technique for the preparation of functional porous scaffolds	21
Guo <i>et al.</i>	(PEG fumarate)/GMP loaded with (TGF- $\beta$ 1)	Rabbit BMSCs	Crosslinked hydrogel	Composite hydrogels for localized delivery of SCs and bioactive molecules	72
Kathuria <i>et al.</i>	CHSN-GEL	Fibroblasts	Cryogels	Enhanced cell adhesion, proliferation, and ECM secretion	73
Ko <i>et al.</i>	COL II/CS/HA crosslinked by genipin	Cartilage repair <i>in vivo</i>	FD	Mimics the natural ECM of AC	74
Buttafocia <i>et al.</i>	COL/elastin crosslinking with (EDC/NHS)	Smooth muscle cells	Electrospinning from aqueous solutions	Suitable scaffold for TE applications	75
Tan <i>et al.</i>	CHSN-COL	K562 cell line	Nanostructured gel	Hybrid scaffold with biological and mechanical potential benefits for use in CTE	76

3D, three-dimensional; AC, articular cartilage; ADCs, adipose tissue-derived cells; ADMSCs, adipose tissue-derived mesenchymal stem cells; AGR, agarose; ALG, alginate; BDCs, bone marrow-derived cells; BMSCs, bone marrow-derived mesenchymal stem cells; COL, collagen; CS, chondroitin sulfate; CT, cartilage tissue; CTE, cartilage tissue engineering; ECM, extracellular matrix; ECPs, epiphyseal chondroprogenitor cells; EDC, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; FD, freeze-drying; GAGs, glycosaminoglycans; GEL, gelatin; GEL-MA, gelatin-methacrylamide; GMP, GEL microparticles; GO-np, graphene oxide nanoparticles; HA, hyaluronic acid; hBMSCs, human bone marrow-derived mesenchymal stem cells; hESCs, human embryonic stem cells; hMSCs, human mesenchymal stromal cells; IFP, infrapatellar fat pad; IPN, interpenetrating polymer network; LDM, low-temperature deposition manufacturing; MA, methacrylamide; MEW, melt electrospinning writing; MSCs, mesenchymal stromal cells; NHS, N-hydroxysuccinimide; PBT, poly(butylene terephthalate); PCL, poly( $\epsilon$ -caprolactone); PEG, poly(ethylene glycol); PEOT, poly(ethylene oxide terephthalate); PLCL, poly(L-lactide-co- $\epsilon$ -caprolactone); PLGA, poly(D,L-lactic acid); PHBHHx, poly(3-hydroxybutyrate-co-3-hydroxyhexanoate); PNIPAAm, poly(N-isopropyl acrylamide); PVA, poly(vinyl alcohol); SC, solvent casting; SF, silk fibroin; SL, salt leaching; TGF- $\beta$ 1, transforming growth factor-beta 1; UV, ultra violet.

TABLE 2. HYALURONAN-BASED HYBRID SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING

Author	Scaffold composition	Biological assessment	Fabrication process	Advantages	Ref.
Jung <i>et al.</i>	Hybrid (MCG-HG-PLGA-PD-B) composite system	ATDC5 prechondrocyte cell line	PLGA microspheres coated by HG	Scaffold can be a promising candidate for CTE	85
Hsieh <i>et al.</i>	PCL/hydroxyapatite/glycidyl-MA-HA	The knees of Lanyu miniature pigs for a period of 12 months	FDM	A new clinical option to be considered alongside current treatments of cartilage injury	86
Zhu <i>et al.</i>	HA/S-ALG	Fibroblasts and keratinocytes <i>in vitro</i>	Hydrogels were prepared crosslinking via epoxy groups on HA and ionic crosslinking on S-ALG	Cell carrier role	87
Karabiyik Acar <i>et al.</i>	Coacervate-based HA/CHSN	MSCs <i>in vitro</i>	Coacervate preparation	Proper cell viability, well-spread morphology	88
Schitavi <i>et al.</i>	ALG/HA	hMSCs <i>in vitro</i>	Hydrogel	Capable <i>in vivo</i> to mimic all depths of chondral defects	89
Teong <i>et al.</i>	HA-MA	hADMSCs <i>in vitro</i>	Hydrogel	Degree of methacrylation can modulate matrix stiffness of a hydrogel, thus affecting chondrogenesis	83
Han <i>et al.</i>	DBCO/HA/PEG	Subcutaneously injected into Balb-c mice	Injectable hydrogel	Hydrogel supported cell survival, regeneration of CT	90
Wang <i>et al.</i>	Hydrazine-modified elastin-like protein/aldehyde-modified HA	hMSCs <i>in vitro</i>	Injectable hydrogel	Significant mechanical protection to encapsulated hMSCs	91
Zhu <i>et al.</i>	Hydrazine-modified elastin-like protein/aldehyde-modified HA	Chondrocyte <i>in vitro</i>	Injectable hydrogel	3D scaffolds with decoupled niche properties	92
Shie <i>et al.</i>	Light-cured polyurethane/HA	Wharton's jelly MSCs	3D printing	Mimics the mechanical properties of AC	93
Raia <i>et al.</i>	SF/HA	hBMSCs <i>in vitro</i>	Hydrogel	Biologically relevant system with controllable temporal stiffening and elasticity	94
Lin <i>et al.</i>	PGA-coated HA	<i>In vivo</i> subcutaneous implantation in rabbit	Electrospinning	HA coating can significantly enhance biocompatibility	95
Chen <i>et al.</i>	HA/RGD-functionalized pectin hydrogel	Mouse subcutaneous implantation	Injectable hydrogel	Acceptable tissue compatibility	96
Kim <i>et al.</i>	Oxidized HA/glycol CHSN	ATDC5 cells <i>in vitro</i>	Injectable hydrogel	Respectable biocompatibility and stability under physiological conditions	97
Park <i>et al.</i>	ALG/HA	ATDC5 cells <i>in vitro</i> derived from mouse chondrogenic cells	Hydrogel	Well-characterized composition and mechanical properties	98
Lynch <i>et al.</i>	PVCL/HA-MA	Fetal bovine calf leg chondrocyte	Thermosensitive hydrogel	ECM production <i>in vitro</i> showed 10-fold increase compared with HA-MA controls	99
Dai <i>et al.</i>	PLGA/HA-MA	BMSCs <i>in vitro</i> and cell-free scaffolds in rabbit knees	Directional cooling of HA-MA solution	Anti-inflammatory properties, bioactivity, and good repair of full-thickness cartilage defect <i>in vivo</i>	100
Shim <i>et al.</i>	ateloCOLagen/HA	<i>In vivo</i> repair in knee joints of rabbits	Hydrogel	3D printing-based platform technology for regeneration	101

(continued)

TABLE 2. (CONTINUED)

Author	Scaffold composition	Biological assessment	Fabrication process	Advantages	Ref.
Tavakoli <i>et al.</i>	PLGA/HA/fibrin/bioactive glass	—	SC and PL	Microstructural and mechanical properties for CTE	102
Bas <i>et al.</i>	GEL-MA/HA-MA hydrogels/highly oriented PCL fibers	hMSCs <i>in vitro</i>	MEW hydrogels/highly oriented fibers	Improvement in mechanical properties	55
Snyder <i>et al.</i>	Fibrin/HA-MA	BMSCs <i>in vitro</i>	Hydrogel	Gene expression of COL-1 was decreased and increased in SOX9 in the presence of a platelet lysate, early chondrogenesis was observed	103
Mintz <i>et al.</i>	HA-based hydrogel/PCL	Dedifferentiated chondrocytes	PL	Mechanical properties same as human cartilage	104
Levett <i>et al.</i>	GEL-MA/HA-MA/CS-MA	Human chondrocytes	Photocrosslinkable hydrogel	Enhanced chondrogenesis and mechanical properties	105
Wang <i>et al.</i>	CS/HA/heparin sulfate	hADSCs	3D hydrogel	Biochemical and biomechanical cues interact with hADSCs in a 3D environment to regulate chondrogenesis	106
Schuurman <i>et al.</i>	GEL-MA/HA	—	Hydrogel	GEL-MA for using in biofabrication processes	107
Murphy <i>et al.</i>	COL-GAG	Wistar rat BMSCs	—	Lowest stiffness showed upregulation of SOX9 expression; highest stiffness showed upregulation of RUNX2 expression	108
Correia <i>et al.</i>	HA/CHSN	<i>In vitro</i>	FD	Noncytotoxic, promotes cell adhesion	109
Coburn <i>et al.</i>	PVA-MA and CS-MA	Cultured with MSCs for 6 weeks in both chondrogenic induction medium and <i>in vivo</i> osteochondral defect of rat model	Electrospinning	Enhancing type II COL synthesis and mechanical strength of tissues	110
Lee <i>et al.</i>	HA/CHSN	Chondrocytes in full-thickness cartilage defects on patellar groove of rabbit knee	—	GAG and COL II gene expression and presence of lacunae were exhibited	111
Jin <i>et al.</i>	HA/PEG	Chondrocytes	Injectable hydrogels	Injectable hydrogels with potential for CTE	112
Jin <i>et al.</i>	HA grafted with dextran-TA	Bovine chondrocytes	Injectable hydrogels via enzymatic crosslinking of the TA residues	Enhanced chondrocyte proliferation and matrix production	113
Fan <i>et al.</i>	PLGA-GEL/CS/HA (GCH)	MSCs <i>in vitro</i> and in full-thickness cartilage defect in rabbits	Incorporating GCH microsponges into PLGA framework and then crosslinked with TGF- $\beta$ 3	The group treated by the scaffold exhibited better chondrocyte morphology, integration with surrounding tissues, continuous subchondral bone	114
Im <i>et al.</i>	HA-atelocollagen/hydroxyapatite-tricalcium phosphate	Seeded chondrocytes, repair of osteochondral defects in minipigs	—	Showed significantly performance for repair	115
Tan <i>et al.</i>	HA/CHSN	Bovine articular chondrocytes	FD	Nontoxic reagents, injectable	116

(continued)



TABLE 2. (CONTINUED)

Author	Scaffold composition	Biological assessment	Fabrication process	Advantages	Ref.
Tan <i>et al.</i>	PLGA/GEL-CHSN-HA	Chondrocytes	FD	Increasing compressive modulus Enhanced ECM secretion and mechanical properties	117
Chen <i>et al.</i>	HA-g-CHSN-PNIPAM	Articular chondrocytes and meniscal cells of rabbit <i>in vitro</i>	Hydrogel		118
Erggelet <i>et al.</i>	Cell-free polyester/HA scaffold	Full-thickness AC defects in sheep	—	Enhancements in histological structure, and COL type-II expression Novel cell carrier for CTE	119
Pereira <i>et al.</i>	Carrageenan/fibrin/HA	Human articular chondrocytes <i>in vitro</i> and <i>in vivo</i>	Injectable hydrogel		120
Kasahara <i>et al.</i>	HA/CHSN	Osteochondral defects in the patellar groove of rabbits	—	Integration with surrounding natural cartilage and normal reconstruction of subchondral bone	121
Kim <i>et al.</i>	HA/COL	Bovine chondrocytes	Electrospinning process combined with SL		122
Solchaga <i>et al.</i>	HA- and polyester (HYAFF-11)-based scaffolds	Polyester-based sponge in rabbit osteochondral defects	SC/PL	Cellular adhesion and proliferation enhanced, chondrocytes maintained chondroblastic morphology Slow degradation of HYAFF-11, delayed cartilage formation and endochondral bone construction	123
Yamane <i>et al.</i>	CHSN-based HA	Rabbit chondrocytes	Fibers		124

DBCO, dibenzocyclooctyl; FDM, fused deposition modeling; GCH, GEL/CS/HA; hADMSCs, human adipose tissue-derived mesenchymal stem cells; hADSCs, human adipose-derived stem cells; HA-MA, hyaluronic acid-methacrylamide; MCG-HG-PLGA-PD-B, multichannel biphasic calcium phosphate granule-hyaluronic acid-gelatin-poly(lactic-co-glycolic acid)-polydopamine-BMP-7; PL, particulate leaching; PVCL, poly(N-vinylcaprolactam); RGD, Arg-Gly-Asp; RUNX2, runt-related transcription factor 2; SOX9, Sry-type high-mobility group box-9; TA, tyramine.

gene expression and stimulates proliferation and differentiation of chondrocytes.<sup>126</sup> The biological activities of CS make it an ideal biomaterial for CTE. The chondroprotective action of CS can be explained by the stimulation of the anabolic progression of cartilage metabolism, and its anti-inflammatory action, delaying numerous inflammation-induced catabolic processes in the tissue.<sup>127,128</sup> The inclusion of CS in composite scaffolds improves their mechanical properties and compressive strength by interacting with cell surface receptors to regulate chondrocyte behavior.<sup>129</sup> Hybrid scaffolds containing CS and synthetic as well as natural polymers have been used for CTE and are listed in Table 3.

#### ECM analog-based scaffolds

The use of decellularized ECM scaffolds and tissues is speedily expanding in TE.<sup>146</sup> There are many reasons for utilization of ECM-based materials in TE applications, including promotion of SC recruitment, cell infiltration, and differentiation without the need of additional biological factors. Cartilaginous ECM may be innovative in orthopedic medicine because of its chondroinductive potential, particularly in hydrogel-based systems.<sup>147</sup> The potential of ECM-based scaffolds to retain growth factors such as transforming growth factor-beta 1 (TGF- $\beta$ 1), fibroblast growth factor, and insulin-like growth factor is one of the major advantages of utilizing these materials as a scaffold.<sup>148</sup> However, the inferior mechanical properties of scaffolds composed completely of natural materials compared with synthetic materials are a major disadvantage for load-bearing tissue applications. Therefore, combinations of these ECM-based materials with suitable synthetic materials can be of advantage for CTE.<sup>147</sup> Hybrid scaffolds composed of ECM analogs and synthetic materials, as well as natural biomaterials, have been used for CTE and are listed in Table 4.

#### Hybrid Scaffold Processing Approaches

A multitude of porous ECM-derived hybrid scaffolds and scaffold processing approaches have been utilized in CTE. The most frequently used types are discussed below.

##### Fibrous scaffolds

Electrospinning of natural and synthetic biomaterials is a promising technique to produce fibrous scaffolds for TE applications.<sup>102</sup> The combination of synthetic polymeric materials and natural components such as COL could increase cell attachment while presenting ideal mechanical properties for TE applications.<sup>56</sup> PCL–COL electrospun meshes were used in autologous chondrocyte implantation as an innovative substitute to conventional grafts, which was the first try to design a mechanically enhanced cartilage resurfacing membrane composed of strong PCL mesh with bioactive COL. MSCs adhered on the surface of the mesh after seeding. More importantly, the mesh induced MSC differentiation into chondrocytes and inhibited a cellular hypertrophic response. This study showed the impact of the use of PCL–COL hybrid mesh as a cartilage patch and showed the importance of incorporation of the ECM-derived component COL into the synthetic PCL.<sup>163</sup> In a similar study, oriented PLA-co-PCL/COL I nanofiber yarn meshes

were fabricated by dynamic liquid electrospinning and aided as a skeleton for a freeze-dried (FD) COL/IHA chondral phase to improve the mechanical properties of the scaffolds. *In vitro* and *in vivo* results demonstrated that the hybrid constructs allowed cell infiltration similar to sponge scaffolds, and repaired the rabbit model osteochondral defects with improved mechanical properties of the newly engineered cartilage.<sup>57</sup> In another approach, COL/PVA nanofiber scaffolds were prepared and seeded with autologous MSCs to repair osteochondral defects of rabbit joints. The hybrid scaffolds induced higher chondrocyte morphology and new cartilage formation compared with the control defect without any treatment. The results showed that the nanofibrous COL/PVA scaffolds provide a supportive environment for cartilage tissue (CT) regeneration over 12 weeks. The histological results demonstrated that the COL/PVA group had better cartilage repair, more new matrix formation, and continuous subchondral bone compared with the control group.<sup>66</sup> In a further approach, oriented PCL fibers were fabricated by melt electrospinning writing (MEW) and combined with gelatin-methacrylamide (GEL-MA) and GEL-MA/HA-MA hydrogels to produce fiber-reinforced GEL-MA/HA-MA composites with enhanced mechanical properties. The results demonstrate that reinforcement of hydrogels with fibers leads to increases in the mechanical properties of the hybrid construct.<sup>55</sup> *In vitro* and *in vivo* studies on a sandwich model of electrospun GEL/PCL membranes, seeded with MSC/chondrocyte cocultures, were performed. To engineer the sandwich model, a GEL/PCL mesh was placed at the bottom of a well and seeded with cell suspension. A second mesh was then stacked on top of the first sheet, followed by cell seeding. The stacking was repeated until ten sheets. After implantation of the sandwich constructs 12 weeks subcutaneously into nude mice, histological analysis, GAG assay, and mechanical property measurement confirmed the formation of mature cartilage-like tissue. The strategy indicated that designed constructs were suitable for SC-based CTE. The constructs showed white appearance, flexibility, and a well-distributed synthesized neomatrix typical of cartilage.<sup>56</sup>

Yamane *et al.*<sup>164</sup> developed hybrid fibers based on CHSN and CHSN-HA by wet spinning. Articular chondrocytes from rabbits were cultured in the sheets of CHSN- and HA-based hybrid fibers. The fibers exhibited potential as an appropriate biomaterial for cartilaginous tissue scaffolds.<sup>124</sup> In a similar study, 3D scaffolds based on novel CHSN-based HA hybrid polymer fibers, which could control porous structure, were fabricated by wet spinning. The results showed that cell adhesion, proliferation, and synthesis of ACAN were higher in the CHSN-HA hybrid fiber than in the CHSN-only group.<sup>164</sup>

Chondroinductive nanofiber composites of PVA-MA and chondroitin sulfate-methacrylamide (CS-MA) were synthesized and used for AC repair. MSCs cultured on these scaffolds were shown to support an increase in cell proliferation, ECM production, and cartilage-specific gene expression (Fig. 1) *in vitro* as well as *in vivo*.<sup>165</sup>

ECM-coated scaffolds display the advantages of both natural and synthetic components. Polymeric scaffolds provide the strength and robustness to support tissue growth, while the ECM coating acts in bioactive signal providing needed cues for differentiation.<sup>160</sup> Electrospun PCL microfibers

TABLE 3. CHONDROITIN SULFATE-BASED HYBRID SCAFFOLDS FOR CARTILAGE TISSUE ENGINEERING

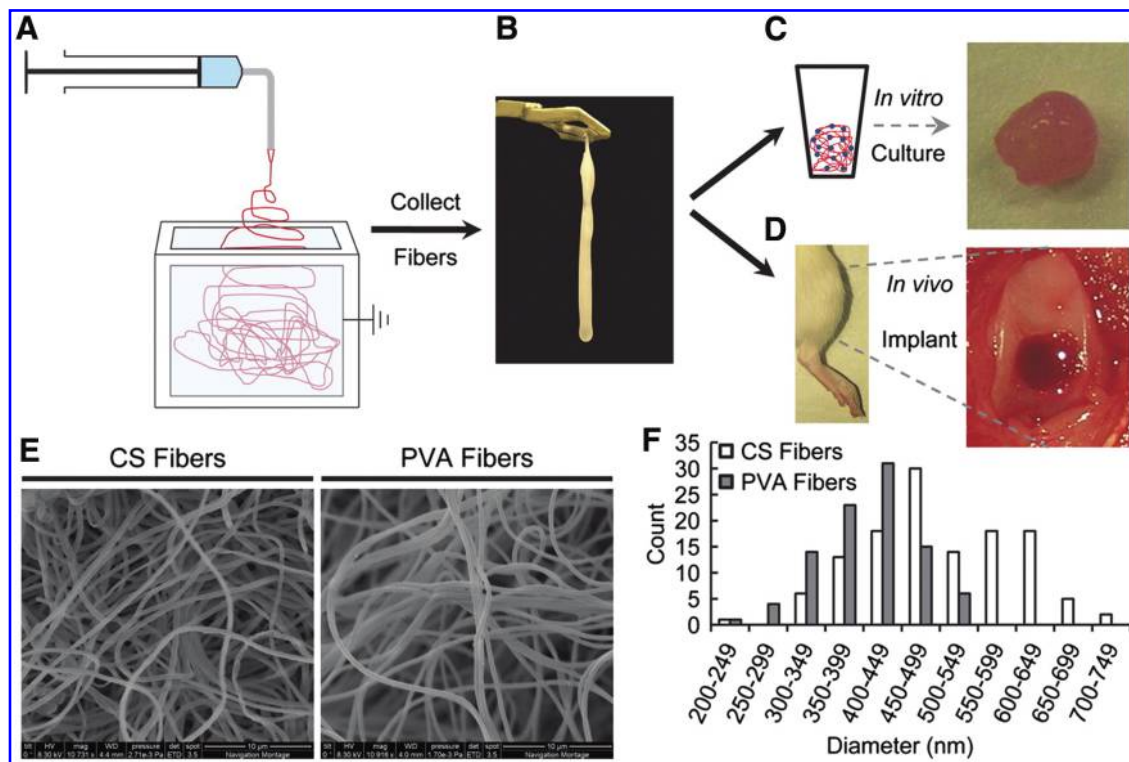
Author	Scaffold composition	Biological assessment	Fabrication process	Advantages	Ref.
Agrawal <i>et al.</i>	SF/CHSN/CS	hMSCs–spinner flask bioreactor	FD	Potential of SF/CHSN/CS scaffolds for hMSC recruitment and directing CT regeneration	130
Bang <i>et al.</i>	CS–A/GEL–TCEP	Fibroblast <i>in vitro</i>	<i>In situ</i> hydrogel	Excellent biocompatibility, mimicking ECM components	131
Li <i>et al.</i>	CS/pullulan	Rabbit articular chondrocytes	Injectable hydrogel	Hydrogel could conserve chondrocyte phenotype and increase chondrogenesis	132
Piai <i>et al.</i>	CS immobilized PCL	Human articular chondrocytes <i>in vitro</i>	Electrospun nanofiber meshes	Effective method for surface functionalization	133
Fan <i>et al.</i>	CHSN/CS	Bovine articular chondrocytes <i>in vitro</i>	Injectable hydrogel	Decreases in swelling ratio and degradation rate	134
Vishwanath <i>et al.</i>	Glucosamine/SF/CHSN	Umbilical cord blood MSCs	FD	Increased cell supportive properties	135
Zhou <i>et al.</i>	SF/CS	Human articular chondrocytes <i>in vitro</i>	Salt-leaching, FD	Anti-inflammatory activities	136
Shahali <i>et al.</i>	Poly-3-hydroxybutyrate scaffolds loaded with glucosamine sulfate	Human chondrocytes <i>in vitro</i>	Electrospinning	Excellent cell viability, cell adhesion, and cell penetration	137
Chen <i>et al.</i>	CMP-TA and CS-TA	Porcine articular chondrocytes, mouse subcutaneous implantation	Enzymatically crosslinked injectable and biodegradable hydrogel	Acceptable tissue compatibility	138
Costantini <i>et al.</i>	GEL-MA/CS amino ethyl MA/HA-MA	MSCs	Hydrogel	Promoted viability and chondrogenic differentiation of MSCs	139
Liao <i>et al.</i>	CS-MA/PECA/GO	CT repair of rabbit hMSCs or chondrocytes evaluated their performance with dynamic compression <i>in vitro</i>	Porous scaffold Hydrogel	Scaffold is applicable in articular CTE	140
Sawatjui <i>et al.</i>	SF/GEL/CS/HA	ADSCs	Hydrogel	The microenvironment provided by the scaffolds and dynamic compression enhanced tissue regeneration	141
Naeimi <i>et al.</i>	SF-CS-S-ALG porous scaffold containing CHSN NPs	Cartilage repair <i>in vivo</i>	Hydrogel	Incorporation of NPs into the scaffold improved compressive modulus	142
Nanda <i>et al.</i>	PVA/CS	Growth of BHK cells	Hydrogel crosslinking with glutaraldehyde	Fill of cartilage defects without inflammation, integration with surrounding tissues	126
Silva <i>et al.</i>	CHSN/CS	hMSCs <i>in vitro</i>	Layer-by-layer technology	Chondrogenic differentiation of hMSCs	143
Chen <i>et al.</i>	CHSN/CS/dermatan sulfate	Chondrocyte	—	Stimulating ECM production and cartilage regeneration	144
Lee <i>et al.</i>	PVA-CS	Growth of BHK cells	Hydrogel crosslinking with glutaraldehyde	Advantages of both PVA and CS	145

BHK, baby hamster kidney fibroblasts; CHSN, chitosan; CMP-TA, carboxymethyl pullulan-tyramine; GO, graphene oxide; MPEG-PCL-AC, poly(ethylene glycol) methyl ether-ε-caprolactone-acryloyl chloride (PECA was used as abbreviation for MPEG-PCL-AC); NPs, nanoparticles; TCEP, tris(carboxyethyl)phosphine.

TABLE 4. HYBRID SCAFFOLDS BASED ON EXTRACELLULAR MATRIX ANALOGS FOR CARTILAGE TISSUE ENGINEERING

Author	Scaffold composition	Biological assessment	Fabrication process	Advantages	Ref.
Kim <i>et al.</i>	MPC/PLGA	NIH/3 T3, KCLB216480, and (RAW 264.7, KCLB40071) cells <i>in vitro</i> , implanted subcutaneously in rats	Solvent-casting/salt-leaching	Cell adhesion and proliferation increased, inflammatory cytokines and cellular ROS reduced, interaction between tissue and scaffolds <i>in vivo</i>	149
Ghosh <i>et al.</i>	DCM encapsulated in PLA microspheres	hMSCs <i>in vitro</i>	Filament	Filaments containing chondroinductive microspheres	150
Jung <i>et al.</i>	CAM-silk bioink	Rabbit MSCs <i>in vitro</i>	3D printing	3D printing of cartilage-shaped scaffolds	151
Ghassemi <i>et al.</i>	Single-wall CNTs/decellularized bovine cartilage	hADSCs	FD	Increased dECM mechanical strength	152
Masaeli <i>et al.</i>	Polyhydroxyalkanoate/dECM	hADMSCs, human primary chondrocytes <i>in vitro</i>	Nanofibrous scaffold	Mimicked the natural motifs of cartilage ECM	153
Rothrauff <i>et al.</i>	MA-ECM	hMSCs	Photocrosslinked hydrogels	Thermoresponsive, photocrosslinkable hydrogels	154
Levingstone <i>et al.</i>	Multilayered biomimetic COL-based scaffolds	Femoral condyle (MC) defects in the caprine stifle joint	FD-EDC/NHS cross-linked	Recruitment of host cells	155
Nogami <i>et al.</i>	ECM-PLGA	Rat MSCs <i>in vitro</i> , implanted into osteochondral defect in rat knees	ECM-coated PLGA	Cell-free scaffolds providing an environment for growth of MSCs and facilitating cartilage repair	156
Beck <i>et al.</i>	MeSDCC gels	Rat MSCs	Photocrosslinked hydrogel	MeSDCC hydrogels may be promising materials for CTE	147
Almeida <i>et al.</i>	Fibrin/ECM	Infrapatellar fat pad-derived stem cells	Injectable hydrogel	Invasive single-stage, cell-based therapies for joint regeneration	157
Sutherland <i>et al.</i>	PLGA surface coated with decellularized cartilage	Rat MSCs <i>in vitro</i>	Microsphere	Bioactive approach to cartilage regeneration with microsphere-based scaffolds	158
Garrigues <i>et al.</i>	PCL/CDM	Human ADSCs	Electrospinning	Role of lower elastic modulus, particularly from CDM, in promoting chondrogenesis	159
Levorson <i>et al.</i>	PCL/ECM	Chondrocytes and MSC coculture	Microfiber scaffolds coated with ECM	PCL/ECM supporting chondrogenic differentiation of MSCs	160
Liao <i>et al.</i>	PCL/ECM	MSCs <i>in vitro</i>	Microfiber scaffolds coated with cartilaginous ECM	Method for fabrication of polymer/ECM composite scaffolds	161
Wang <i>et al.</i>	Demineralized BMG/fibrin	Rabbit chondrocytes <i>in vitro</i>	BMG scaffolds soaked in a chondrocyte–fibrin suspension	Potentially cell carrier vehicle and a structural basis for CTE	162

BMG, bone matrix-GEL; CAM, cartilage acellular matrix; CDM, cartilage-derived matrix; CNTs, carbon nanotubes; DCM, decellularized cartilage matrix; dECM, decellularized ECM; MeSDCC, MA-solubilized decellularized cartilage; MC, Femoral condyle; MPC, microporous porcine cartilage; NIH/3 T3 cells, mouse embryo fibroblasts; KCLB216480; RAW 264.7, KCLB40071, mouse leukemic monocyte macrophage cell line; ROS, reactive oxygen species.



**FIG. 1.** (A, B) Nanofiber composites of PVA-MA and CS-MA were synthesized using electrospinning system in which the nanofibers were collected into an ethanol bath. (C) *In vitro* chondrogenesis of MSCs was performed over 42 days. (D) Nanofibers were implanted into osteochondral defects in the rat hind limbs for 6 weeks. (E) Scanning electron microscopy imaging showed the morphology and (F) size distribution of the fibers.<sup>165</sup> CS, chondroitin sulfate; MA, methacrylamide; MSCs, mesenchymal stromal cells; PVA, poly vinyl alcohol. Reprinted with permission from Coburn *et al.*<sup>165</sup> Color images are available online.

were coated with cartilaginous ECM to fabricate PCL/ECM composite scaffolds. Briefly, chondrocytes were cultured in a flow perfusion bioreactor, and then, the cellular constructs were decellularized. The composite scaffolds in the presence of TGF- $\beta$ 1 exposure showed upregulation of ACAN and COL II gene expression.<sup>161</sup> In a similar approach, coculture of chondrocytes and MSCs on electrospun fibrous scaffolds was performed to produce polymer/ECM hybrid constructs. The results indicated the capacity of cocultures to deposit cartilaginous matrix within a polymeric scaffold. Thus, cocultures of MSCs and chondrocytes can be used to reduce the needed chondrocytes to fabricate polymer/ECM hybrid scaffolds.<sup>160</sup> An innovative technique was performed to fabricate PCL and PCL/cartilage-derived matrix scaffolds by the serial collection of 60 electrospun single-layer scaffolds, which were then seeded with human adipose-derived stem cells (hADSCs). The results indicated that multilayer hybrid constructs improved homogeneous cell seeding and showed chondrogenesis-related bioactivity.<sup>159</sup> Recent studies demonstrated that electrospinning can produce fibrous scaffolds with a high surface/volume ratio, resembling the natural ECM pore structure, thus useful in terms of cell adhesion, proliferation, and differentiation. To improve cell-scaffold interactions, a range of natural and synthetic biomaterials can be blended into the fibrous scaffolds. In view of the fact that the biodegradation rate of scaffolds can influence cell behavior and the following tissue regeneration, a proper biodegradation rate of the hybrid me-

shes can also be produced to match and control the rate of tissue regeneration.

#### Hydrogel scaffolds

Hydrogels are composed of hydrophilic polymer chains with natural or synthetic origin. They offer many advantages, including biodegradability, easy processing, minimally invasive delivery manner, and modulating ability by regulation of crosslink density. For these reasons, hydrogels have been widely used in TE application and especially in CTE.<sup>79</sup> COL is one of the most common ECM derivatives used for culturing cells *in vitro*. COL can be reconstructed to form hydrogel to mimic the connective tissue *in vitro*. Numerous ECM-like composites that combine 3D COL hydrogels with synthetic and natural polymers have been examined.<sup>76</sup> Hybrid gel matrices composed of COL and CHSN had been examined for their capacity to regulate cellular activity. The K562 (a human hematopoietic cell line) cells were cultured in 3D gels to examine cell proliferation and differentiation.<sup>76</sup> In completion of the previous studies, elastic cryogels composed of CHSN-GEL were prepared via crosslinking with glutaraldehyde. Hybrid cryogels exhibited efficient cell adherence, proliferation, and ECM deposition by culturing a fibroblast cell line (Cos-7). The results showed the potential of the hybrid hydrogel for TE applications.<sup>73</sup> To achieve proper biomechanical and biological properties, researchers tried to fabricate scaffolds based on an optimized ratio of polymer solutions (CHSN,

Aga, and GEL) and glutaraldehyde as the crosslinker, via cryogelation technology with incubation at subzero temperature. The fabricated scaffolds showed proper biodegradation, mechanical, and biological properties for effective cell interaction in subsequent tissue development. *In vivo* biocompatibility examination of the scaffolds suggested the potential of these cryogels as a 3D scaffold for CTE.<sup>166</sup> Human chondrocytes were encapsulated in GEL-MA-based hydrogels, and combined with small amounts of photocrosslinkable CS-MA and HA-MA. According to the results, the incorporation of HA-MA to GEL-MA resulted in further round cell morphologies, improved chondrogenesis, and increased synthesized neomatrix throughout the hydrogel. As a result, the compressive modulus of the hydrogel containing HA-MA and CS-MA increased to 114 kPa compared with the control GEL-MA (26 kPa) after 8 weeks of culture.<sup>105</sup>

Entrapment of ECM molecules within a synthetic hydrogel is ultimately the most comprehensive method to create hydrogels containing biological signals. The ultra violet (UV) photopolymerized PVA hydrogels were prepared via methacrylated functionalization, and the effects of incorporation of heparin and GEL within a PVA hydrogel were examined.<sup>167</sup> Chen *et al.* advanced the novel double network PEG/COL, whose mechanical strength could be modified by changing the rigidity, molecular weight, and crosslinking density of the two components. Results indicated that the incorporation of COL significantly enhanced the strength and toughness of hydrogels. The resulting hydrogels could offer a proper environment for cell attachment and proliferation.<sup>38</sup>

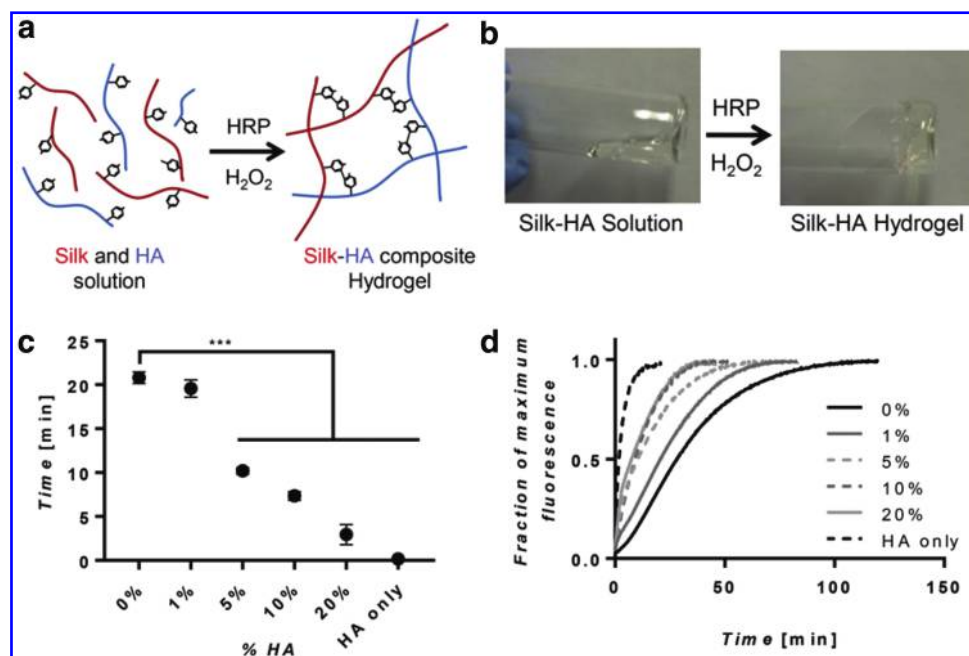
Injectable hydrogels can be presented by minimally invasive procedures and activated by environmental conditions, including pH, temperature, ultrasound, ionic strength, or electric fields, to undertake a shape compliant to the surrounding defect site. Moreover, biological cues such as cells and growth factors could be coinjected with the hydrogels.<sup>80</sup> Injectable synthetic/natural hydrogel composites containing oligo(PEG)-fumarate (OPF), and GEL microparticles (GMP), were synthesized. OPF/GMP TGF- $\beta$ 1-loaded composites, hydrogel encapsulating rabbit MSCs, supported osteochondral tissue generation in rabbit osteochondral defects at 12 weeks.<sup>72</sup> Novel biodegradable, biocompatible, and tough elastomeric hybrid hydrogels based on photocrosslinkable GEL and ionically crosslinkable ALG were engineered. These hydrogels offer an exciting venue to investigate the effect of mechanical stimulation on SC proliferation and differentiation.<sup>46</sup> *In situ* CS/GEL hydrogels were achieved by simple mixing of aqueous solutions of both GEL-tris(carboxyethyl)phosphine and CS-acrylate via click chemistry strategy. *In vitro* studies showed excellent biocompatibility and potential of the hydrogel in various biomedical applications, including TE and drug delivery.<sup>131</sup>

Various ECM derivative molecules, such as CS and HA, have been used as the 3D hydrogels to support SC chondrogenesis. However, because of the lack of proper mechanical properties and matrix stiffness, it is difficult to explain the relative contribution of matrix stiffness on SC fate using ECM derivative hydrogels. Improvement of mechanical properties of these hydrogels can occur by incorporation of polymeric biomaterials.<sup>106</sup> Three major types of cartilage ECM derivatives, HA, CS, and heparan sulfate, were incorporated to fabricate biomimetic hybrid scaffolds. The degree of methacrylation of these ECM molecules was

a key factor to produce ECM-based hydrogel scaffolds. To investigate the effects of mechanical and biochemical cues on chondrogenesis, ADSCs were encapsulated in different hydrogel compositions with various ECM derivative concentrations [0.5%, 1.25%, 2.5%, and 5% (w/v)] and different matrix stiffnesses (3, 30, and 90 kPa). The results indicated that the influence of matrix stiffness on chondrogenesis is dependent on the composition of hydrogels in a nonlinear manner.<sup>106</sup> A chondrogenic hydrogel composed of fibrin/HA-MA seeded with MSCs was further evaluated. The results indicated that the hydrogel could be considered a proper vehicle for MSC delivery and chondrogenesis induction.<sup>103</sup>

Regarding the disadvantage of using crosslinking agents in biological systems, a novel biocompatible hybrid hydrogel was prepared by an oxidized HA/CHSN solution in the absence of a crosslinker. This composite hydrogel supported the survival of encapsulated bovine articular chondrocytes, which retained a chondrocytic morphology.<sup>116</sup> Novel injectable mixtures were synthesized under physiological conditions with PEG vinylsulfone macromers and thiol functionalized HA, which were crosslinked via Michael addition. On mixing with chondrocytes, these hydrogels afforded a homogeneous distribution of cells.<sup>113</sup> Another injectable hydrogel, using blending of thermoresponsive engineered proteins and a dynamic covalent crosslinking system, was fabricated. By mixing aldehyde-modified HA and hydrazine-modified elastin-like protein via dynamic covalent hydrazone bonds, hydrogel formation occurred. This hydrogel represented proper mechanical support to encapsulated human mesenchymal stromal cells (hMSCs) during injection.<sup>91</sup> In a recently published study by Raia *et al.*,<sup>94</sup> HA and SF were enzymatically crosslinked to fabricate biocompatible hydrogels with mechanical strength comparable with the native tissues. The SF proteins crosslinked via horseradish peroxidase (HRP) by formation of di-tyrosine bonds, and tyramine (TA)-substituted HA was synthesized by the same reactions. Consequently, HA was covalently crosslinked with silk to form a composite hydrogel (Fig. 2). The results show that synthesized hydrogel can provide a proper biological system with controllable mechanical properties for TE applications.<sup>94</sup> According to previous works, HA-MA with different degrees of methacrylation were synthesized. The degree of methacrylation modulated matrix stiffness of the hydrogels, therefore affecting the ability of hADSC chondrogenesis.<sup>83</sup>

PVA is one of the most studied polymers in biomedical application, because of its great biocompatibility in combination with a variety of appropriate biomechanical properties, swelling capacity, and crosslinking opportunities. Nanda *et al.*<sup>126</sup> crosslinked the PVA-CS hydrogels with glutaraldehyde and used them as a scaffold in TE.<sup>144</sup> Also, hydrogel scaffolds of PVA/CS were fabricated to imitate the ECM to provide an environment for improved AC cartilage repair *in vivo*.<sup>126</sup> Recently, based on CS, enzymatically crosslinkable, injectable, minimally invasive, and biodegradable hydrogels have been described under physiological conditions. The hydrogels consisted of carboxymethyl pullulan-TA (CMP-TA) and CS-TA conjugates, which were enzymatically crosslinked by horseradish peroxidase and hydrogen peroxide. According to the results, hydrogels were cytocompatible. The CMP-TA/CS-TA composite hydrogels



**FIG. 2.** (a) The silk-HA gel formation by covalent crosslinking of tyramine side chains on HA and tyrosine residues on silk. (b) The images of silk-HA gelation through a vial inversion test. (c) Vial inversion test results to evaluate gelation times. (d) The increasing of HA concentration affected crosslinking kinetics ( $n=7$ ).<sup>94</sup> ( $n=4$ ,  $***p<0.001$ ). Reprinted from Biomaterials, Raia *et al.*,<sup>131</sup> Enzymatically cross-linked silk-hyaluronic acid hydrogels, pp. 58–67, 2017, with permission from Elsevier. HA, hyaluronic acid; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide. Color images are available online.

enhanced cartilaginous ECM synthesis and cell chondrogenesis.<sup>138</sup> Injectable and self-crosslinkable hydrogels were fabricated based on COL I and activated CS with N-hydroxysuccinimide by chemical and physical crosslinking without catalysts. The results suggest that these hydrogels were suitable candidates for applications in the fields of cell delivery and TE.<sup>37</sup>

Synthetic and natural compounds can be used to produce hydrogels taking advantage of their complementary and sometimes synergistic properties. Hydrogels consisting of ECM analogs can be similar to the natural cartilaginous ECM. Through the elastic network, they facilitate transport of nutrients and cellular metabolites, and they can be applied by simple, minimally invasive procedures to fill large and irregular complex defects.<sup>138,147</sup> Similarly, injectable hydrogel based on methacrylated solubilized decellularized porcine cartilage was prepared by methacrylation and UV photocrosslinking modifications. *In vitro* studies showed that these hydrogels induced chondrogenic gene expression and new ECM cartilage formation with mechanical characteristics of native cartilage.<sup>147</sup> In conclusion, hydrogels can be prepared from both synthetic and natural biomaterials. Synthetic biomaterials have the ability to control the chemical composition, mechanical properties, and biodegradation rate of the composite hydrogels. In contrast, hydrogels composed of natural biomaterials have the ability of providing biological cues and signals to enhance cell adhesion, proliferation, and differentiation. However, hybrid hydrogels composed of both synthetic/natural biomaterials can be an effective tool to achieve optimal cartilage regeneration.

### 3D printing and biofabrication

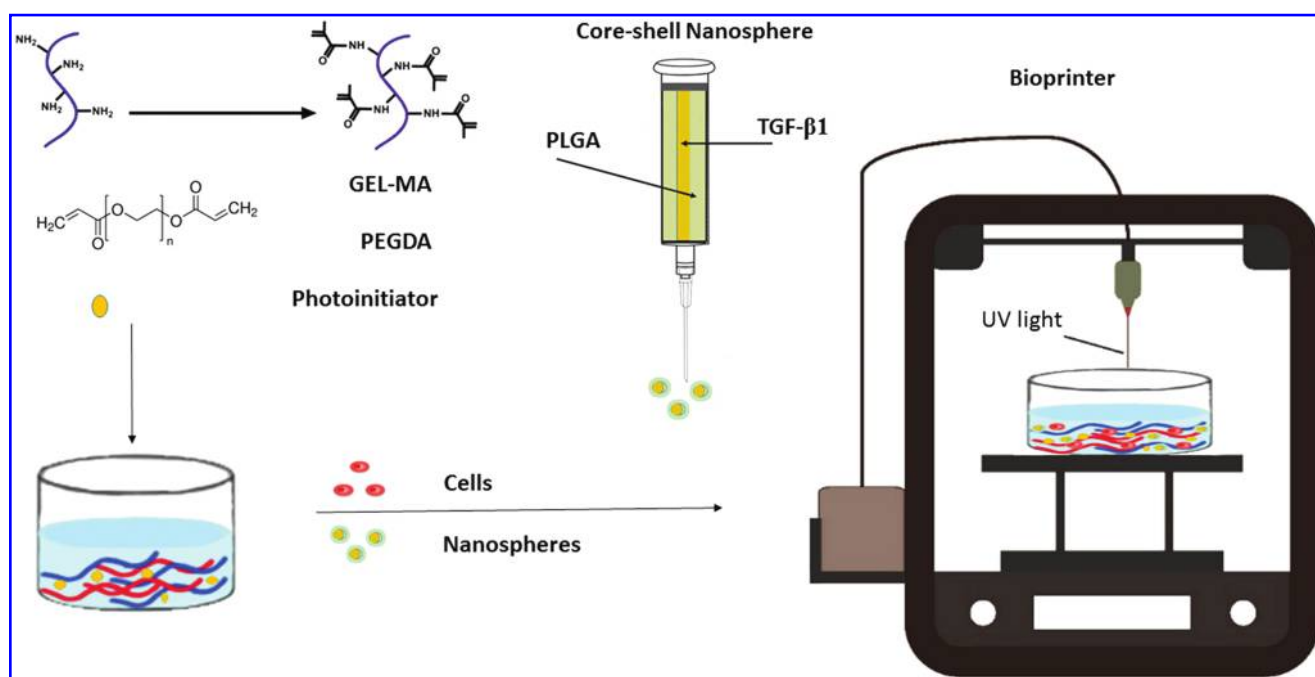
Additive manufacturing tools are based on the development of computer science and manufacturing technologies. The potential to fabricate highly complex constructs such as whole organs directly from a computer model is one

of the main advantages of these approaches.<sup>168</sup> Additive manufacturing offers a different potential to understand the structural constraints for TE scaffolds. On the contrary, it is also critical to develop the methods allowing efficient scaffold cellularization independent of shape and porosity.<sup>169</sup> Frequently due to the absence of biological cues and hydrophobicity of the synthetic biomaterials used in scaffold fabrication by additive manufacturing, these scaffolds generally offer a limited environment for cell attachment and growth. Conversely, in those biofabrication techniques using living cells and biological materials, tissues are directly produced with ECM derivatives by controlling their 3D structures. However, inferior mechanical properties of such biofabricated constructs are considered a main limitation of these approaches.<sup>170</sup> Recently, to overcome these restrictions, multihead deposition systems with the ability of bio-printing different row constituents consisting of synthetic and natural biomaterials, proteins, and cells have been developed.<sup>171</sup> Xu *et al.*<sup>62</sup> tried to use inkjet-based cell printing in conjunction with electrospinning to fabricate constructs with improved mechanical properties. After spinning of a PCL layer, a rabbit chondrocyte/fibrinogen/COL solution was deposited onto the electrospun PCL fibrous layer. After gelation of the cell-printed solution, PCL was spun another time and followed by inkjet cell printing again. A final construct consisting of five layers of 1 mm thickness was fabricated. Cells showed >80% viability a week after culture.<sup>62</sup> Before using GEL-MA as a biomaterial for biofabrication purposes, several crosslinking parameters consisting of UV exposure time, polymer concentration, and thermal gelation before UV exposure were investigated to control the swelling and mechanical properties of the hydrogel. The opportunity to control mechanical properties, swelling behavior, and high cell compatibility and the ability to synthesize cartilaginous matrix make GEL-MA a suitable material for CTE. Results showed that when GEL-MA is combined with HA as a viscosity-enhancing additive, it could be printed into layered hydrogel structures. The results

confirmed that the engineered constructs allow matching the natural functional variations in cartilage biomechanical properties.<sup>107</sup> In a more recent approach, Visser *et al.*<sup>172</sup> reinforced soft GEL-MA hydrogels with high porosity and a highly organized 3D-printed PCL network that was fabricated via the MEW technique. The mechanical properties of the gel/scaffold composites improved compared with microfiber scaffolds or hydrogels alone. Chondrocytes embedded in the hybrid construct were viable, and retained their round morphology and physiological behavior *in vitro*.<sup>172</sup> A novel hybrid scaffold based on PLA/photopolymerizable cell-laden hydrogels has been established. A hydrogel precursor was prepared from a solution of MA-modified GEL and the photoinitiator Li-TPO-L in cell culture medium. The fabricated TE constructs merged both advantages, synthetic additive manufactured constructs and a natural hydrogel matrix.<sup>169</sup> Recently, a bioink consisting of GEL-MA, CS-amino ethyl-MA, and HA-MA was loaded with MSCs to fabricate 3D biomimetic hydrogel scaffolds for CTE. Two coaxial needles were used to establish a proper system to bioprint hybrid constructs with high cell viability, high cell density, and high bioprinting resolution. The results confirmed that this method is a valuable candidate for advanced CTE.<sup>139</sup> By combining SF and GEL with MSCs and a specific-affinity peptide E7, a functionally and structurally improved scaffold was designed via an indirect 3D printing method. Briefly, The SF-GEL mixture solution was dispensed to a 3D computer-aided design mold. After decreasing the temperature to form the gel, the mold was dissolved, and the scaffold then crosslinked with genipin. The scaffold showed efficient recruiting capacity for MSCs and provided a mechanical support and proper microenvironment for proliferation, differentiation, and neocartilage tissue production.<sup>49</sup> Fabrication of gradient structures has been made by using the

layer-by-layer inherent fabrication of additive manufacturing technologies. Gradient functionalization with controlled geometry and porosity on the surface of an additive manufactured PCL scaffold was investigated. First, the surface of PCL scaffold was aminolysed using a continuous gradient of amine concentration; second, by using EDC reaction, a COL gradient was formed via protein grafting. The results showed that for the construction of 3D scaffolds with chemical gradients and controlled structural properties, a combination of surface modification and additive manufacturing is an appealing strategy.<sup>45</sup> A tabletop stereolithography-based bioprinter has been used for a new cell-laden CT construct fabrication. The bioink was composed of GEL-MA, various concentrations of PEG diacrylate, 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone as a photoinitiator, and TGF- $\beta$ 1-embedded nanospheres (Fig. 3). Cell growth, viability, and chondrogenesis were explored to develop an optimized 3D-bioprinted construct for CTE. A significant increase in chondrocyte-specific gene expression on printed constructs containing TGF- $\beta$ 1 nanospheres over 3 weeks showed that cell-laden bioprinting is a promising strategy for CTE.<sup>32</sup> A bioprintable natural bioink based on cartilage acellular matrix (CAM) for bioprinting of irregular shape tissues has been developed. As a support of the CAM powder, SF was used because of its physical crosslinking ability and controllable viscosity. Bioprinting of a cartilage-shaped scaffold using this CAM-SF bioink has been done successfully.<sup>151</sup> The results of *in vitro* culture showed that a printed CAM-SF construct provided better cell morphology and neomatrix synthesis from rabbit BMSCs compared with a printed PCL construct.

According to recently published articles, bioprinting approaches have shown great potential in CTE applications. However, several limitations are still remaining in achieving



**FIG. 3.** Schematic design of cartilage construct printing via stereolithography-based bioprinter and synthesized bioink.<sup>32</sup> GEL-MA, gelatin-methacrylamide; PEGDA, poly ethylene glycol diacrylate; PLGA, poly(DL-lactic-co-glycolic acid); TGF- $\beta$ 1, transforming growth factor-beta 1; UV, ultra violet. Color images are available online.



clinical applicability of these methods. The most challenging limitations are technical improvement and standardization, bioink formulation, and more closely mimicking the native cartilage mechanical properties.<sup>151</sup> However, when different synthetic and natural biomaterials are associated within bioinks, they appear to mimic the cartilage microenvironment and to enhance cell viability and chondrogenic ability.

### Freeze-drying

In FD, the material is frozen in a hydrogel shape and the water forms pockets of ice throughout the matrix. Consequently, these ice pockets are sublimated and removed under vacuum to produce the porous network. The size of pores can be controlled by the freezing temperature, thus producing porous structures with varying pore sizes and interconnectivities, due to varying heat transfer coefficients. Interconnected network composite scaffolds based on incorporating type II COL with CS and HA were fabricated utilizing chemical crosslinking and FD procedures, to mimic the native ECM of AC and upregulate cartilage ECM biosynthetic activities.<sup>74</sup> The 3D porous hybrid scaffolds were prepared with incorporation of PLGA microspheres into GEL/CHSN/HA scaffolds and crosslinking with EDC, using the simple FD method. Cell culture confirmed that chondrocytes could secrete ECM and proliferate similar to the control (GEL/CHSN/HA scaffolds).<sup>117</sup> The FD method was also used to prepare interconnected PVA/GEL/nanohydroxyapatite/polyamide-6 bilayered hybrid scaffolds for *in situ* osteochondral defect repair.<sup>68</sup> In another study, COL/PLA, CHSN/PLA, and COL/CHSN/PLA hybrid scaffolds were fabricated via the combination of FD of the natural biomaterials COL and CHSN and PLA meshes. The 3D PLA meshes provided mechanical properties and the natural biomaterials mimicked the natural niche of chondrocytes.<sup>59</sup> GEL scaffolds with interconnected pore structures and with good mechanical strength were fabricated using ice particulates and FD. Bovine articular chondrocytes were cultured on these scaffolds, for CT formation *in vitro*.<sup>173</sup> The 3D porous scaffold-based cellulose nanofibers, stabilized using a genipin crosslinked matrix of Gel and CHSN, were prepared using FD. The results showed that the scaffolds have interconnected and homogenous pores, which supported chondrogenesis, thus highlighting the impact and efficiency of FD in the fabrication of hybrid scaffolds for CTE application of CHSN.<sup>53</sup> Open and interconnected pore structures are considered the most important characteristic of the TE scaffolds.<sup>35</sup> Uniform multidirectional COL-based scaffolds were fabricated by unidirectional freeze casting of COL/HA and COL/hydroxyapatite suspensions. The scaffolds were joined by a lyophilization bonding process. With the arrangement of these compositional and architectural biomimetic cues, the scaffolds hold great capacity for zonal CTE.<sup>43</sup> A novel class of hybrid scaffolds based on CHSN as a structural material and low portion of HA to mimic cartilage ECM were fabricated using FD. The results showed that incorporation of HA to the CHSN scaffolds enhanced the biological properties of the scaffolds, which had a superior porous structure network and exhibited higher cartilage ECM deposition.<sup>109</sup> To fabricate porous hybrid constructs, CS was blended with SF to fabricate SF/CS scaffolds via FD. The scaffolds showed a pore size of 37–

212  $\mu\text{m}$ , contact angle 46.2–50.38, biodegradation, and controlled swelling. Biocompatibility was confirmed by implantation of scaffolds subcutaneously in a mouse. The results indicated that incorporation of CS to the scaffolds promoted proliferation, cell attachment, and metabolic activity of hMSCs *in vitro*.<sup>130</sup> Despite the great advantage of using the FD technique in fabrication of hybrid constructs in CTE, a small and nonhomogeneous pore size can be considered the main limitation of this method for several materials.

### Solvent casting and particulate leaching techniques

In particulate leaching (PL) techniques, pore size and porosity of the scaffolds can be controlled by a selection of appropriate porogen materials. Using this method, numerous porous structures have been fabricated from varied synthetic and natural biomaterials for TE applications.<sup>79</sup> Electrospinning combined with salt leaching (SL) has also been used to fabricate macroporous and nanofibrous HA scaffolds. HA and COL were electrospun into a nanofiber mesh by the deposition of salt particles during electrospinning and following crosslinking and SL. Cytocompatibility of the scaffold was evaluated by culturing bovine chondrocytes on the HA/COL scaffolds. The results demonstrated that cell proliferation was improved and COL content enhanced.<sup>122</sup> Gas foaming/SL was used for the fabrication of PLGA/HA hybrid scaffolds. The PLGA scaffolds were fabricated by blending PLGA with varying amounts of amine-terminated PLGA-PEG diblock copolymers. Gene expression results, biochemical assays, and histological analysis showed that HA-modified scaffolds supported higher cartilage ECM formation, COL II gene expression, and morphological characteristics.<sup>174</sup> Porous scaffolds based on poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) tubes were fabricated using a dipping method followed by SL. Culture of hMSCs on hybrid scaffolds showed early chondrogenic differentiation by expression of SOX9 in the presence of a proper induction medium.<sup>60</sup> Nanocomposite scaffolds composed of PLGA/HA/fibrin/bioglass were prepared using solvent casting and PL (SC-PL) techniques. The scaffolds showed proper porosity percentage ( $87.01 \pm 3\%$ ) with interconnected pore morphologies and pore size between 100 and 200  $\mu\text{m}$ . The nanocomposite scaffold was cytocompatible, and the human adipose tissue-derived mesenchymal stem cells attached and proliferated on the scaffold. These scaffolds can be used for CTE applications.<sup>102</sup> To overcome the possible disadvantages of PLGA, such as its hydrophobicity, limited support of nutrient exchange, and induction of inflammatory responses, micronized porcine cartilage (MPC) was added to the scaffolds to reduce the inflammatory effects and improve cell attachment and proliferation. PLGA and MPC/PLGA scaffolds were fabricated by the SC-PL technique. The results showed that incorporation of bioactive materials of MPC had constructive effects for enhancing PLGA scaffold biocompatibility.<sup>149</sup>

### Future Outlook

About 132 articles published between 2000 and 2018, describing ECM-based hybrid and composite scaffolds used in CTE, were reviewed in the present study (Tables 1–4). A statistical analysis demonstrates which materials and methods are the most frequently used for ECM-based

hybrid scaffold fabrication. To evaluate frequency of the different materials and methods, the number of published articles for each material or method was divided by total number of published articles during these years [frequency percentage = (number of published articles for each material or methods/total number of published articles)  $\times 100$ ]. The results indicated that between cartilage ECM derivatives, the most frequently used materials were COL (40%), HA (34%), CS (14%), and cECMa (12%). According to the results, the most used synthetic materials to design hybrid and composite scaffolds in CTE are PLGA (18%), MA (16%), PCL (15%), PEG (9%), PVA (9%), and PLA (8%), and other polymeric materials consisting of poly-N-(vinylcaprolactam), other polyesters, polyurethanes, and PGA (25% totally). Among natural biomaterials, CHSN (41%), SF (21%), ALG (14%), fibrin (11%), and AGR (5%), respectively, were the most frequently used in CTE. Finally, among scaffolding methods for fabrication of hybrid scaffolds at CTE applications, hydrogels (38%), fibrous scaffolds (22%), FD (15%), 3D printing (10%), SC-PL (6%), and other methods consisting of the microsphere, mesh-microsponge, fiber-hydrogel (9% totally) were more familiar, respectively. COL and HA have been mostly used in the fabrication of hybrid scaffolds in comparison with other ECM derivatives. These two polymers have been thoroughly investigated because of their biocompatibility, biomimetic properties, and abundance. As shown in the beginning, synthetic polymers, PLGA, PCL, and MA, were more frequently used hybrid scaffolds than PVA, PLA, and PEG polymers. PLGA and PCL are biodegradable, biocompatible, and their rate of biodegradation can be controlled by the degree of hybridization with other synthetic and natural biomaterials. Results demonstrated that CHSN has been used the most frequently as a natural biomaterial in the fabrication of hybrid scaffolds. CHSN biostability is due to its large number of reactive amino groups that play a useful role as sites for specific crosslinking. CHSN is miscible at the molecular level and it exhibits hydrogen bonding or electrostatic interactions that contribute to mechanical stability. Furthermore, adding CHSN to ECM derivatives increases the number of crosslinking sites. When crosslinked, these hybrid scaffolds prevent access to hydrolytic enzymes to the sensitive cleavage sites of ECM derivatives. Biostability on the one hand and degradation rates on the other hand can be controlled by the extent and type of crosslinking. Finally, among the fabrication methods, hydrogels and fibrous approaches were the most common in CTE application.

As the results show, collagens and HA were used most frequently in the fabrication of hybrid scaffolds. However, according to articles published in recent years, the use of ECM analogs in the preparation of hybrid scaffolds for CTE applications has been expanding due to the potential to induce SC differentiation.

Among natural biomaterials, CHSN has been widely used due to its miscibility with ECM derivatives, and the presence of functional groups for crosslinking with ECM derivatives, all important features for cartilage tissue repair. With regard to fabrication, hydrogels and fibers are used most frequently in hybrid scaffold fabrication due to their similarity to natural cartilage structure. There is also a growing trend with the use of injectable hydrogels and biofabrication methods for CTE.

## Conclusions

In hybrid scaffold fabrication, ECM-derived biomolecules are combined with natural or synthetic biomaterials. Because of their specific biophysical and biochemical properties, hybrid scaffolds provide superior interactions with cells and better control of cell adhesion, spreading, proliferation, and differentiation. ECM-derived hybrid scaffolds are promising materials for cartilage and other TE needs. Hybrid scaffolds consisting of cell-derived ECM and synthetic materials have superior mechanical properties compared with acellular tissues and pure ECM scaffolds. Based on the reviewed publications, it is possible to conclude that COL and HA are the most frequently used in hybrid scaffolds for CTE, and that from the perspective of fabrication or process or material format, fibrous and hydrogel scaffolds are the most popular.

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