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Functionalized Hydrogels for Articular Cartilage Tissue Engineering

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ABSTRACT

Articular cartilage (AC) is an avascular and flexible connective tissue located on the bone surface in the diarthrodial joints. AC defects are common in the knees of young and physically active individuals. Because of the lack of suitable tissue-engineered artificial matrices, current therapies for AC defects, especially full-thickness AC defects and osteochondral interfaces, fail to replace or regenerate damaged cartilage adequately. With rapid research and development advancements in AC tissue engineering (ACTE), functionalized hydrogels have emerged as promising cartilage matrix substitutes because of their favorable biomechanical properties, water content, swelling ability, cytocompatibility, biodegradability, and lubricating behaviors. They can be rationally designed and conveniently tuned to simulate the extracellular matrix of cartilage. This article briefly introduces the composition, structure, and function of AC and its defects, followed by a comprehensive review of the exquisite (bio)design and (bio)fabrication of functionalized hydrogels for AC repair. Finally, we summarize the challenges encountered in functionalized hydrogel-based strategies for ACTE both *in vivo* and *in vitro* and the future directions for clinical translation.

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1. Introduction

Articular cartilage (AC) is a materially nonlinear, heterogeneous, anisotropic, poro-viscoelastic, and highly specialized connective tissue physiologically without lymphatic channels, blood vessels, and innervations [1]. AC can serve to reduce the friction and bear the load in synovial joints, allowing for translation and rotation, which are crucial for body movements [1]. It consists mainly of an extracellular matrix (ECM) and a few chondrocytes (less than 10% of the volume in humans) in a hierarchical structure. Collagen fibers impart high strength and elasticity, and chondrocytes contribute to producing, secreting, organizing, and maintaining organic components in the ECM [2,3]. AC has four anatomically and functionally distinct zones. These four zones collectively endow the AC with several highly specialized functions. Without timely and proper therapies, severe AC damage will ultimately progress toward joint dysfunction and disability, due to its limited intrinsic healing. To date, the true global incidence of AC lesions remains inconclusive. Approximately 900 000 US citizens annually suffer from knee AC damage, and more than 200 000 of these require surgical interventions [4,5]. Nowadays, clinical therapies for AC injuries, including non-surgical and surgical approaches (microfracture, osteochondral autografts and allografts, autologous chondrocyte implantation (ACI), and matrix-assisted ACI (MACI)),

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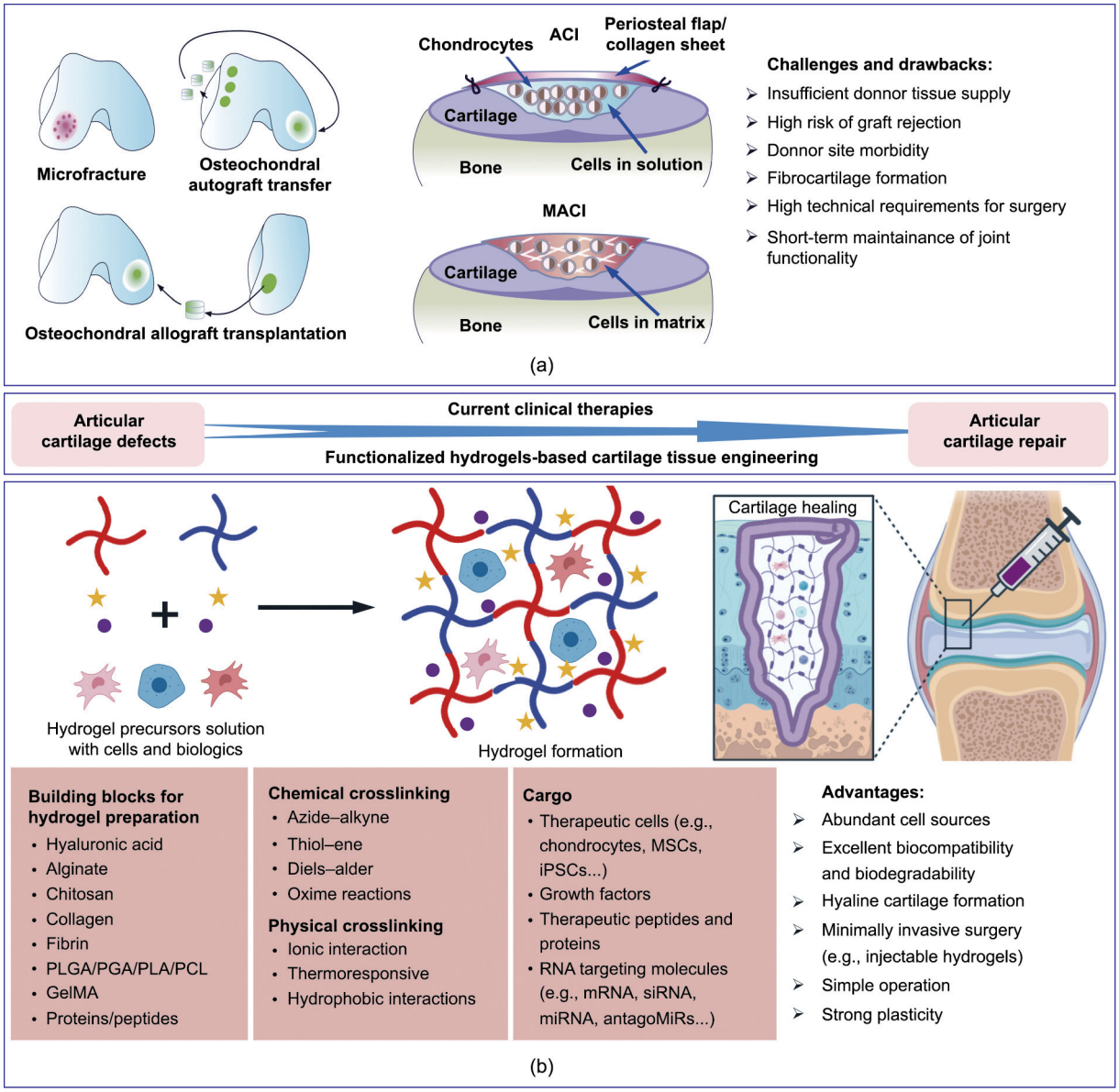


Fig. 1. Schematic diagram of conventional commonly-used surgical therapies and functionalized hydrogels-based tissue-engineered strategies for AC repair, and their advantages and disadvantages. (a) Graphical illustration of conventional surgical therapies for AC repair, including microfracture, osteochondral autografts and allografts, ACI, and MACI, and their challenges and drawbacks. (b) Emerging functionalized hydrogel-based strategies for ACTE and their advantages. PLA: polylactide; PLGA: poly(lactide-*co*-glycolide); PGA: polyglycolide; PCL: polycaprolactone; GelMA: gelatin methacryloyl; MSC: mesenchymal stem cell; iPSC: induced pluripotent stem cell; mRNA: messenger RNA; siRNA: small interfering RNA; miRNA: microRNA; antagoMiRs: a class of antisense oligonucleotides function as anti-miRNAs. (a) Reproduced from Ref. [1] with permission of John Wiley and Sons, © 2020; (b) partially created from BioRender.

continue to encounter formidable challenges with inconsistent long-term results (Fig. 1(a)). Their indications, strengths, and weaknesses have been previously summarized [6,7]. Existing limitations have vastly upgraded ACTE to a higher level by integrating material science, engineering, and biomedical science to generate neocartilage for restoring and repairing damaged AC. At present, numerous tissue-engineered strategies have emerged for (bio)fabricating cartilage constructs with desirable mechanical and biochemical capabilities [1]. Hydrogels have become promising materials among these strategies because of their extensive characteristics and capability to entrap cells [8]. The (bio)design and (bio)fabrication of functionalized hydrogels have recently achieved substantial improvements, reinforcing their validity for AC repair and regeneration (Fig. 1(b)). These advancements include, but are not limited to, the progression of hydrogel structural design (e.g., additional components, surface ultrastructure, inner structure and three-dimensional (3D) architecture, mechanical properties, structural flexibility, degradation profile, and zonal organization), novel fabrication techniques (e.g., four-dimensional (4D) (bio)printing), and cargos (e.g., therapeutic cell loading and spatiotemporally controlled presentation and release of bioactive factors and RNA-targeting molecules). Although AC is predicted to be successfully engineered as one of the first batches of tissues, tremendous challenges remain, and few research directions have advanced to the clinical trial stage. One of the key reasons for this is the biomechanical mismatch between adjacent native cartilage

and implanted engineered constructs. Biochemically coherent neocartilage can be easily fabricated *in vitro*, but its immature structure (e.g., isotropic) results in inadequate biomechanics compared with native mature AC [9,10]. As a member of AO Research Institute Davos Collaborative Research Program of Osteochondral Defect, which has been committed to AC repair for many years, our specialty and research emphasis involve both *in vivo* and *ex vivo* AC regeneration using different biomaterial-based strategies, including various functionalized hydrogels. This review summarizes the current "state-of-the-art" approaches to engineer innovative functionalized hydrogels for AC repair, with endeavors for successful clinical applications.

2. AC in the human body

AC is hyaline-like and located in synovial joints, including the shoulder, elbow, hip, and knee (Fig. 2(a)). Owing to its unique composition and structure, repairing or restoring the osteochondral interface in full-thickness AC defects is challenging [7]. AC injuries can result in severe musculoskeletal morbidities. Therefore, timely and proper diagnosis and treatment of AC are paramount for protecting joint health. Healthy AC relies heavily on maintaining stratified biological, structural, and biomechanical properties.

2.1. Composition, structure, and function of AC

The main function of AC is to provide a lubricated and smooth surface to articulate and facilitate load transmission with low friction (Fig. 2(b)). AC mainly consists of the ECM and chondrocytes. Apart from water (65%–80% of the total weight), the ECM primarily consists of collagen, proteoglycans, and a low proportion of other glycoproteins and non-collagenous proteins [11]. This composition is conducive to retaining water inside the ECM, which is crucial for sustaining its special biomechanical features. Within the ECM, rod-like tropocollagen molecular structures (1.4 mm in diameter and 300 nm in length) polymerize to form collagen fibers with diameters 25 to 40 nm.

AC has four distinct zones (Fig. 2(c)). The superficial zone (SZ) is the thinnest layer (approximately 10%–20%) in the AC. Within the SZ, three important macromolecules—lubricin, aggrecan, and hyaluronic acid (HA)—are involved in AC lubrication. Maintaining very low friction of the AC surfaces is attributed to the boundary lubrication and fluid film (Fig. 2(d)). Additionally, the SZ contains flattened ellipsoid chondrocytes and collagen fibrils (primarily type II and IX collagens) that pack tightly and align parallel to the AC surface, reinforcing its tensile strength and resistance to shear and compressive forces. Therefore, disruption of the SZ can lead to biological and biomechanical changes in the AC, indicating the initial degenerative pathologies during osteoarthritis (OA)

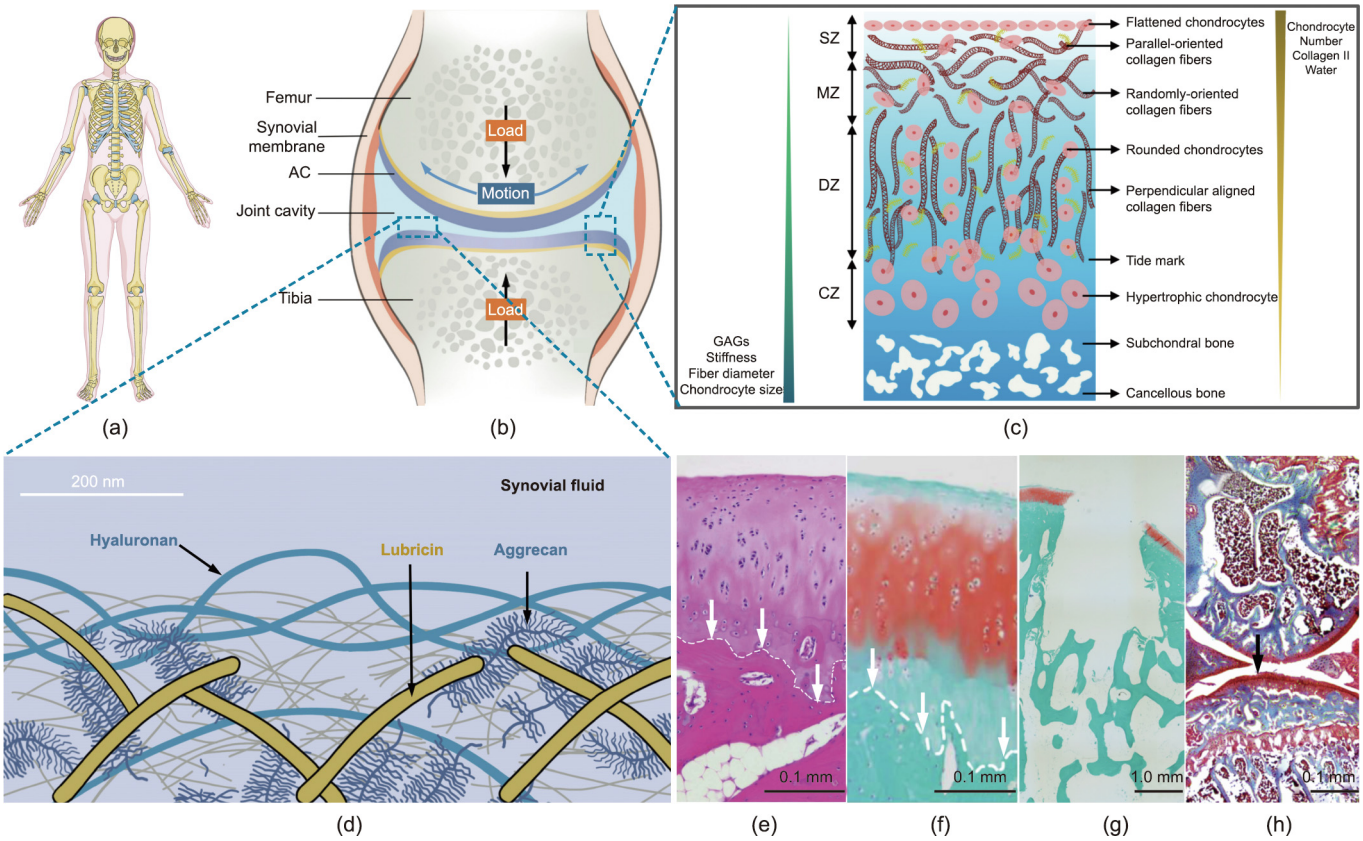


Fig. 2. The physiology of AC in knee joints. (a) AC is located in synovial joints, including the shoulders, elbows, hips, and knees. (b) Brief diagram of a human knee joint, including femur, synovial membrane, AC, joint cavity, synovial fluid, and tibia. AC covers the ends of the bones in the knee joint and plays a vital role in transmitting loads. (c) AC is organized into four main zones: the superficial zone (SZ), the middle zone (MZ), the deep zone (DZ), and the calcified zone (CZ). The number, size, and shape of chondrocytes, collagen II, and glycosaminoglycans (GAGs); water contents within AC; and mechanical properties of AC display a depth-dependent manner. (d) The structural diagram of the most superficial layer in AC. Three dominant macromolecules, including lubricin (yellow), bottle-brush-like aggrecan (dark blue), as well as linear hyaluronic acid (HA, blue) involved in AC lubrication. (e) Hematoxylin–eosin (H&E) staining and (f) safranin O/fast green staining of the AC on the formal condyle from a 16-week-old New Zealand rabbit. The white dash line demonstrates the tidemark. (g) Safranin O/fast green staining of an osteochondral defect (OCD) in the formal condyle of a 16-week-old rabbit. (h) Safranin O/fast green staining of the late stage of knee OA progression in a Sprague-Dawley rat. The black arrow indicates the degenerated AC. (c) Reproduced from Ref. [1] with permission of John Wiley and Sons, © 2020.

progression. Underneath the SZ is the middle zone (MZ), which serves as a functional and anatomical border between the deep zone (DZ) and the SZ. In general, MZ accounts for 40%–60% of AC and consists of large diameter collagen fibrils with oblique organizations, a high concentration of proteoglycans, and low-density and spherical chondrocytes embedded in abundant ECM. Functionally, the MZ provides a transition between the compressive forces of the DZ and the shearing forces in the SZ. The DZ accounts for approximately 30% of the total AC. Within the DZ, the collagen fibrils deposit radially with the largest diameters, and the proteoglycan content is the highest, but the water concentration and chondrocyte density are the lowest. The chondrocytes are spheroid-shaped, and the collagen fibers are aligned perpendicular to the AC surface. The functions of the DZ are facilitating load distribution, and empowering the greatest compression resistance. The tidemarks (Figs. 2(e) and (f)) represent a boundary between the DZ and the calcified zone (CZ), identifying the transition to a less resilient subchondral bone (SB). Through anchoring to the collagen fibrils of the DZ and the hydroxyapatite crystals of the SB, CZ plays an integral role in securing the cartilage to the bone. The volume percentage of CZ varies from 3.23%–8.80% [12]. In the calcified matrix, scarce hypertrophic chondrocytes demonstrate a very low level of metabolism. The CZ acts as a barrier to prevent blood vessel invasion from the SB.

2.2. The biomechanical properties of AC

The prominent roles of human AC involve avoiding abrasive wear between the bone extremities, safeguarding the SB from overloading, as well as bringing low-friction joint surfaces, which are dependent on the biomechanical properties of the AC. Moreover, the biomechanical properties of AC are highly attributed to its unique and complicated structure and its ECM composition including a fluid phase and a solid matrix. During mechanical loading, interstitial fluid is redistributed through the pores of the permeable matrix, leading to predominantly poro-elastic conduction. AC displays properties such as aggregate modulus (0.1–2.0 MPa), compressive strength (14–59 MPa), stiffness ($\geq$1 MPa), tensile resistance (15–35 MPa), tensile elongation at break (80%), and Poisson's ratio (0.06–0.30) [13,14].

2.3. AC defects

AC injuries contain a broad scope of damage, ranging from partial AC defects to osteochondral defect (OCD) (Fig. 2(g)) and end-stage degenerative OA (Fig. 2(h)), which is largely aggravated and perpetuated by inflammatory flares. AC defects usually derive from traumatic destruction or degenerative joint diseases and most commonly occur in the knee joint. Patients with AC defects experience inflammation, stiffness, and restricted mobility. A comprehensive grading system has been formulated by the International Cartilage Regeneration & Joint Preservation Society (ICRS) for the evaluation of focal cartilage lesions [5]. Among them, the two major categories are partial-thickness and full-thickness AC defects. The difference between them is whether the damage involves the underlying SB. The former defects only cause injuries in zonal AC and result in insufficient self-healing responses. However, the latter have injuries that penetrate both the AC and SB. Therefore, defect sites have full access to blood cells, macrophages, and mesenchymal progenitor cells, bringing to the spontaneous immune responses coupled with healing processes. Unfortunately, a common result at the defect site is fibrocartilages with undesirable biomechanics and permeability, triggering a transient spontaneous repair process and subsequent AC degeneration. Although sporadic chondrogenesis may still occur, perfect resurfacing is rare, resulting in bone-to-bone articulation accompanied by significant pain, inflammation, and disability.

3. Tissue-engineered cartilage for AC regeneration

At present, numerous AC repair and regeneration techniques have been extensively used, and some have achieved desirable clinical results to some degree, according to properly chosen indications [15,16]. Nevertheless, every prevailing approach possesses its pros and cons, and none, individually or in combination, can provide most patients with reliable long-term efficacy. Therefore, the research and development (R&D) of innovative technologies and further optimization of existing therapeutic strategies are of paramount importance. As an emerging interdisciplinary area, tissue engineering (TE) fully exploits the basic theories and methods of various subjects (e.g., material science, engineering, chemistry, and biomedical science), aiming to (bio)fabricate biomimetic substitutes for restoring, maintaining, or improving the functionality of impaired tissues [17]. TE involves using various cell sources, scaffolds, and bioactive factors, leading to an exponential increase in the number of possible combinations [17] (Fig. 3(a)). Besides, advances in (bio)printing, mechanobiology, induced pluripotent stem cells, decellularized organs, immunomodulation, biorthogonal chemistry, and gene-editing technology have tremendously promoted the development of TE (Fig. 3(b)). The ever-increasing incidence of challenging aging-related musculoskeletal disorders (e.g., knee OA) and advances in technologies for AC repair are critical drivers for nurturing the prosperity of the global market for ACTE [1,18]. The market was estimated at 4.2 billion USD in 2016, and it is now on track to achieve a compound annual growth rate of 5.4% during 2016–2025, despite the coronavirus disease 2019 (COVID-19) pandemic. To date, several tissue-engineered cartilage products have obtained clinical approval worldwide; however, US-approved therapies based on TE are still ACI and MACI [19]. Meanwhile, hundreds more are presently in different stages of clinical trials[†].

4. Functionalized hydrogels as instructive scaffolds for ACTE

Because AC is a complicated tissue with abundant water content and favorable viscoelastic characteristics, hydrogels are considered ideal biomaterial matrices for AC repair. Over the past decade, researchers have published over 2000 scientific papers on the topic of "cartilage and hydrogel" (Fig. 3(c)) to markedly foster the development of ACTE. Hydrogels are crosslinked polymeric or nonpolymeric networks containing enough water to enable these optimal biomaterials to engineer tissues rich in moisture content, for example, AC [21]. At the molecular level, the molecular weight, mesh size, and polymer chain between the crosslinks are the three most prominent parameters to define the structures of the hydrogels (Fig. 4(a)) [22]. Hydrogels are generally classified into different categories based on different aspects, such as material sources, pore size, interchain crosslinking methods, and electrical charge [23] (Fig. 4(b)). Several natural and synthetic polymers (Fig. 4(c)), inorganic substances, and composite biomaterials are typically used to form hydrogels for cartilage repair via many different approaches (Fig. 4(d)), including physical crosslinking, chemical crosslinking, self-assembly, enzymatic crosslinking, as well as photo-crosslinking [21]. Among the polymers, owing to their excellent tunable property profiles regarding degradability, biomechanics, controllability, reproducibility, and molecular weight, synthetic polymer-derived hydrogels can be self-reinforced to improve their mechanical performance for bearing

[†] https://www.clinicaltrails.gov.

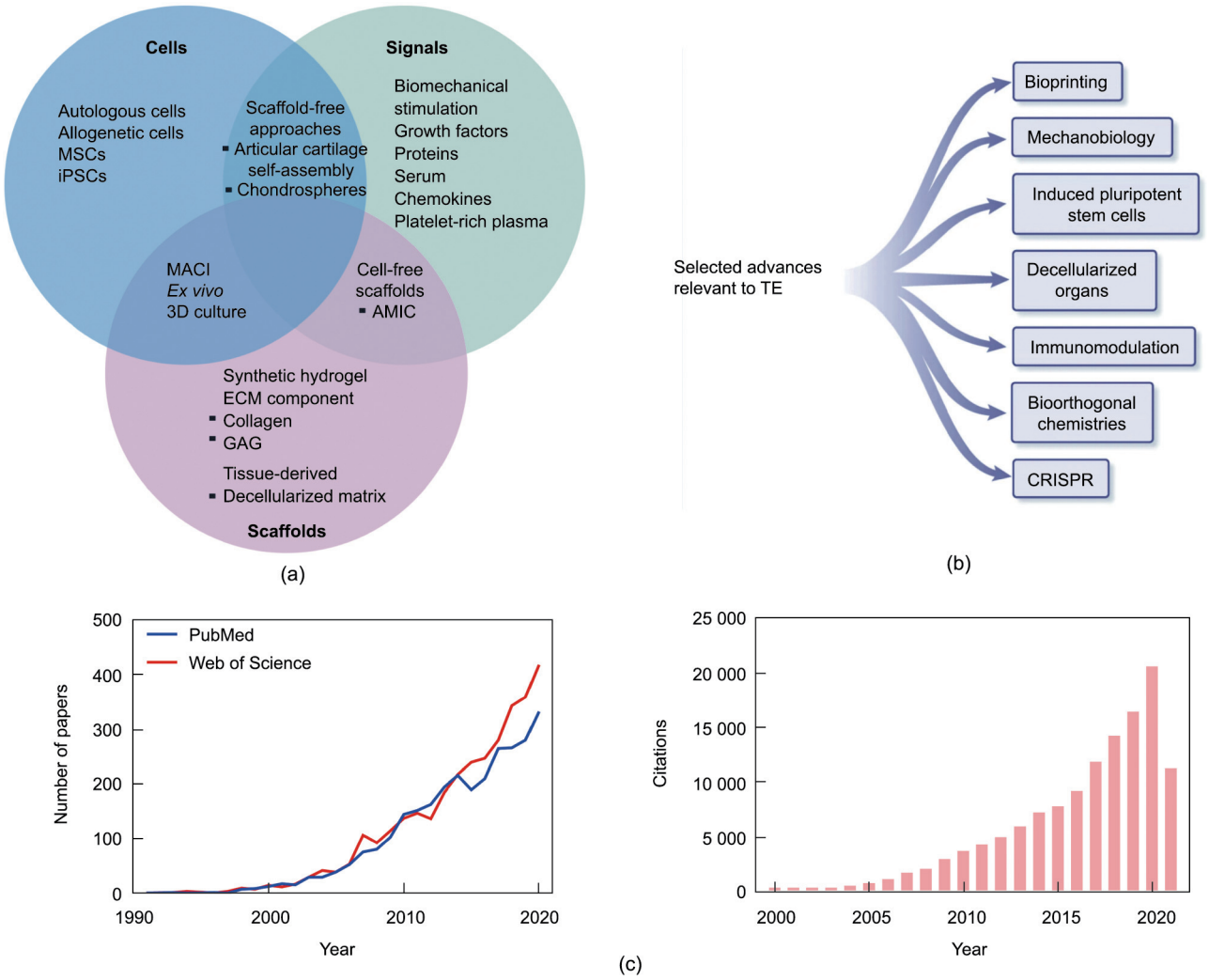


Fig. 3. The basic concept and recent progress of TE and tissue-engineered cartilage for AC regeneration. (a) The three key elements for TE. (b) Recent advances related to TE. (c) The number of papers published and citations on the topic of "hydrogel and cartilage" have been increasing significantly in the past two decades (as of July 1, 2021). AMIC: autologous matrix-induced chondrogenesis; CRISPR: clustered regularly interspaced short palindromic repeats. (a) Reproduced from Ref. [18] with permission of Springer Nature, © 2014; (b) reproduced from Ref. [20] with permission of Springer Nature, © 2016.

loads at synovial joints. Nevertheless, synthetic polymers can elicit unfavorable immune responses and severe toxicity and exhibit lower biological activities [24]. In contrast, hydrogels derived from natural polymers (polysaccharide-based and protein-based biomaterials) have some intrinsic advantages, such as high biocompatibility, biodegradability, and macromolecular similarity to native ECMs, which allow for improving bioactivity and cell adhesion [25]. However, rapid degradation, inconsistent hydration, unfavorable elastic properties, and poor stability hinder the further applications of natural hydrogels [25]. At present, a variety of hybrid hydrogels have been developed to surmount the shortcomings of both synthetic and natural hydrogels. The advantages and disadvantages of several commonly utilized hydrogels for AC repair and regeneration were compared and summarized in Refs. [26] and [27].

Hydrogels are beneficial for encapsulating chondrocytes or stromal cells rather than merely enhancing adhesion. They help maintain the round morphology and chondrogenic phenotype of these cells. Also, hydrogels are able to create a favorable 3D local microenvironment for cells. In this microenvironment, cells and networks mutually affect each other. Cells can sense numerous biochemical and biophysical cues within hydrogels, and these signals can guide cell behaviors (e.g., migration, proliferation, and differentiation) [28]. Meantime, cell-mediated remodeling of biomimetic encapsulating hydrogels can affect their structures, mechanical properties, and degradation profiles [28,29]. Besides, as a permeable matrix, hydrogels can function as the controlled delivery and release of soluble biochemical factors. Cell and hydrogel mixtures can be directly injected into the defect sites to allow gelation *in situ* [30], eradicating the steps of cell encapsulation and post-scaffold (bio)fabrication procedures, and offering the option of minimally invasive surgery. Alternatively, these mixtures can be precisely (bio)designed and (bio)fabricated *in vitro* to form implantable hydrogels using 3D-(bio)printing (3DBP) platforms.

Traditional chemical hydrogels are crosslinked via covalent bonds and may have disadvantages (such as brittleness, difficulty in injectability after gelation, and limited cell proliferation and migration). However, some functional hydrogels, for example, a novel class of supramolecular hydrogels, may show the advantages of effective cell delivery, dispersion and survival, self-healing, and shear-thinning properties, which are beneficial for cartilage repair. Functionalized hydrogels can chemically, mechanically, and electrically mimic the functions of biological tissues [31]. Among these, functionalized hydrogels engineered via modern manufacturing

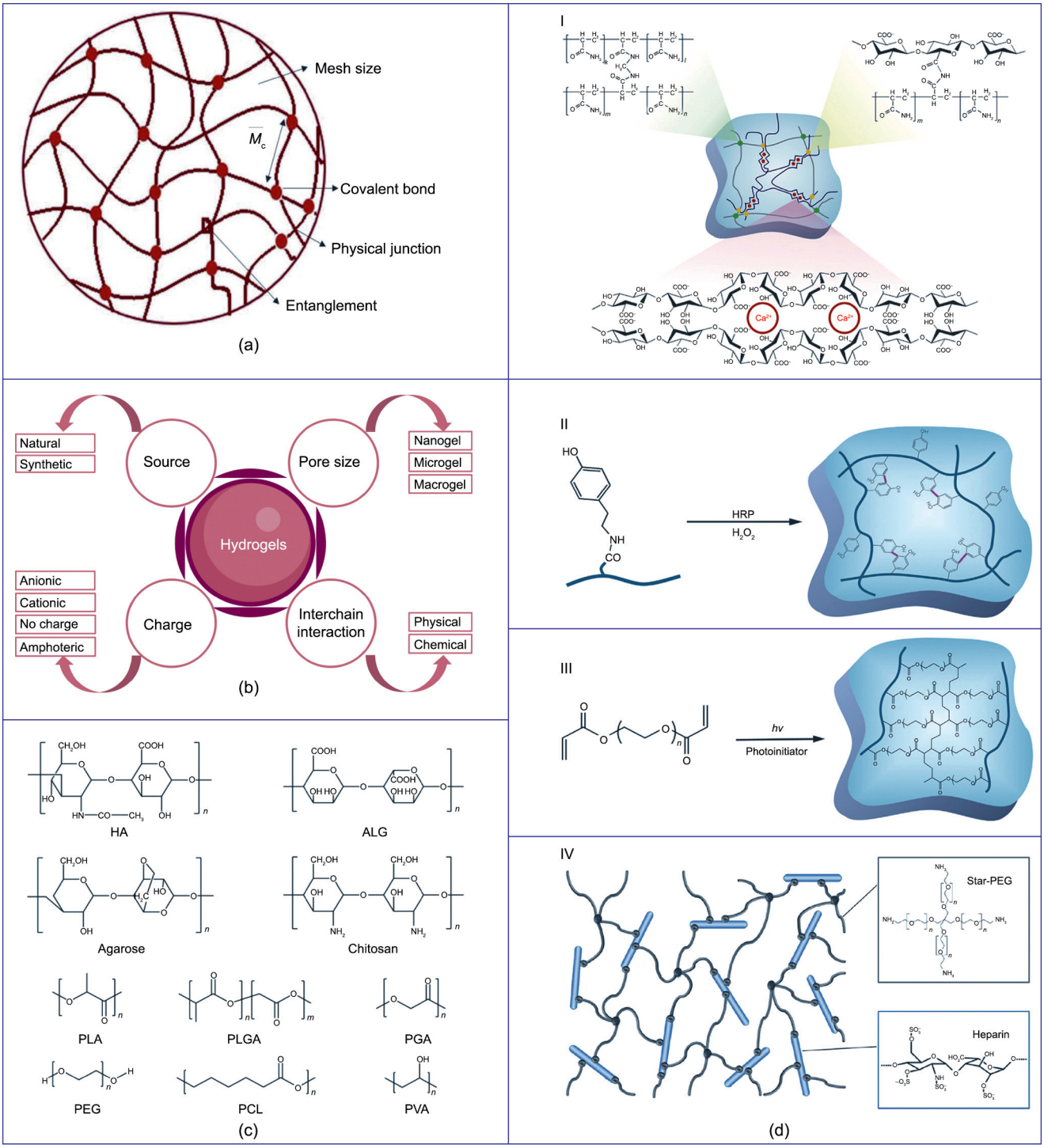


Fig. 4. Functionalized hydrogels as optimal scaffolds for ACTE. (a) Structure of hydrogels at the molecular level. (b) Physical and chemical aspects for the classification of polymer hydrogels. (c) Chemical structures of some natural polymers (HA, alginate (ALG), agarose, and chitosan) and synthetic polymers including PLA, PLGA, PGA, poly(ethylene glycol) (PEG), PCL, and poly(vinyl alcohol) (PVA). (d) Examples of hydrogel formation through several different methods. (d-I) *In situ* gelation through ionic interaction between Ca^{2+} and ALG, and the chemical crosslinking strategy. (d-II) *In situ* gelation through the enzymatic crosslinking reaction between H_2O_2 and horseradish peroxidase (HRP). (d-III) *In situ* gelation through the photo-crosslinking approach. (d-IV) Heparin and star-PEG are crosslinked to form a hydrogel. $\overline{M}_c$: molecular weight of polymer chain between the crosslinks; *hv*: light energy. (a) Reproduced from Ref. [22] with permission of Elsevier, © 2020; (d) reproduced from Ref. [21] with permission of IntechOpen, © 2016.

technologies have led to various functional biomaterials with versatile surface effects, bringing great potential in numerous applications. Some conclusions can be drawn from here, especially for stimuli-responsive hydrogels. In the scenario of ACTE, the functionalized hydrogels can be defined as "a class of biomimetic hydrogels formed by many innovative (bio)design and (bio)fabrication technologies to achieve similar structural, mechanical, and biological properties of native cartilage tissues for treating

cartilage defects." Functionalized hydrogels can serve as drug/nutrition carriers, elastomers, and scaffolds to enable cartilage regeneration. Functionalized hydrogels have evolved into a research hotspot for AC repair owing to their bio-inspired tunable macro-and micro-structures and favorable biochemical and biophysical functions, particularly under the circumstances of minimally invasive therapies [32,33].

5. (Bio)design of functionalized hydrogels for cartilage regeneration

5.1. Addition of ECM components into functionalized hydrogels

Many components, such as exogenous cells, growth factors [34], therapeutic peptides [35], prochondrogenic molecules [36], and RNA-targeting molecules (e.g., small interfering RNA (siRNA), microRNA (miRNA), messenger RNA (mRNA), and a class of antisense oligonucleotides function as anti-miRNAs (antagoMiRs)) [37] can be added into hydrogel precursors to function as traditional biological cues for cartilage regeneration. Apart from the above components, ECM has drawn widespread attention to be utilized in hydrogels as a functional factor for cartilage regeneration, due to the intrinsic advantages of natural biomimetic materials. Usually, ECM is prepared as solubilized or particle-based components into functionalized hydrogels for repairing AC defects [38,39].

5.1.1. ECM components as solubilized form

The two main ECM components of cartilage, collagen and glycosaminoglycans (GAGs), are widely applied in hydrogels to restore the ECM of cartilage. Solubilized collagen and GAGs alone can function as hydrogels without other polymers or additional chondrogenic components in composite hydrogel systems [40]. Although collagen II dominates the collagen components in cartilage, several prior studies have used collagen I-based hydrogels for cartilage repair because of its availability and homogeneity. Adding collagen II to composite hydrogels enhances cartilage-specific ECM production, promotes chondrocyte proliferation, and improves cartilage repair [41]. In a seminal study by Kilmer et al. [42], a blend of collagen I/II hydrogel promoted GAG production and AC repair in the femur cartilage defect area of rabbits. In addition, hybrid hydrogels with a combination of GAGs and collagen, closer to the natural cartilage matrix, showed superiority and could replace collagen hydrogels as the "gold standard" for ACTE [40]. However, GAGs are the only side chains of proteoglycans, which are the main functional units of the non-collagen component of cartilage. Adding 0.25% proteoglycan mimics into 2.25% agarose hydrogels improved their compression and stress relaxation properties [43]. Surprisingly, it also decreased the metabolic activity and viability of human adipose-derived mesenchymal stem cells (MSCs). Further modification to maintain proteoglycan integrity and cell viability simultaneously might enhance the mechanical properties and biocompatibility of hydrogels for cartilage regeneration.

Another strategy for reconstructing natural cartilage ECM is the application of decellularized ECM (dECM) to hydrogels. The decellularized natural cartilage matrix, which mainly contains collagen, is enzymatically degraded, solubilized, and then re-polymerized; however, the bioactivity of enzyme-digested dECM remains controversial [44]. Furthermore, the solubility of dECM is quite low (up to 3% w/v [39]) with enzymatic and acidic digestion, resulting in the inability to reach the normal proportion of collagen within healthy cartilage. Through the digestion–lyophilization–redissolution method, dECM was significantly enriched with a concentration of up to 6% (w/v) [45]. Devitalized cartilage (DVC), which lacks additional chemical processes to remove cell contents, is thought to be more chondroinductive than cartilage dECM [46]. However, solubilized DVC has similar limitations (e.g., low concentration and bioactivity). The ability of DVC to elicit an immune response while maintaining cell composition is also a challenge that requires further investigation.

5.1.2. ECM components as particle-based form

In contrast to the poor mechanical properties and low porosity of hydrogels formed by solubilized ECM components, hydrogels formed by particle-based ECM components present advantages in terms of biological and mechanical properties for cartilage regeneration [47]. In addition, tissue structure components can be pulverized and freeze-dried into particles. The natural architecture and topology can be largely preserved in dECM/DVC particles, making it possible to fabricate tissue-specific hydrogels with varying geometries and porosities. The preservation of GAGs during decellularization is crucial to maintain the mechanical properties and to retain growth factors in the dECM particles of hydrogels [48]. Collagen I hydrogels incorporated with dECM particles enhanced chondrogenic gene expression of MSCs, which was further amplified by transforming growth factor β3 (TGF-β3) [49]. To retain dECM particles inside a hydrogel and mimic the natural cartilage composition, dECM particles could be chemically crosslinked with modified GAGs [50] (Fig. 5(a)). DVC particles appeared to be superior to dECM in both chondroinductivity and mechanical strength [46]. Moreover, Levinson et al. [51] incorporated autologous cartilage particles into fibrin/collagen hydrogels to support chondrocyte outgrowth and survival. However, limited tissue sources restrict the extensive application of autologous cartilage particles.

In addition to designing hydrogels with monotonous additional components, many studies have focused on diverse ingredients with various physical forms. Our recent study showed that embedding microparticles (MPs) within a continuous hydrogel matrix was an effective approach to the improvement of shear strength and compressive moduli [52]. Owing to the physical interactions between the polymer chains, such as hydrogen bonding or intramolecular entanglements, MPs impart a drag force within the hydrogel network, improving the resistance to deformation. These results were also consistent with other reports that two-phase polymer composites containing encapsulated MPs demonstrated notably improved toughness [53], stiffness, and elasticity [54]. Solubilized collagen and GAGs hydrogels incorporated with dECM particles were preferred and considered close to the natural matrix [48]. Furthermore, Beck et al. [55] added non-solubilized DVC particles to a solubilized DVC hydrogel, in which solubilized DVC might open up more reactive sites on the cartilage ECM, and DVC particles mimicked the natural structure of cartilage ECM. The data demonstrated improvements in the mechanical properties and chondrogenic bioactivity of the hydrogels both *in vivo* and *in vitro*.

To maintain the natural function and components of the cartilage matrix, a hybrid of dECM particles and native cartilage-derived intact proteoglycans at physiological concentrations might be a promising functionalized hydrogel-based strategy for cartilage regeneration. Moreover, hydrogels fabricated by ECM from immune-exempt tissues are also very promising. Taking the advantages of naturally intact and unmodified structure and composition, Lindberg et al. [56] presented highly permissive and bioactive ECM hydrogels from vitreous humor tissue and ECM-based hydrogels significantly augmented the proliferation and chondrogenic differentiation of human MSCs.

5.2. Lubrication design of functionalized hydrogels

Healthy AC has a low-friction coefficient due to the lubrication effect of the joint surface and synovial fluid. One tissue-engineered

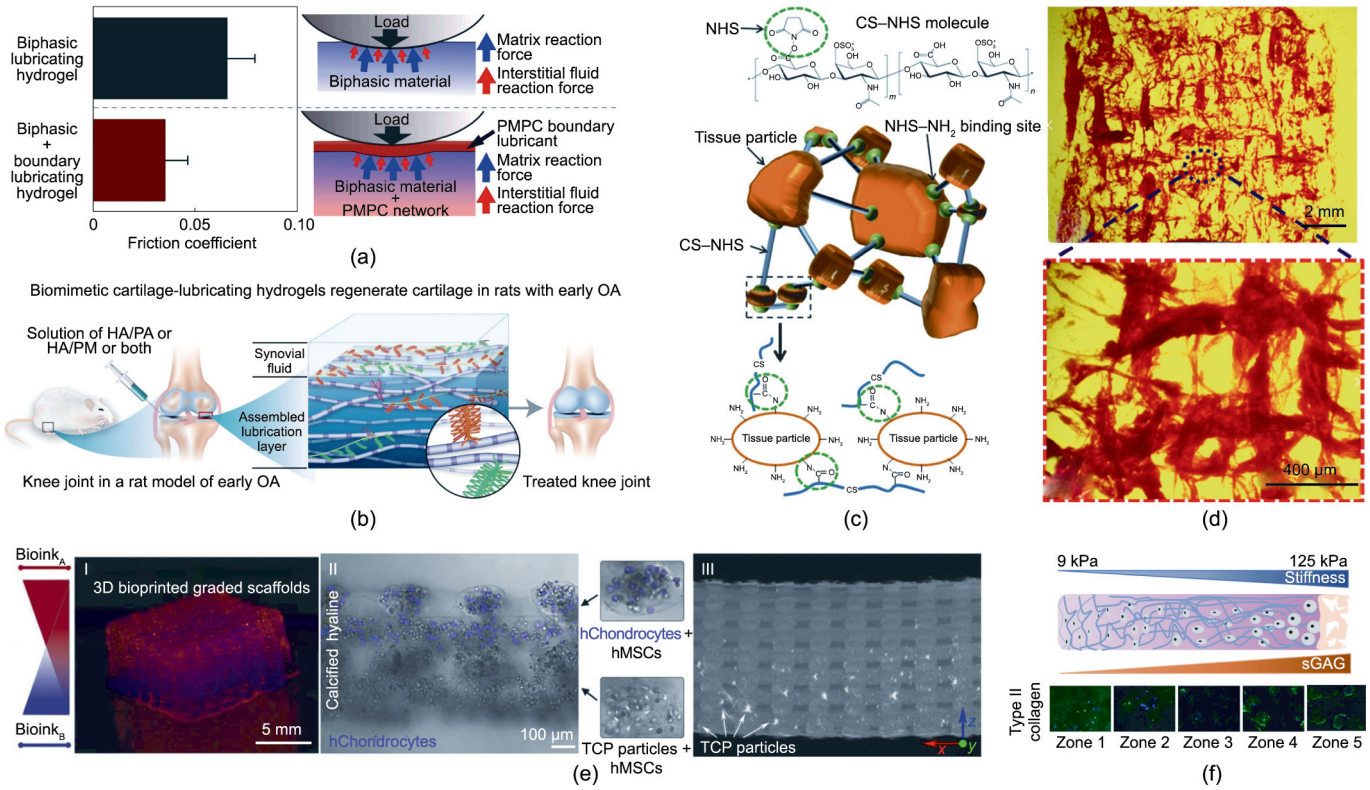


Fig. 5. The representative (bio)design of functionalized hydrogels-based strategies for cartilage regeneration. (a) Low-friction, biphasic, and boundary lubricating triple-network hydrogel for AC repair. (b) Biomimetic cartilage-lubricating hydrogels for cartilage regeneration in a rat model with early OA. (c) The chondroitin sulfate (CS) molecules are chemically modified with *N*-hydroxysuccinimide to facilitate the binding with the amine groups on tissue particles. (d) Collagen fiber orientation inside the extrusion-printed filaments of hydrogels. (e) Spatial distribution of the two different bioinks and cells in a 3D-(bio)printed construct. (e-I) Zonal distribution of bioinks in the layered scaffolds. (e-II) Longitudinal cross-section of the bioprinted layered scaffolds in which chondrocytes (blue) are confined within the top layers while unlabeled human MSCs (hMSCs) and β-tricalcium phosphate (TCP) particles (white arrows in the magnified calcified cartilage area) occur at the bottom of the scaffold. (e-III) TCP MP distributions along the *z*-axis in the acellular scaffold visualized through micro-computed tomography (μ-CT). (f) Gradient stiffness and type II collagen formation after the cartilage-mimicking zonal hydrogel organization. PMPC: poly(2-methacryloyloxyethyl phosphorylcholine); HA/PA: a synthesized lubricant is formed by covalently grafting poly(2-acrylamide-2-methylpropanesulfonic acid) sodium salt (PAMPS) to the HA main chain; HA/PM: a synthesized lubricant is produced by covalently linking poly(2-methacryloyloxyethyl phosphoryl choline) (PMPC) to the HA main chain. (a) Reproduced from Ref. [58] with permission of Elsevier, © 2018; (b) reproduced from Ref. [62] with permission of Springer Nature, © 2021; (c) reproduced from Ref. [50] with permission of Wiley Periodicals, Inc., © 2017; (d) reproduced from Ref. [79] with permission of Springer Nature, © 2019; (e) reproduced from Ref. [83] with permission of IOP Publishing, © 2019; (f) reproduced from Ref. [87] with permission of ACS, © 2018.

strategy of reduction in friction for cartilage repair is to construct an inherently slippery surface. Means et al. [57] established double-network hydrogels that mimic the modulus and strength of cartilage to trapped liquids to form a slippery surface for lubrication. Furthermore, Milner et al. [58] constructed a low-friction, biphasic, and boundary lubricating triple-network hydrogel with no decrease in chondrocyte viability and proliferation (Fig. 5(a)). Inspired by the natural brush-like lubrication layer of cartilage, lubrication with polymer hydrogels can also contribute to an inherent slippery surface [59]. However, natural cartilage exhibits ultralow friction, even at high squeezing pressures. A novel cartilage-mimicking bilayer hydrogel system using thick hydrophilic polyelectrolyte brushes entangled into the subsurface of a load-bearing hydrogel was developed [60]. Low-friction coefficients (order of 0.010) under heavily loaded conditions (contact pressure of 10 MPa) were attained even when subjected to 50 000 reciprocating cycles, which presented a performance incredibly close to that of a natural AC. It is significantly meaningful that hydrogels can retain low friction under harsh conditions.

The use of lubricants can also reduce surface friction during ACTE. Lin et al. [61] incorporated trace lipid concentrations into synthetic hydrogels to create a cartilage-inspired lipid-based boundary layer. The continuously exuded lipids can form a slippery layer on the hydrogel surface and significantly reduce the friction and wear of the hydrogels, which was observed even after the hydrogels were dried and rehydrated. This strategy is promising to sustain extreme lubrication of hydrogels for cartilage repair. Another *in vivo* study also showed that natural cartilage-mimicking hyaluronan backbones grafted with lubricin-like sulfonate-rich polymers or lipid-like phosphocholine-rich polymers enhanced cartilage regeneration in a rat model of early OA [62] (Fig. 5(b)). The friction coefficient was significantly lowered to the ultralow levels of native AC. The *in vivo* results showed that cartilage regeneration and abrogation of OA could be achieved within eight weeks. These approaches might provide practical strategies for clinical translation.

5.3. 3D architecture of functionalized hydrogels

5.3.1. Porous structures

Hydrogels with porous structures provide sufficient space for cell attachment, ingrowth, proliferation, and differentiation. Many studies suggested a recommended pore size of 100–500 μm for chondrocyte and MSC proliferation and chondrogenesis [63-66]. Pores that are too small would prevent cell infiltration [66], whereas hydrogels with relatively larger pore sizes could be beneficial for cell infiltration, proliferation, and chondrogenesis [67]. The pore size and porosity of hydrogels can be dramatically affected by the type of biomaterials and crosslinking densities. Al-Sabah et al. [68] compared alginate (ALG) and nanocellulose

hydrogels with different crosslinking densities, and the results revealed that $CaCl_2$ crosslinker concentrations significantly altered the overall pore size and porosity of hydrogels. Gao et al. [69] lyophilized ALG hydrogel and found that the pore size increased dramatically to 200–300 μm. Another study [70] showed that the dECM fiber diameter and pore size of hydrogel decreased with increasing concentration from (0.094 ± 0.005) μm and (0.128 ± 0.017) μm^2 at low concentrations to (0.069 ± 0.005) μm and (0.059 ± 0.001) μm^2 at high concentrations, respectively. Generally, pure solubilized hydrogels with high crosslinking densities preserve a compact structure with low porosity. However, Qi et al. [71] constructed a pure solubilized sericine hydrogel by photocrosslinking methods, and the hydrogels obtained a favorable pore size up to (193.51 ± 7.68) μm with porosity up to 97%. Particles are widely used to increase the pore sizes of hydrogels. Almeida et al. [67] successfully prepared hydrogels with pore sizes ranging from (32 ± 12) μm to (65 ± 20) μm by altering the concentration of the dECM particle slurry. With the addition of particles, the number of hydrogel pores significantly increased. Porogens can also achieve controlled pore size for chondrogenesis [72]. However, considering the single function of porogens, one should be very careful to seek a balance between the pore structure and other properties of this material. Stimuli-responsive pore formation can be achieved with the initiation of specific stimuli. Moreover, sequential pore formation through the sequential activation of multiple stimuli-responsive porogens is practical. Han et al. [73] dynamically controlled the porosity of stimuli-responsive tri-porogen hydrogels by syngenetic stimulation of chelation, temperature, and enzymatic activity.

5.3.2. Networks

In general, networks of a single polymer result in a very limited adjustment range for the hydrogel structure. An increased crosslinking density may compromise the viability, filtration, and bioactivity of the encapsulated cells. Networks formed by a combination of multiple polymers (e.g., interpenetrating network, double-network, dual network, and guest–host network) can contribute to improved mechanical properties and different porosities. Independent networks with partial bonding (not covalent) can form interpenetrating networks. Dinescu et al. [74] created an interpenetrating hydrogel with three components (gelatin, ALG, and polyacrylamide) and achieved a highly ordered porosity pattern and well-defined interconnected pores during long-term culture, which largely promoted the proliferation and chondrogenesis of stem cells. However, a double network is composed of two separate networks in the same hydrogel system. In general, one network is rigid and the other is flexible. For a double-network hydrogel, its mechanical properties and surface structure can be potentially approached close to native cartilage [58,75]. Two materials are covalently crosslinked in the same network within a dual-network hydrogel. Beachley et al. [50] fabricated a dECM particle–GAG dual-network hydrogel with tunable gelation kinetics and mechanical properties (Fig. 5(c)). Noncovalent bonds between two polymers give rise to a reversible guest–host network. One shear-thinning hydrogel with guest–host interaction between *O*-carboxymethyl chitosan and a 3D dynamer was designed for repairing cartilage with desirable results, which were mainly attributed to the flexibility of its networks [76].

5.3.3. Biomimetic hydrogels with cartilage-specific zonal structures

A key aspect of ACTE is the simulation of the highly hierarchical structure, ECM composition, and mechanical features of the stratified AC. Generally, hydrogels are devoid of internally oriented structures. Scanning electron microscopy (SEM) of the two types of dECM hydrogels showed that the networks of both dECM hydrogels obtained no angular alignment with a normalized orientation index close to 0% [70]. Aiming to replicate the collagen fiber distribution within the SZ of AC, Owida et al. [77] produced an HA-based hydrogel that enveloped specifically oriented nanofiber meshes by electrospinning. With the accessibility to control the orientation of the printing matrix composition, 3DBP provides great potential to rebuild the complexity of cartilage. Schwab et al. [78] achieved shear-induced alignment of collagen fibrils by 3DBP technology. The collagen fibrillar alignment was highly patterned with horizontal orientation in the SZ, isotropic orientation in the MZ, and vertical orientation in the DZ. Moncal et al. [79] printed collagen fibers with low anisotropy using a thermally controlled extrusion-based (bio)printing platform (Fig. 5(d)). Moreover, the inclusion of magnetic particles in hydrogels can also align the composition of molecules in a magnetic-field-guided manner [80]. The agarose–collagen I bioink embedded with iron nanoparticles was exposed to a magnetic field during (bio)printing, and the collagen fibers were aligned. Alignment was most prevalent in less concentrated hydrogels, with a maximum agarose concentration of 0.5% (w/v).

Apart from the distribution and orientation of collagen fibers, the cartilage-specific layered structure formed by different cell types, cell densities, and cell alignments [81–84] should be taken into consideration when designing biomimetic hydrogels for cartilage TE (Figs. 5(e) and (f)). Several zonal-hydrogel-based strategies have been developed for AC regeneration (Table 1 [77,81–90]). With this approach, cells loaded into hydrogels are expected to exhibit zonal-specific properties. A homogenous collagen II hydrogel was used to encapsulate chondrocytes by depth-dependent density. Different biosynthetic activities of loaded cells and gradient distribution of ECM have been observed [81]. Additionally, modulating cell alignment and elongation in hydrogels can also regulate the biosynthesis of GAGs and collagen [82]. Chondrogenesis has been significantly enhanced by mixing MSCs with articular chondrocytes in hydrogel-formed cartilage constructs [83]. Another approach for engineering cartilage grafts is to construct layers by hydrogels (homogenous or heterogeneous) with different compositions. Using this approach, different cell-laden hydrogel materials were applied in different layers, and desirable results for OCD regeneration were obtained in a rat model [83].

Furthermore, the gradient distribution of oxygen is a critical cue for chondrocyte bioactivity in different cartilage zones. Increased oxygen production from the lowermost to the uppermost region was achieved, which opened a new field for zonal hydrogel-based ACTE [85]. Designing hydrogels with gradient mechanical properties is critical for fabricating hydrogel-based constructs [86,87]. Despite many strategies in terms of using hydrogels to simulate cartilage-specific zonal structures and properties, so far none of them can biofabricate implanted cartilage grafts, which are equivalent to native AC tissue.

5.3.4. Structural variability

Hydrogels can absorb massive amounts of water up to thousands of times of their dry weight [91]. Degradable hydrogels experience an increase in the swelling ratio along with degradation [92]. Controllable swelling can be achieved through the modification of the crosslinking density. The PEG hydrogel swelling ratio can be lowered by increasing the concentration of the PEG solutions, decreasing the molecular weight of the PEG macromers, or using branched PEG structures instead of linear structures [93].

Notably, cell-mediated contraction leads to potential risks for cell-laden hydrogels [94]. The properties of the hydrogel change with the macromolecule contraction. Furthermore, the integration of the surrounding tissues is significantly hindered owing to the loss of contact between hydrogels and the surrounding tissues *in vivo*. Enhanced crosslinking can counteract cell-mediated contractions. However, an increased crosslinking intensity reduces cell

Table 1
Biomimetic hydrogels with the cartilage-specific zonal structure for cartilage defect repair.

Strategy	Hydrogel	Approach	Aim	Key outcomes	Reference
Zonal cells	Collagen II	3DBP	Cell density gradient	• Gradient distribution of ECM • Affected the biosynthetic ability of chondrocytes through the cell distribution pattern and total cell density	[81]
	CS-*g*-PNIPAAm	Micro-molding	Cell alignment and elongation	• Aligned cells in the SZ and unpatterned cells in the MZ • Increased secretion of GAGs and total collagen	[82]
	ALG	Cell culture insert in the designed magnetic field	Zonal cell arrangement	• Vertical cell arrangement • Upregulated expression of Col2a1 and aggrecan • Zonal ECM organization	[84]
Fiber orientation	HA	Nanofiber mesh	Zonal nanofiber orientation	• Low production of collagen II and GAGs, elongated cell morphology, high production of collagen I, and high cell proliferation activity in the SZ • Clustered cells and high expression of collagen II in the MZ • Highest GAG production, lowest collagen I expression and cell proliferation in the DZ	[77]
Zonal polymers	ALG + GelMA + CS-AEMA + HAMA	3DBP	Hyaline + calcified cartilage, zonal cell types	• High cell viability • Upregulated expression of hypertrophic biomarkers in the homogenous equivalent of calcified cartilage but not in the gradient heterogeneous construct • The bioprinted scaffolds were beneficial for OCD regeneration in rats • The mineralized matrix consisted of hypertrophic proteins, GAG, osteocalcin, and collagen type X was formed	[83]
Gradient components	Agarose + gelatin	Circular silicon mold	CS/BG gradient	• Chondrocytes secreted hyaline-like matrix with higher sulfated GAG, aggrecan, and collagen II on CS fibers • Enhanced mineralization on BG fibers • Continuous opposing gradients of GAG enriched and mineralized ECM in response to the physical gradients of raw materials CS and BG	[88]
Gradient oxygen supply	Pectin + fibroin	Mixing chamber	The depth-dependent gradient of oxygen releasing	• The increased amount of produced oxygen from the lowermost to the uppermost section	[85]
Gradient osteochondral unit	PEGDMA	3DBP	Osteochondral gradient	• Precise cell distribution • Increased cell viability • Firm attachment with adjacent tissue and more proteoglycan deposition at the interface between implanted construct and native cartilage • Elevated GAG contents	[86]
Hybrid approaches	Chitosan–gelatin hydrogel/PLGA	Glass tube orifice	The graded transition from the hydrogel to PLGA scaffold and graded variation in the amount of BMP-2 and TGF-β1	• Promote bone marrow MSCs toward chondrogenesis and osteogenesis, respectively • Better integration of the regenerated hyaline-like cartilage and SB with the surrounding tissues by utilizing the BMP-2 and TGF-β1 double-loaded hydrogel/PLGA graded scaffold *in vivo*	[89]
	PEG + CS	Interconnected chamber	CS gradient + mechanical gradient	• Depth-dependent stiffness and gradient biochemical properties • Increased expression of hyaline cartilage markers, and upregulated collagen deposition and chondrocyte proliferation in a zonal-dependent manner • Enhanced zonal organization by chondrocytes	[87]
	GelMA	Custom-made Teflon injection mold	Zonal growth factors + superficial/deep cartilage + SB + depth-dependent fiber organization	• Downregulated osteogenesis and chondrogenesis of MSCs, and cellular phenotype and matrix accumulation profiles resembling those of the native tissue • The defects have been repaired by the formation of neocartilage with a more lubricating and wear-resistant surface and the denser SB	[90]

CS-*g*-PNIPAAm: chitosan-*g*-poly(*N*-isopropylacrylamide); CS-AEMA: chondroitin sulfate chains through the *N*-ethyl-*N*′-(3-(dimethylamino)propyl)carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) coupling reaction with 2-aminoethyl methacrylate; HAMA: methacrylated hyaluronic acid; BG: bioactive glass; PEGDMA: poly(ethylene glycol) dimethacrylate; BMP-2: bone morphogenetic protein-2; Col2a1: a1 subunit of collagen II.

infiltration and bioactivity. The balance between crosslinking intensity and cell-mediated contraction is of significance. Cheng et al. [95] prepared matrix-derived contraction-free hydrogels by various genipin (natural biological crosslinker) concentrations and found that with 0.05% genipin, the moderately crosslinked hydrogels were chondroinductive without significant cell-mediated contraction during the culture period. Also, particles were usually used in hydrogels to avoid unfavorable contraction. The methacrylated solubilized DVC hydrogel containing DVC particles did not show a significant volume change, whereas the methacrylated solubilized DVC hydrogel alone contracted by 18% at six weeks [55].

Self-healing properties allow hydrogels to dynamically change and restore the framework, which endows the hydrogels with injectability for clinical applications. Hydrogels with dynamic covalent reactions (chemical crosslinking) and/or non-covalent reactions (physical crosslinking) can achieve self-healing properties [32,96]. Yu et al. [76] synthesized a novel dynamic hydrogel based on *O*-carboxymethyl chitosan and a soluble compound synthesized by the reaction of benzene-1,3,5-tricarbaldehyde with jeffamine for ACTE scaffolds, indicating excellent pH-sensitive swelling and self-healing properties. Zhang et al. [97] further proposed an aldehyde-functionalized cellulose nanocrystal/collagen hydrogel crosslinked under physiological conditions and obtained superior shear-thinning and self-healing characteristics. The proposed self-healing hydrogel could protect cells from high shearing stress during injection into irregular cartilage defects.

5.4. Tailored shape and size for irregular cartilage defects

In situ polymerization of hydrogels has been widely applied in TE owing to their flexibility and plasticity in matching irregular tissue defects [98]. The shape and size of *in situ* polymerized hydrogels, for example, some injectable hydrogels, can precisely match the anatomy of cartilage defects and appropriately fill irregularly shaped defects, providing satisfactory contact with the surrounding tissue. However, some traditional strategies cannot produce ideal-shaped AC grafts with a high resolution in damaged areas. 3DBP technology enables the fabrication of hydrogels-based cartilage constructs with heterogeneous composition and complex architectures with high shape fidelity [83,99]. 3D shapes and custom-made complex structures can be designed and applied through precise spatial control [100]. This is important for mimicking the shape of the defect and the heterogeneous and anisotropic cartilage architecture during hydrogel construction.

5.5. Mechanical properties

Functionalized hydrogels have been extensively used for cartilage repair. Among these applications, the mechanical behavior of hydrogels plays a crucial role because AC is a load-bearing tissue with a fracture strength of tens of MPa, a modulus of 1 MPa, and an elongation at a break of 100% [13]. Many studies have focused on identifying hydrogels that mechanically mimic cartilage ECM. In general, a higher concentration of gel components or a higher level of crosslinking will make the gels stiffer and/or more brittle. The network structures and inclusion of additional biomaterials and cells can also help alter the mechanical performance of hydrogels. Poly(vinyl alcohol) (PVA) hydrogels have been extensively characterized and demonstrated to be closer to cartilage than other hydrogels in terms of their compressive modulus, shear modulus, tensile modulus, and permeability [92]. DeVolder et al. [54] showed that collagen hydrogels incorporated with poly(lactide-*co*-glycolide) (PLGA) MPs could modulate their stiffness and elasticity. The mechanical properties of hydrogels can also be enhanced by continuous fibrous reinforcement [101]. Qiao et al. [90] reinforced an inherently weak gelatin methacryloyl (GelMA) hydrogel with high-modulus fiber polymers and acquired a compressive modulus comparable to that of native cartilage. Furthermore, double-network hydrogels have attracted wide attention because they are excellent structural platforms to integrate different mechanical properties into a single biomaterial. Yan et al. [102] synthesized gelatin/polyacrylamide double-network hydrogels and achieved superior mechanical properties (high elastic modulus, failure of tensile stress, strain, and fracture energy) compared to traditional single network hydrogels. Besides, attempts have been made to alter the mechanical properties of gels dynamically, for example, by constructing hydrogels that stiffen over time [103]. Apart from optimizing toughness, strain, stiffness, and dynamic mechanical response, the mechanical homogeneity of hydrogels has been widely overlooked. Xue et al. [104] engineered maleimide–thiol crosslinked PEG hydrogels with well-defined and controllable homogeneity and showed that nanoscale variation in matrix stiffness could considerably regulate cell fate.

5.6. Tunable degradability and biocompatibility

Degradation-associated neotissue remodeling is critical for functionalized hydrogels during cartilage regeneration. The major challenge is designing hydrogel scaffolds with appropriate time-dependent degradation properties and mass-loss profiles. Such degradation not only disaggregates the networks of hydrogels but also decreases their mechanical properties. Therefore, via adjusting the degradability of the hydrogels, the time-dependent ultrastructure and mechanical properties can be modulated to some degree. The degradation process of hydrogels can be regulated by several approaches (such as hydrolysis, protease-mediated degradation, and external stimuli-triggered degradation). Ideally, *in vivo* degradation of implanted hydrogels is proportional to matrix deposition and neocartilage formation. Degradability that is too fast or too slow would result in undesirable mechanical support and hamper the ingrowth of cartilaginous tissue, respectively. Up to now, many strategies have been applied to manipulate hydrogel degradation both *in vivo* and *in vitro*. Kloxin et al. [105] reported controlled hydrogel degradation through the light duration, intensity, and area by incorporating a photodegradable composition into PEG-based hydrogels. Temporal variation in the composition of the photodegradable gel was utilized to promote chondrogenesis of the encapsulated stem cells. However, this approach is not applicable *in vivo*. A PEG-based bioresponsive hydrogel modified by matrix metalloproteinase groups can balance cell-mediated degradation and cartilage formation [106]. Wang et al. [107] developed a novel double-network hydrogel with fish-derived self-assembled collagen and self-crosslinked PVA. A controllable degradation rate of such hydrogels was achieved by adjusting the ratio of PVA to collagen.

Additionally, for ACTE, ideal functionalized hydrogel-based cartilage grafts require excellent biocompatibility, which is usually defined as the capability of an implanted biomaterial to cause a proper host response. Recently, various hydrogels have been chemically modified and prepared to improve their biocompatibility for AC repair and regeneration [108].

5.7. Controlled delivery and release of biochemical factors

Hydrogels are materials of choice for drug delivery. Given their high water content, tunable mesh size, and viscoelastic properties, hydrogels can be loaded with small and biological drugs, or with cells. The 3D environment provided by hydrogels can protect their payload, whereas spatiotemporal control over their release can be modulated by physicochemical interactions, mesh size, viscoelastic properties, or by active mechanisms in "smart" hydrogels.

Research on hydrogels for drug delivery is well established and has been successfully translated into clinical use, with the most notable example being Infuse®, a collagen fleece perioperatively reconstituted to form hydrogels for bone morphogenetic protein-2 (BMP-2) delivery [109]. Other clinically approved hydrogel-based drug delivery systems include those for delivering anti-cancer drugs, antibiotics, and wound dressings. The range of applications explored in R&D is much wider, and there is abundant literature describing the physicochemical principles governing drug release from hydrogels [110].

The targeted delivery achieved with hydrogels prevents off-target systemic effects and increases the local concentration without exposing the rest of the organisms to massive doses. The biomaterials used for drug delivery act as a physical barrier and protect their payload, or slow down the penetration of degrading enzymes, thus increasing half-lives, especially for biological drugs. These hydrated polymeric networks can entrap, present, and release biochemical factors similar to the physiological cartilage ECM.

Considering the physicochemical aspects of drug delivery from hydrogels, the main parameters to consider are mesh size and specific functional group-based chemical interactions. Mesh size is fundamental for determining the diffusion rate throughout the hydrogel. For most hydrogels, the typical mesh size is 5–100 nm [111]. Bovine serum has a hydrodynamic radius of approximately 5 nm and a molecular weight of 66 kDa [112]. For globular proteins, the hydrodynamic radius scales with the molecular weight. The active forms of TGF-β and BMP-2 have molecular weights of about 25 and 26 kDa, respectively, implying that for proteins of this size, a hydrogel mesh is typically significantly larger than the molecular size and the diffusion is mainly unhindered. Larger proteins, such as antibodies, in combination with relatively small mesh-sized hydrogels, can exploit the mesh size to limit the diffusion of the drug payload. For example, Hiemstra et al. [113] compared the release of lysozyme (hydrodynamic radius 4.1 nm) and immunoglobulin G (IgG) (hydrodynamic radius 10.7 nm) from PEG/PLA hydrogels formed via stereo-complexation. The release followed first-order kinetics for the smaller lysozyme, whereas IgG displayed a nearly zero-order release, in agreement with the release modulated by matrix degradation rather than diffusion throughout the mesh.

Although in most instances, the mesh size alone is insufficient to modulate the release of small or biological drugs, hydrogels can be used as carriers of particles or cells. The molecules released from hydrogels can be controlled by specific functional group-mediated chemical interactions, such as ionic or hydrophobic interactions. A typical example is an injectable heparin-conjugated hyaluronan hydrogel for the local delivery of TGF-β1, which promotes successful chondrogenesis [114]. Applying wall-to-wall macroscopic hydrogels with a minimally invasive approach can be implemented using three different approaches: shear-thinning, *in situ* forming, and shape-memory. Shear-thinning materials display relatively low viscosity when subjected to high shear, for example, during injection through a cannula, but they recover this high viscosity once the mechanical stimulus is removed. This behavior confers the capability of being injected similarly to liquid substances while simultaneously remaining localized at the injection site and cohesive after injection. Water-soluble polymers above a certain molecular weight and concentration or lightly crosslinked molecular networks intrinsically display shear thinning, making them viable candidates for drug delivery systems. Shear-thinning hydrogels can also originate from guest–host interactions or dynamic covalent chemistry [115].

In situ-formed hydrogels are injected as liquid precursors and undergo transition to a gel once implanted, which is usually driven by a crosslinking reaction. Typical systems for *in situ* gelation are based on two components that are initially separate and are mixed during or after injection, for example, with static mixer cannulas or co-axial needles where the components are in contact only at the end. Alternatively, if the kinetics of the reaction can be modulated to be sufficiently slow to permit clog-free injection and sufficiently quick to obtain a cohesive material at the implantation site, the two components can be mixed before injection. This approach is illustrated in a previous study [116], where a thiol derivative of HA was combined with PEG vinyl sulfone. Upon mixing, these macromers underwent a Michael addition reaction with gelation times varying between less than one minute and 14 min. Chondrocytes were loaded into the composite gel, illustrating the potential of this approach for ACTE.

Shape-memory hydrogels can undergo significant deformation, for example, during extrusion through a cannula but fully recover their shape [117,118]. Macroporous hydrogels are examples of this category, in which the water phase occupying the macropores can be reversibly expelled implementing a reversible mechanical collapse [119]. Cryogels have been investigated in ACTE for building ECM-derived microporous cartilage scaffolds [120], hydroxyethyl methacrylate–lactate–dextran cryogels [121], PEG-based scaffolds with interconnected porosity [122], and hyaluronan–gelatin scaffolds [123]. Micro- or nano-sized gels are useful systems for drug and cell delivery in the cartilage. Microbeads can function as a template for ACTE [124,125]. They are usually produced in size considerably smaller than the needle dimension, are easily injectable, and have been used to deliver small molecules and proteins for cartilage repair [126]. PLGA microspheres were laden with BMP-2 and TGF-β1 into a bilayered system for OCD repair [127]. In another study, MSCs and polylactic acid microcarriers were 3D bioprinted for osteochondral repair [128]. Another interesting approach is the use of nanocrystal-polymer particles, where the crystalline character allows the extension of the drug release window to months. This concept was illustrated by delivering the p38α/β mitogen-activated protein kinase (MAPK) inhibitor PH-797804 in a murine model of inflammatory antigen-induced arthritis [129,130]. Nanostructures for intra-articular drug delivery were also produced via self-assembly of hyaluronan derivatized with thermoresponsive moieties, undergoing spontaneous self-arrangement into nanoparticles upon exposure to body temperature [131]. Other systems for intra-articular drug delivery for OA treatment are summarized in Ref. [132].

6. (Bio)fabrication of functionalized hydrogels for cartilage regeneration

6.1. In situ *polymerization*

Apart from perfectly matching irregular AC defects, the other advantage of *in situ* polymerization of hydrogels is the improved integration with surrounding tissues. Functional modification of hydrogel polymers with peptides is a traditional method for improving the adhesion of hydrogels to living tissues. Tamesue et al. [133] reported an adhesive hydrogel system, which could be used and applied easily, using *in situ* polymerization of linear polymers interpenetrated into hydrogel networks. Lee et al. [134] demonstrated that light-triggered *in vivo* activation of cell-adhesive Arg–Gly–Asp (RGD) peptides on implanted hydrogels could promote *in vivo* cell adhesion and tissue integration. Recently, a novel photoinduced-imine-crosslinking hydrogel was fabricated in which HA modified by *o*-nitrobenzyl alcohol moieties generated light-initiated aldehyde groups. These aldehyde groups subsequently bind to the amino groups of the tissue surfaces. Through this method, the adhesion performance of the hydrogel and *in situ* seamless tissue integration were markedly improved

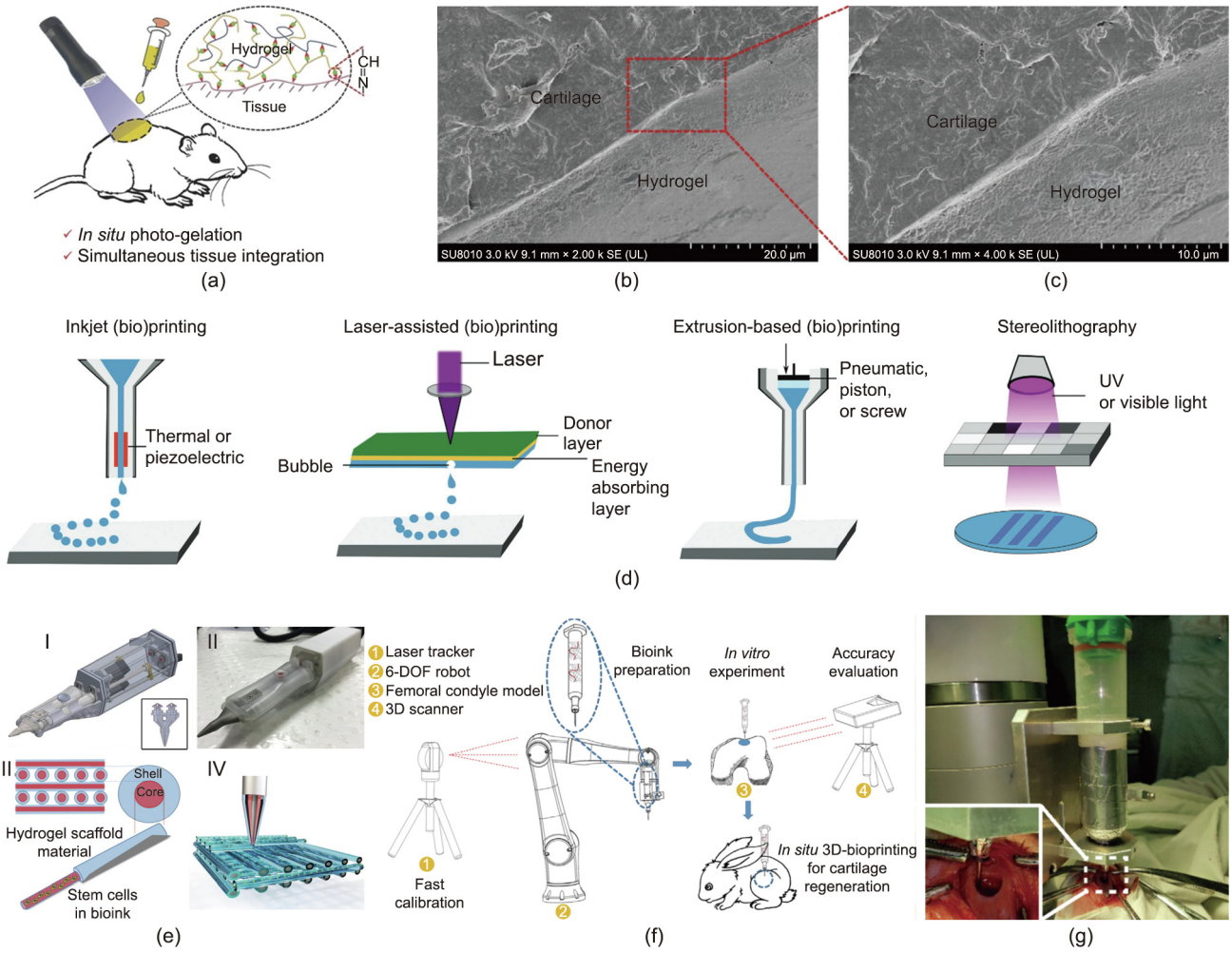


Fig. 6. The representative (bio)fabrication approaches of functionalized hydrogels for cartilage repair. (a) Schematic illustration of *in situ* photo-gelation hydrogel to enhance tissue integration. (b, c) SEM images show the integration of GelMA hydrogel with cartilage after *in situ* polymerization. (d) Four classic 3D-(bio)fabrication methods involving hydrogel bioinks. (e-I) Design and (e-II) appearance of the handheld 3D printer, (e-III) the schematic diagram of the distribution of core and shell, and (e-IV) the multiple layers of 3D-printed block in a criss-cross pattern. (f) Schematic process of robotic-assisted *in situ* 3DBP for AC regeneration. (g) A 6-DOF robotic-assisted *in situ* 3DBP platform for AC repair in a rabbit model. UV: ultraviolet; SLA: stereolithography; DOF: degree of freedom. (a) Reproduced from Ref. [135] with permission of WILEY-VCH, © 2016; (b, c) reproduced from Ref. [136] with permission of Elsevier, © 2018; (d) reproduced from Ref. [139] with permission of WILEY-VCH, © 2018; (e) reproduced from Ref. [150] with permission of John Wiley & Sons, © 2018; (f, g) reproduced from Ref. [160] with permission of Elsevier, © 2020.

[135] (Fig. 6(a)). Furthermore, Zhou et al. [136] prepared a double-network hydrogel with light-initiated aldehyde groups composed of gelatin methacrylate, oxidized dextran, and gelatin. Superior integration between the neotissue and native cartilage and improved mechanical properties were achieved simultaneously (Figs. 6(b) and (c)).

6.2. Molding

Gel molding is a remarkably flexible and simple (bio)fabrication technique for functional hydrogels in ACTE. Plastics, polymers, and metals can be employed to fabricate hydrogel-based constructs, of which polymers and elastomers are the most widely used [137]. At present, various custom-designed molds can be used to manufacture hydrogels for cartilage repair. The molding process helps construct cartilage-biomimetic hydrogels with zonal structure and specified cell and fiber orientations at high resolution. A photomask with parallel strips has been utilized to guide cell arrangement, which could potentially mimic the zonal cell phenotype in the native cartilage [82]. The microengineered 3D MSC-laden hydrogel mimicked the cell shape and organization in the SZ of cartilage, with the significantly upregulated secretion of GAGs and total collagen after four weeks. Micropatterning is an effective method for mimicking the zonal arrangement of cells and provides a convenient and precise strategy for the (bio)fabrication of hydrogels. Owida et al. [77] fabricated zonal-specific 3D hybrid scaffolds (HA hydrogel with aligned polylactic acid nanofiber meshes) to simulate collagen orientation in the cartilage matrix, inducing the formation of zonal-specific morphology of chondrocytes and ECM synthesis. Owing to the rapid development of electroconductive [138] and magnetic conductive [80] composite biomaterials, functionalized hydrogels can combine several inherent advantageous properties of conductive materials with their own highly tunable physical and biochemical features. The (bio)fabrication resolution by hydrogel molding can reach the microscale or even nanoscale.

6.3. 3DBP

3DBP, a form of additive manufacturing that involves building a tissue or organ layer-by-layer using the bottom-up approach, has drawn increasing attention for ACTE. Unlike 3D-printing,

3D-bioprinting includes additional complexities (e.g., choice of biomaterial types, cell sources, bioactive factors, and technical concerns relevant to the sensitivities of living cells and the formation of neo-tissues). To regenerate hyaline AC, many novel functionalized hydrogels acting as (bio)inks have been widely explored in 3DBP technologies, such as inkjet, laser-assisted, and extrusion-based (bio)printing [1,139] (Fig. 6(d)).

(1) Inkjet (bio)printing can print functional hydrogel-based constructs at high speed through the precise control of droplets and simultaneously obtain high cell viability and low cost at the same time [140]. The drawbacks of inkjet (bio)printing include a limited variety of bioinks, clogging of the nozzle, restriction of structural integrity, acoustic and thermal stresses on cells, and low cell density [141]. Gao et al. [142] fabricated a human MSCs laden poly(ethylene glycol) dimethacrylate (PEGDMA)–GelMA hybrid hydrogel for ACTE with good mechanical properties by 3D inkjet (bio)printing. Recently, a microreactive inkjet (bio)printing system enabled freestanding 3D ALG hydrogel microstructures based on the in-air collision of the precursor and crosslinker microdroplets, which addressed the deficiency of conventional inkjet (bio)printing methods to some degree [143].

(2) Laser-assisted (bio)printing is a modified version of the laser-induced forward-transfer technique. In general, this technique allows high cell viability, density, and printing resolution, and a wide range of viscosities. But it is relatively expensive and restricted by fabricating thin structures, and has limited availability of materials with viscosities of up to 300 MPa·s^{-1} [140,141]. Gruene et al. [144] produced an MSC-laden ALG hydrogel construct by laser-assisted (bio)printing, and laser-printed MSCs grafts could be differentiated into cartilage *in vitro* with high expression of chondrogenic markers.

(3) Extrusion-based (bio)printing (robotic dispensing) is the most widely used method with distinct advantages over cost, viscosity range (from 30 to 6 × 10^7 MPa·s^{-1}), cell density, and multimaterial printing [140]. Pati et al. [39] fabricated cartilage grafts by dECM hydrogels through extrusion-based (bio)printing, and the printed dECM structures achieved high cell viability and desirable functionality. However, there are several disadvantages, including nozzle mechanical or shear stress on cells, low printing speed, and relatively low resolution [141]. In other words, the major concern of 3DBP is the balance between the optimization of print parameters and the control of material properties to yield biomimetic constructs with high biological activities [145]. For extrusion-based (bio)printing, it is challenging to provide high-resolution and structurally reliable printed constructs while protecting cells from shear forces during (bio)printing. Zandi et al. [146] developed three types of nanocomposite hydrogels based on silicate nanomaterials, laponite, and GAGs nanoparticles, respectively, and found the shear-thinning behavior of nanocomposite hydrogels could prevent encapsulated cells from aggressive shear stress during (bio)printing. A novel strategy named freeform reversible embedding of suspended hydrogels (FRESH) has been established through extrusion-based (bio)printing in a support bath [147]. FRESH extrudes bioinks within a yield-stress support bath, holding the bioinks in a targeted place until cured [148]. The support bath can be a solution with crosslinkers or soft pregels with self-healing abilities. The concern of cell viability can be completely relieved if the cells are suspended in the support bath. Further release or enhancement of the support bath can obtain the final 3D hydrogels.

Co-axial extrusion (bio)printing can be handled using a new bioink with gentle, freestanding bioassembling tissue strands. O'Connell et al. [149] designed a co-axial extrusion (bio)printing system that allowed two different bioinks in a core/shell distribution. Multiple ink formulations with a collinear geometry were obtained using custom-made titanium nozzles. This system provides a handheld device for surgical printing of cartilage repair [150,151]. They further improved the "Biopen" and rebuilt it to add a 405 nm light-emitting diode near the tip of the nozzle [152]. This advancement allowed a soft or liquid bioink to be retained by the rapidly photocrosslinked shell hydrogels. Moreover, Yu et al. [153] successfully fabricated cartilage tissue patches by ALG bioinks through robotic-assisted co-axial extrusion (bio)printing. The *in vivo* results showed enhanced chondrogenesis and integration with the surrounding cartilage tissues. In addition, to improve the printing resolution of extrusion-based (bio)printing, Castilho et al. [154] developed novel hydrogel-based bioinks (based on gelatin and silk fibroin) for cell electrowriting, and the diameters of printed well-organized cell-laden fiber structures ranged from 5 to 40 μm, providing fascinating opportunities for the reproduction of intrinsic functions and morphologies of living tissues.

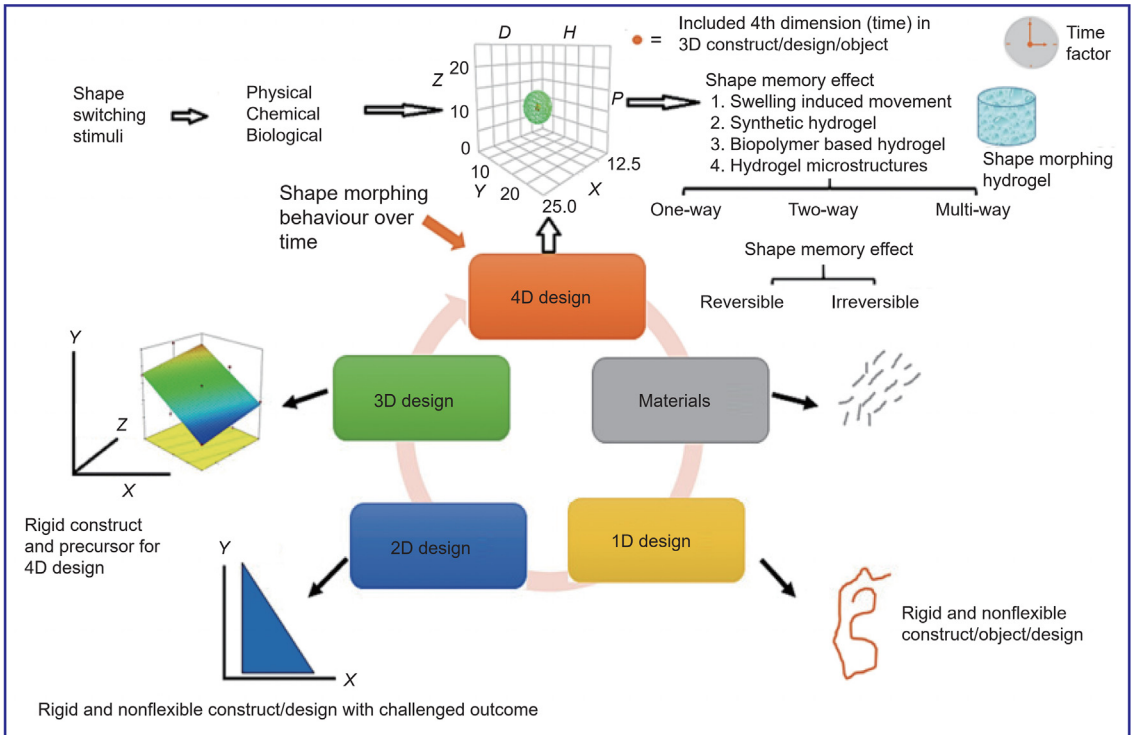
(4) Stereolithography (SLA) is a 3DBP process with the light crosslink (e.g., ultraviolet (UV) light) of a photosensitive bioink on the polymerization plane [155]. Only photocrosslinkable polymers with a small viscosity range can be (bio)printed through SLA, which hampers its application. Recently, Hong et al. [156] used a silk fibroin hydrogel for digital light processing technology 3DBP and promoted chondrogenesis both *in vitro* and *in vivo*. Aisenbrey et al. [157] printed a hybrid scaffold that combined an SLA-based 3D-printed support structure with an injectable and photopolymerizable hydrogel for delivering chondrocytes to repair focal chondral defects.

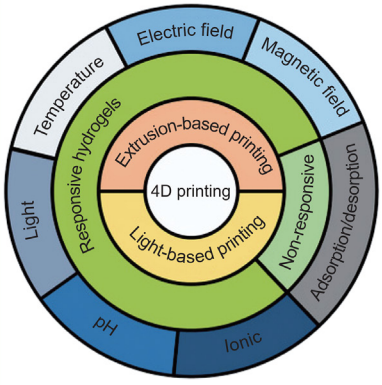
6.4. In situ *3DBP*

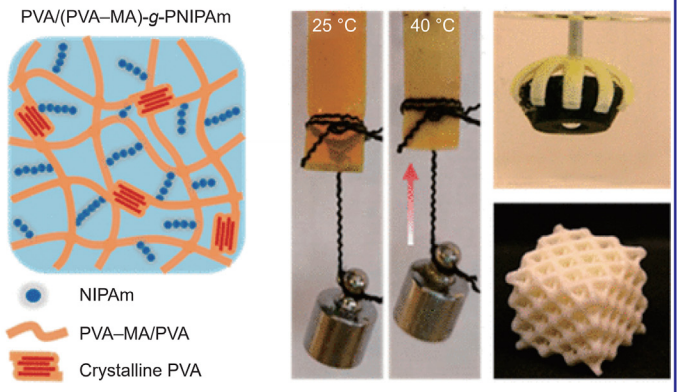
In vitro 3DBP technologies exist several weaknesses that impede their clinical translation, including mismatches with patient-specific defects, multistep procedures, risk of contamination, and post-processing manipulation requirements. However, *in situ* 3DBP, the next frontier for 3DBP, attempts to manufacture new tissues *in vivo* directly in patients undergoing surgical treatments [158].

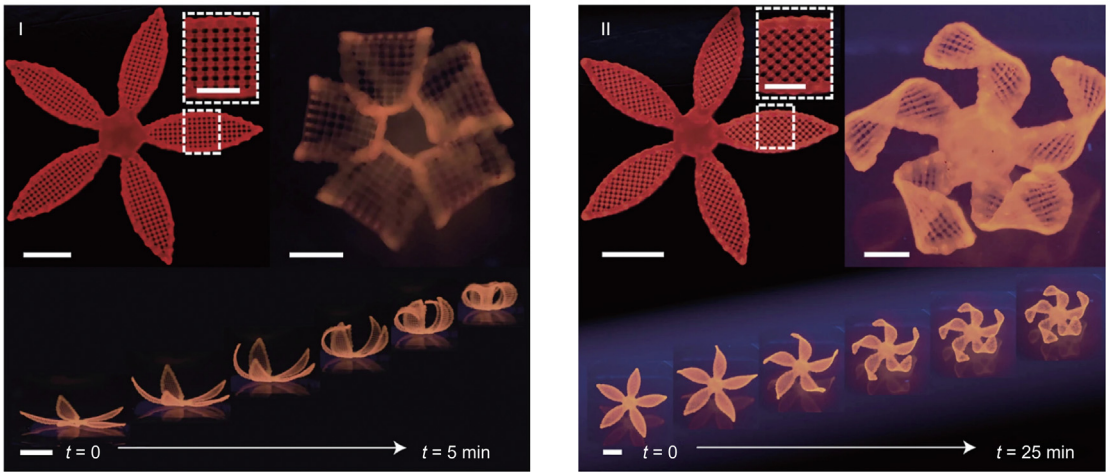
Recently, a novel *in situ* 3D printer "Biopen" was applied to reconstruct a standardized critical-sized full-thickness chondral defect of a sheep *in vivo* [150] (Fig. 6(e)). The flexibility of *in situ* (bio)fabrication through "Biopen" allowed surgeons to fill irregular cartilage defects with different hydrogel bioinks in a freeform style. As a portable medical device for manual manipulation, it can print a functionalized hydrogel-based 3D construct in a layer-by-layer fashion at a higher resolution than *in situ* polymerized hydrogels, providing high plasticity in geometry and morphology of cartilage defect areas and high fidelity. Through a handheld 3D scanner, Li et al. [159] used *in situ* 3DBP to repair cartilage defects and demonstrated the feasibility and high efficacy of dynamic imaging evaluation for real-time amendments. Ma et al. [160] further established a six-degree-of-freedom robot-assisted *in situ* 3DBP platform for *in vivo* cartilage regeneration by utilizing HA methacrylate bioink (Figs. 6(f) and (g)). A fast center-point calibration tool on a resin model was successfully developed to improve the robot's movement and *in situ* 3DBP accuracy. After calibration, the surface error was < 30 μm. Meanwhile, micro-computed tomography (μ-CT) was applied to monitor the robot-assisted 3DBP process. An *in vivo* rabbit experiment indicated that the well-established *in situ* 3DBP system could repair ICRS grade IV cartilage defects after 12 weeks. Cartilage injury was repaired after 12 weeks. This study revealed the potential of this technology for clinical applications.

To facilitate *in situ* 3DBP towards clinical applications, the formulation and characterization of functionalized hydrogel-based inks need to be improved. Moreover, some concerns should be addressed to achieve high shape fidelity, fast gelation, excellent


Shape switching stimuli
Physical
Chemical
Biological
D
H
Z
P
X
Y
20
10
0
12.5
25.0
= Included 4th dimension (time) in 3D construct/design/object
Time factor
Shape memory effect
1. Swelling induced movement
2. Synthetic hydrogel
3. Biopolymer based hydrogel
4. Hydrogel microstructures
Shape morphing hydrogel
One-way
Two-way
Multi-way
Shape morphing behaviour over time
Shape memory effect
Reversible
Irreversible
4D design
3D design
Materials
2D design
1D design
Rigid construct and precursor for 4D design
Rigid and nonflexible construct/object/design
Rigid and nonflexible construct/design with challenged outcome
(a)


Electric field
Magnetic field
Temperature
Responsive hydrogels
Extrusion-based printing
4D printing
Light-based printing
Non-responsive
Adsorption/desorption
Light
pH
Ionic
(b)


PVA/(PVA–MA)-g-PNIPAm
NIPAm
PVA–MA/PVA
Crystalline PVA
25 °C
40 °C
(c)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(d)

mechanical and biocompatible properties, and fewer post-processing processes for *in situ* 3DBP [158].

6.5. 4D-(bio)fabrication

As mentioned before, 3D-(bio)fabrication, especially 3DBP, permits the biomimetic construction of objects by layer-by-layer deposition of biomaterials, leading to precisely controlled dimensions and characteristics of printed tissues with complicated structures. However, 4D-(bio)fabricated objects are 3D-(bio)fabricated structures whose shapes, properties, and/or functions can self-transform over time when exposed to predetermined external stimuli (e.g., temperature, electric field, magnetic field, light, pH, and ions) after fabrication [161-163] (Figs. 7(a) and (b)). The emerging field of 4D printing has grown in interest from both academia and industry since its introduction in 2013. Stimuli-responsive hydrogels have become a competitive and versatile group of biomaterials for 4D printed devices because of their excellent deformability, favorable biocompatibility, low cost, and simple manufacturing processes. Hua et al. [164] presented a biomaterial design that combined thermoresponsive and tough components in a single hydrogel network by 4D printing, which enabled the synergistic realization of high toughness (100 times higher toughness $\sim$10 MJ·m^{-3}) and actuation performance (20 times higher actuation stress $\sim$10 kPa) compared to conventional poly(*N*-isopropylacrylamide) (PNIPAm) hydrogels (Fig. 7(c)). Gladman et al. [163] created a series of functional folding flower architectures (petals in floral form) to demonstrate the capabilities of 4D printing by combining patterns that generated simple curved surfaces (Fig. 7(d)).

In ACTE, 4D-(bio)fabricated hydrogels are able to change their previous shapes, structures, functions, and properties over time as designed, which are beneficial for the cartilage tissue healing process. For example, structure- and degradability-responsive hydrogels can lead to internal structural changes and controlled degradation rates, which are crucial for coordinating the dynamic structural support and tissue ingrowth space. The fast degradability or non-degradability of hydrogels can result in unfavorable consequences, such as insufficient mechanical support or undermining the growth of neocartilage. Thus, the degradation and structural changes of functionalized hydrogels should be carefully balanced against the speed of cartilage formation. However, it is difficult to control the properties of hydrogels accurately. In cases, when the hydrogels can sense dynamic changes and adjust their properties in real-time with the regenerated neotissue, it is possible to sustain the desired properties throughout the regeneration process over time, not only in a specific period. Shape-memory materials have the potential to achieve this goal [165]. Almeida et al. [166] proposed a shape-morphing ALG hydrogel to support the development of complex cartilaginous tissues *in vitro*. This biomaterial can sense newly synthesized collagen and GAGs in hydrogels and dynamically change their structure to sustain the architecture and mechanical properties for ACTE. As yet, there have been very few cases of 4D printing for cartilage repair, although many 3D-printed functional hydrogels have been widely reported to enhance AC repair and regeneration both *in vitro* and *in vivo*. Thus, more preclinical studies on 4D-(bio)fabricated hydrogels, especially 4D-printed functionalized hydrogels, are anticipated soon.

7. Conclusions and future directions

During the past two decades, tremendous progress has been achieved in ACTE by utilizing functionalized hydrogels, which can provide unlimited possibilities for the future; however, much more remains to be done to develop truly biomimetic cartilage regenerative therapies. The translation of functionalized hydrogels into the market depends on various parameters. Currently, there still exist several major challenges to overcome. Apart from the material sources of hydrogels, the distribution of cells and/or bioactive factors within the functional hydrogels, the response of cells (e.g., endogenous and exogenous cells) to biophysical and biochemical cues, mechanical properties (e.g., elasticity, stiffness, and confinement) of hydrogels, and insufficient integration with adjacent cartilage/bone tissues should be considered. In other words, functionalized hydrogels should be biocompatible and non-immunogenic to prevent detrimental inflammatory responses at implantation sites. Innovative strategies should increase the capacity of implanted hydrogel-based constructs to survive, adapt, and withstand the biomechanically arduous joint environment, suggesting that increased use of *ex vivo* multiaxial bioreactors may be needed before progressing to *in vivo* studies. In addition, hydrogels should be suitable for forming neocartilages with customized shapes. From the angle of translation, a streamlined international standardization of the approval procedures, development of more appropriate animal models with AC defects for *in vivo* evaluation, standardized biomechanical and biochemical assessment to confirm the quality of the generated neocartilage compared with adjacent tissues, as well as well-designed clinical studies play vital roles. An integrated quantification system for AC repair and its specified parameters should be identified. If possible, we would better employ non-invasive, quantitative measurements throughout the entire process to acquire information on long-term results. Such information will be of great significance to verify which hydrogel-based strategies truly regenerate neocartilage comparable to healthy and native AC. These grand challenges require unprecedented close cooperation from scientists and researchers with backgrounds in biomedical sciences, materials, chemistry, and engineering, along with surgeons, companies, and regulatory bodies.

To date, an optimal functionalized hydrogel-based solution has not yet been proposed for the formation of hyaline neocartilage with long-term maintenance of joint functionalities. So, future studies should focus on the following goals: ① exploring novel (bio)fabrication technologies (e.g., *in situ* polymerization, 3DBP, and 4D-(bio)fabrication) to synthesize functional hydrogels with desirable 3D architecture, degradability, biomechanics, plasticity, adhesiveness, cytocompatibility, and chondroinductive properties; ② encapsulating cells, presenting and delivering biochemical factors within hydrogels in a spatiotemporally controlled manner to restore cartilage defects with specific zonal structure and mechanical function, and elucidating the underlying molecular mechanisms of cell migration and differentiation, phenotype maintenance, cell-hydrogel interactions, as well as the anti-inflammatory, immunomodulatory, and reparative effects of cells and bioactive factors; ③ devoting to clinical translation and developing functionalized hydrogel-based bioproducts for healing AC lesions.

Fig. 7. Schematic representation of 4D-(bio)fabrication of functionalized hydrogels and their stimuli-responsive properties, such as high toughness and complex morphologies. (a) The evolutionary concept of 1D transformation to 4D transformation in hydrogels. 4D design of functionalized hydrogels under several shape-switching stimuli to achieve favorable temporary shape-memory effects (reversible or irreversible). (b) 4D printing of hydrogels. (c) 4D printable tough and thermoresponsive hydrogels. (d) Complex flower morphologies generated by biomimetic 4D printing (scale bars, 5 mm, inset = 2.5 mm). PVA–MA: poly(vinyl alcohol)–methyl acrylate; NIPAm: *N*-isopropylacrylamide. (a) Reproduced from Ref. [161] with permission of MDPI, © 2021; (b) reproduced from Ref. [162] with permission of John Wiley & Sons, © 2020; (c) reproduced from Ref. [164] with permission of American Chemical Society, © 2021; (d) reproduced from Ref. [163] with permission of Springer Nature, © 2016.

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Authors' contributions

Liangbin Zhou and Ling Qin conceptualized and outlined the content. Liangbin Zhou, Peng Guo, Matteo D'Este, Wenxue Tong, Jiankun Xu, Hao Yao, Martin J. Stoddart, Gerjo J.V.M. van Osch, Kevin Ki-Wai Ho, Zhen Li, and Ling Qin discussed the content of the paper, collected the literature, and drafted the manuscript collectively. All authors revised and commented on the manuscript.

Compliance with ethics guidelines

Liangbin Zhou, Peng Guo, Matteo D'Este, Wenxue Tong, Jiankun Xu, Hao Yao, Martin J. Stoddart, Gerjo J.V.M. van Osch, Kevin Ki-Wai Ho, Zhen Li, and Ling Qin declare that they have no conflict of interest or financial conflicts to disclose.

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