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TOPICAL REVIEW

Engineering three-dimensional cell mechanical microenvironment with hydrogels

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Abstract
Cell mechanical microenvironment (CMM) significantly affects cell behaviors such as spreading, migration, proliferation and differentiation. However, most studies on cell response to mechanical stimulation are based on two-dimensional (2D) planar substrates, which cannot mimic native three-dimensional (3D) CMM. Accumulating evidence has shown that there is a significant difference in cell behavior in 2D and 3D microenvironments. Among the materials used for engineering 3D CMM, hydrogels have gained increasing attention due to their tunable properties (e.g. chemical and mechanical properties). In this paper, we provide an overview of recent advances in engineering hydrogel-based 3D CMM. Effects of mechanical cues (e.g. hydrogel stiffness and externally induced stress/strain in hydrogels) on cell behaviors are described. A variety of approaches to load mechanical stimuli in 3D hydrogel-based constructs are also discussed.

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

1. Introduction

Mechanical cues of cell microenvironment play a significant role in regulating cell behaviors such as cell spreading, migration, proliferation and differentiation [1–4]. Cells sense the mechanical microenvironment via transmembrane molecules (e.g. integrins) and regulate the physiological processes through mechanotransduction [5, 6]. Cells such as fibroblasts, chondrocytes, endothelial cells (ECs), smooth muscle cells (SMCs) and mesenchymal stem cells (MSCs) exhibit different mechano-responsive behaviors, depending upon the mechanical properties of the extracellular matrix (ECM), the mechanical loading modes, and the cell development stages. For instance, fibroblasts adhere to stiffer substrates more strongly, spread farther and migrate faster than those cells on softer substrates [7]. Human MSCs (hMSCs) exhibit neurogenic, myogenic and osteogenic phenotypes when cultured on collagen-coated polyacrylamide (PAAm) hydrogels with stiffness similar to brain (0.1–1 kPa), muscle (8–17 kPa) and nascent bone (>34 kPa), respectively [8]. However, most of these studies have been performed on two-dimensional (2D) substrates, which may not represent the situation *in vivo*. Cells naturally reside in a three-dimensional (3D) niche, and accumulating evidence indicates

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that cells may respond differently when situated in 3D and 2D mechanical microenvironments [9–11]. For example, the vinculin expression of SMCs encapsulated in poly(ethylene glycol) (PEG)-conjugated fibrinogen is lower compared to that in 2D [12]. The focal adhesion (FA) composition and function of fibroblasts encapsulated in a 3D matrix were significantly different from those on 2D substrates [13]. Therefore, it is of great interest and importance to study the cell response to mechanical cues in a biologically relevant 3D microenvironment.

To mimic cell behavior in natural microenvironment, various 3D culture models have been established based on hydrogels, which offer several advantages such as high water content, biocompatibility, as well as tunable chemical and physical properties [10, 14–16]. These hydrogel-based 3D culture models are also utilized to engineer a 3D cell mechanical microenvironment (CMM). For example, cardiomyocytes encapsulated in PEG-fibrinogen hydrogels [17], cancer cells encapsulated in Matrigels [18] or fibrin hydrogels [19], and stem cells encapsulated in methacrylated hyaluronic acid (MeHA) hydrogels [20] have been developed to study the responses of tissue contraction, tumor growth and metastasis, and stem cell differentiation to mechanical stiffness, respectively. These hydrogel-based CMM models with tunable mechanical properties offer valuable opportunities to study fundamental biological mechanisms, which can in turn facilitate biomedical applications in stem cell engineering, cancer therapy, and tissue engineering.

In this review, we start with discussion of mechanical cues of hydrogels and their effects on cell behaviors in engineered 3D CMM. We then review different approaches to engineer hydrogel-based 3D CMM *in vitro*. Lastly, we highlight the challenges and future directions for engineering hydrogel based 3D CMM.

2. Mechanical cues from hydrogels for engineering 3D CMM

Hydrogels are 3D polymer networks swollen with high percentage of water, which are similar to native ECM. This beneficial property, in addition to their tunable mechanical nature, makes hydrogels ideal candidates for engineering 3D CMM. Both naturally derived (e.g. collagen, hyaluronic acid (HA), fibrin) and synthetic hydrogels (e.g. PEG, PAAm) have been employed to engineer 3D CMM. Collagen is a group of proteins found in native ECM, where collagen type I is often used in hydrogels for cell encapsulation and for the study of cell traction [21]. Another widely used hydrogel is HA, a non-sulfated glycosaminoglycan, which can be found throughout connective, epithelial, and neural tissues [22]. Hydrogels derived from natural polymers are usually mechanically weak and have limited controllability of mechanical properties. To overcome this challenge, incorporation of functional groups (e.g. acrylate [23], thiolate [24], tryamine [25]) and addition of other composites (e.g. collagen-alginate [26], agarose-PEG [27], alginate-PAAm [28]) have been attempted. In contrast, synthetic hydrogels have well-defined compositions and possess better controllability of mechanical properties. For example, the mechanical properties of PEG diacrylate (PEGDA) can be adjusted by changing its monomer molecular weight, polymer concentration, light intensity and exposure time during ultraviolet (UV) light-induced crosslinking. Moreover, synthetic hydrogels can be easily modified with bioactive molecules such as peptides, growth factors and biodegradable units [29].

Generally speaking, there are two main types of mechanical cues that cells may experience from hydrogels: the stiffness or elasticity of hydrogels, and the stress/strain from hydrogel-based constructs. We here describe them in details, especially their effects on cell behaviors.

2.1. Stiffness of hydrogels

The most common mechanical cue that cells experience is stiffness, which ranges from 0.1 kPa (e.g. brain) to 40 kPa (e.g. osteoid) for native soft tissues [30] (figure 1(*A*)). It has been found that most cells exhibit *in vivo*-like behaviors when cultured in a mechanically compatible microenvironment [30, 31] (figures 1(*B*) and (*C*)). For instance, cardiomyocytes showed optimized contractibility, striated myofibrils and rhythmic beating on 10 kPa PAAm hydrogels that mimic the stiffness of adult cardiac muscle; while they exhibited overstrained morphology, with lost striated myofibrils and rhythmic contraction on stiffer hydrogels (e.g. similar to the stiffness of post-infarct fibrotic scar) [31]. The osteogenic differentiation of murine MSCs (mMSCs) was found to occur prevalently in 3D Arg-Gly-Asp (RGD)-modified alginate (figures 1(*C-a*) and (*C-d*)), RGD-modified agarose (figures 1(*C-b*)) and RGD-modified PEGDA (figures 1(*C-c*)) hydrogels with intermediate stiffness (11–30 kPa) [32].

Many native tissues are mechanically heterogeneous, such as tendons, heart valves and calcific vascular tissues [33–35]. To investigate the effects of mechanical heterogeneity on cells, several hydrogel systems with patterned or gradient mechanical properties have been developed. For example, PEGDA hydrogels with selectively photopolymerized mechanical patterns were fabricated to guide cell growth [36]. Acrylated HA hydrogels with patterned mechanical regions were created via sequential crosslinking to control 3D cell spreading [23]. Hydrogels with gradient PEGDA and constant Arg-Gly-Asp-Ser concentration were also fabricated to study cell morphology on a gradient mechanical surface [37]. It was found that cells tended to migrate toward increasing stiffness, termed as durotaxis or mechanotaxis [7].

Native CMM may dynamically change with time during development, aging, pathological processes and tissue-biomaterial interface remolding [38–41]. A well-known example is that fibrotic scars show stiffening behavior during the wound-healing process [42]. The dynamic change of stiffness can favor cell migration, proliferation, and differentiation [8, 43–45]. In these cases, hydrogels with constant stiffness may not mimic the native dynamic CMM. To address this, a thiolated-HA hydrogel system was developed to model the mechanical stiffening of developing myocardium, with the stiffness changing from ∼1 to ∼8 kPa over a period

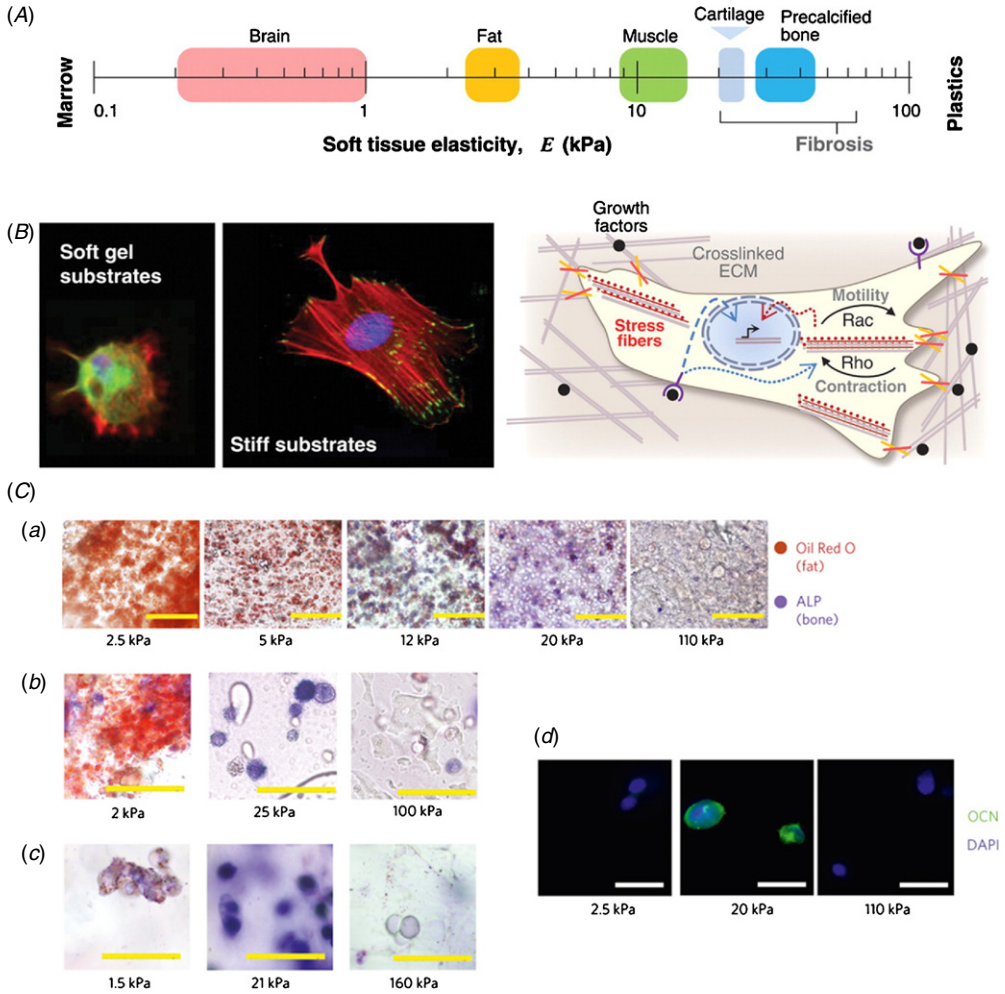

Figure 1. Soft-tissue stiffness and cell response to mechanical stiffness. (*A*) The stiffness of soft tissues ranges from 0.1 to 100 kPa. From [30]. Reprinted with permission from AAAS. (*B*) Stiff substrates enhance cell anchor and spreading. From [30]. Reprinted with permission from AAAS. (*C*) MSC differentiation determined by stiffness of 3D hydrogels: (*a*)–(*c*) alkaline phosphatase (ALP) activity (fast blue; osteogenic biomarker, blue) and neutral lipid accumulation (oil red O; adipogenic biomarker, red) of mMSC after one week of culture in RGD-modified alginate (*a*), RGD-modified agarose (*b*) and an RGD-modified PEGDA (*c*) hydrogels with different stiffness; (*d*) osteocalcin (OCN, green) and nuclear counterstain 40′,6-diamidino-2-phenylindole (DAPI, blue) staining in cell-laden alginate cryosections. Scale bars for (*C*) are (*a*) 100 μm, (*b*)–(*c*) 50 μm and (*d*) 20 μm, respectively. Reprinted by permission from Macmillan Publishers Ltd: *Nature Mater.* [32], copyright 2008.

of 300 h [24]. In another study, a DNA crosslinked PAAm hydrogel with temporal dynamic stiffness was synthesized by controlled DNA delivery [43]. It was demonstrated that fibroblasts responded differently, depending on the magnitude and range of dynamic stiffness of hydrogels. In addition, stiffness softening of hydrogels was also found to affect fibroblast morphology [42]. The softening of hydrogels can be induced by swelling, degradation or de-crosslinking of hydrogel networks. It has been widely proved in tissue engineering that the degradation speed of hydrogels should match the ECM secretion speed by cells encapsulated in hydrogels [46–49]. Thus, to obtain appropriate dynamic CMM for cells, the time-dependent stiffening and softening behaviors of hydrogels should be optimized according to specific cell types.

2.2. Stress/strain from hydrogel-based constructs

Besides stiffness of ECM, some cells can sense and respond to stress/strain of the microenvironment *in vivo*. Fibroblasts in ligament, chondrocytes in cartilage, and ECs in vascular tissues experience tension, compression and shear stress, respectively, and accordingly regulate their morphology, migration, proliferation, and differentiation [50–53].

Among the different loading modes, mechanical stretch plays an active role in many physiological processes such as muscle contraction and heart beating. It has been utilized to stimulate cells in many engineered tissues (e.g. muscle, ligament, tendon and vasculature) to enhance their organization, strength and functionality [50, 54]. For instance, SMCs showed directed cell alignment and migration in collagen along the direction of mechanical stretch [55], down-regulated expression of bone-associated genes, and decreased deposition of calcium [56]. For myocardial cells in collagen, cyclic stretch promoted the formation of interconnected and longitudinally oriented cardiac muscle bundles [57]. Additionally, cardiac cells in fibrin and fibroblasts in collagen tend to increase collagen secretion under cyclic stretch [58, 59].

While mechanical stretch favors cell growth (e.g. SMCs, myocardial cells) in engineered tissues, compression and shear stress selectively affect cells such as chondrocytes, MSCs and ECs. Dynamic compression greatly increased equilibrium modulus, glycosaminoglycan and hydroxyproline content of chondrocytes encapsulated agarose hydrogels [60]. Dynamic compression also enhanced production and uniform distribution of the cartilage matrix in MSC-laden HA hydrogels, reduced hypertrophic maker expression and suppressed calcification [53]. Another interesting work from Bryant *et al* [61] showed that chondrocytes encapsulated in PEG hydrogels exhibited heterogeneous deformation under static compression, even though the deformation of hydrogels was uniform. This result indicated that the cell population and development stage may affect cell behavior under mechanical stimulation. Mechanical compression can have comprehensive effects on cell behavior when combined with some chemical factors. For instance, dynamic compression up-regulated the expression of cartilage-specific genes and the secretion of ECM for both marrow-derived MSCs and human embryoid body-derived (hEBd) cells in PEGDA independently of the presence of transforming growth factor-beta 1 (TGF-β1) [62]. Meanwhile, chondrogenic differentiation was inhibited by mechanical compression without TGF-β1 [62]. It was also demonstrated that RGD played either a negative or an active role in the phenotype expression of chondrocytes encapsulated in PEGDA, depending on the absence or presence of dynamic compression [63].

ECs in the human vascular system experience shear stress from blood flow, which plays an important role in vascular stability and functionality [64, 65]. Recently, hydrogels with endothelialized microfluidic channels were developed to engineer vascularized tissue constructs [66–68]. These endothelialized microfluidic hydrogels may also provide an excellent *in vitro* vascular model with an *in vivo*-like 3D microenvironment to study vascular functionality and diseases. However, to accomplish this goal, many factors should be further investigated, including hydrogel types, microchannel structure and inducible chemical molecules (e.g. second messenger cyclic adenosine monophosphate), as well as flow velocity [14, 69].

The same type of cells may respond differently to various mechanical stimulations [59, 70, 71]. For example, MSCs in ligaments experience stretch, compression and shear stress as induced by body movement. It was shown that MSCs exhibited distinct morphology and proliferation behavior when subjected to dynamic stretch, pressure, and shear stress *in vitro* with varying magnitude, frequency and duration [71].

3. Methods for engineering 3D CMM with hydrogels

A variety of methods have been developed to engineer 3D CMM with hydrogels, by varying hydrogel stiffness or inducing stress/strain in hydrogel-based constructs. Specifically, these mechanical stimulations have been achieved by using different hydrogels, or by changing polymer concentration, crosslinking density [17] or environmental conditions (e.g. pH, temperature, electric and/or magnetic field) [72, 73]. Alternatively, incorporating other mechanically enhanced or degradable units [46], applying a scaffold constraint [74] or external stretch/compression [50, 53], and controlling fluid flow [75, 76] have also been demonstrated.

3.1. Control of hydrogel mechanical properties

The most commonly used method to modulate hydrogel mechanical properties (e.g. stiffness) is to change polymer concentration. The resultant stiffness of hydrogels can range from ~Pa to ~MPa, especially for some synthetic hydrogels such as PEG. By increasing polymer concentration from 10% to 20%, Bryant *et al* [77] created PEG-based hydrogels with stiffnesses ranging from 60 to 500 kPa to facilitate cartilage production. In addition, it has been shown that the introduction of an interpenetrating polymer network (IPN) may greatly enhance the stiffness of hydrogels [27, 78, 79].

Another method to modulate hydrogel stiffness is to change the crosslinking density or molecular weight of polymer networks without increasing polymer concentration. HA-tyramine hydrogels were explored to encapsulate MSCs for engineering cartilage [25]. The stiffness of hydrogels can be increased from 5.4 to 11.8 kPa by increasing the concentration of hydrogen peroxide (H_2O_2) from 500 to 1000 μM, which was associated with the crosslinking degree. Such hydrogels can enhance cellular condensation and chondrogenesis at low crosslinking densities. Alternatively, porous MeHA hydrogels, with tunable stiffness ranging from ~1.5 to 12.4 kPa, were fabricated by changing crosslinking density with a sequential UV polymerization [20]. The morphology, proliferation and differentiation of hMSCs on such hydrogels depended on the stiffness of MeHA hydrogels.

To mimic the mechanical heterogeneity of native tissues, microengineering methods (e.g. photopatterning) have been incorporated to fabricate mechanically heterogeneous hydrogels [80–82]. Photopatterning is widely used to create microgels of various shapes, and it has been recently explored to create hydrogels with patterned and gradient stiffness by using specially designed masks [82, 83]. Marklein *et al* [84] fabricated MeHA hydrogels with patterned stiffness to investigate the response of hMSCs to local stiffness variations. PEGDA hydrogels with different stiffness patterns were formed by UV photopolymerization [82, 85], figure 2(*A*). Mechanical gradient PEGDA hydrogels were also formed using gradient masks (i.e. masks with gradient UV light transmitted patterns) or microfluidics method [82, 86].

CMM is dynamically changing, either softening or stiffening, during tissue development, wound healing, regeneration and tumor invasion [87, 88]. It is of great interest to investigate and control these biological processes *in vitro*. Biodegradable hydrogels are typical softening systems and they have been widely used for 3D cell culture and tissue engineering [46]. The degradation of hydrogels can facilitate molecular diffusion and formation of new tissues in hydrogels. The degradation process in cell-laden hydrogel systems may be mediated by the encapsulated cells. For example, hMSCs can secrete matrix metalloproteinase to degrade PEG-peptide hydrogels via thiol-ene photopolymerization, thus enhance

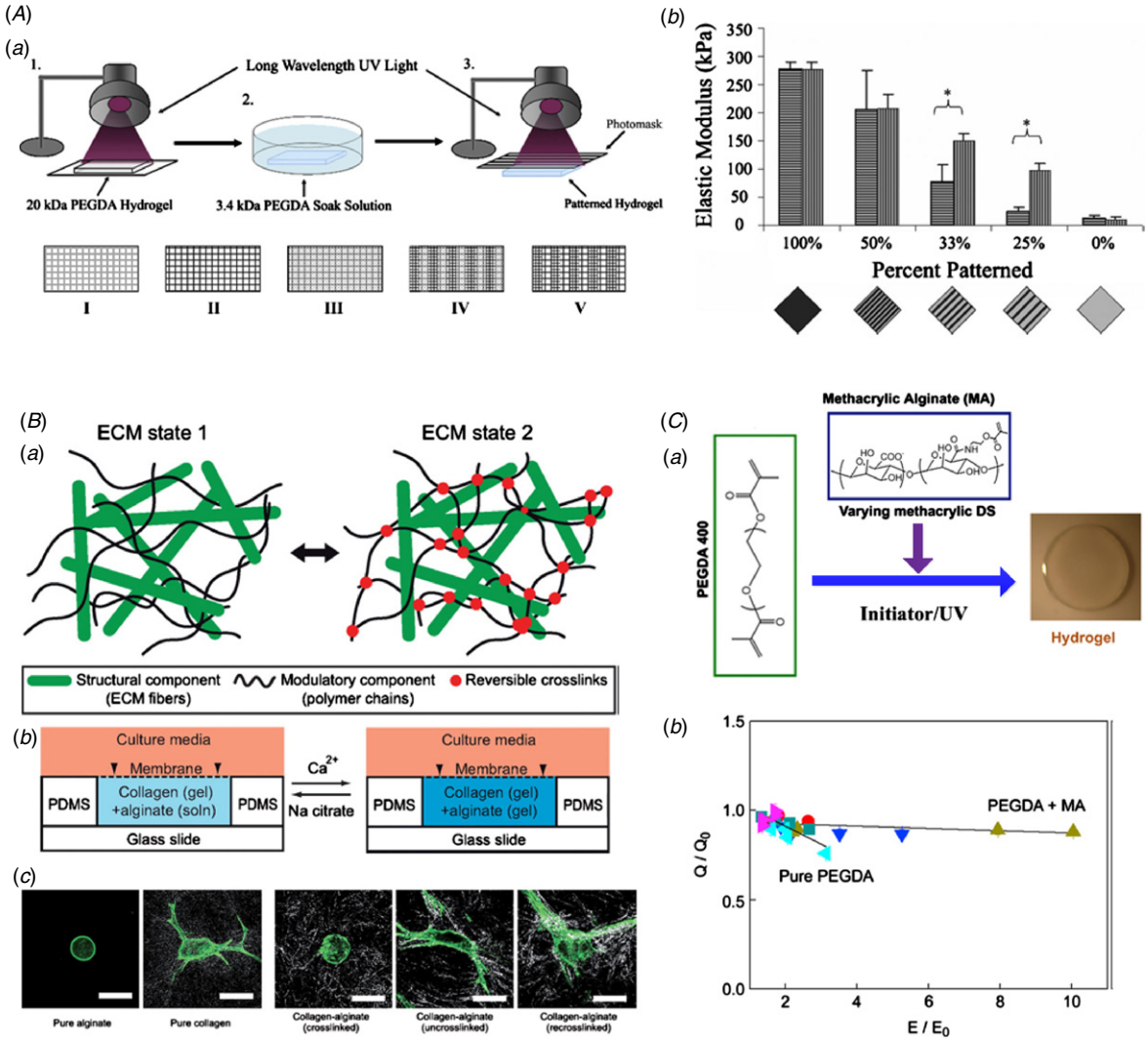

Figure 2. Tuning stiffness of hydrogels. (*A*) Fabrication of mechanical patterned and gradient hydrogels: (*a*) schematic diagram of mechanical patterned hydrogel formation and (*b*) stiffness of patterned hydrogels. Reprinted with permission from [82]. Copyright 2009 Wiley Periodicals, Inc. (*B*) Hydrogels with dynamic changed microenvironment: (*a*) a composite ECM system composed of a structural component (ECM fibers) and a modulatory component (polymer chains), (*b*) schematic illustration of alternating the crosslinking state of alginate in collagen-alginate hydrogels and (*c*) confocal microscopy observed cellular actin (green) of encapsulated fibroblasts and collagen fibers (gray scale) in hydrogels (scale bars, 20 μm). Reprinted with permission from [26]. Copyright 2010 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (*C*) Decoupling the inverse dependency between stiffness and permeability of hydrogels: (*a*) schematics of PEGDA hydrogels crosslinked by MA with varying degrees of methacrylic groups (methacrylic DS) and (*b*) the change of swelling ratio is independent from stiffness of MA-crosslinked PEGDA. Reprinted from [95], copyright 2010, with permission from Elsevier.

their directed differentiation [89]. However, this process is slow and not controllable. The mechanical stiffness of biodegradable hydrogels can gradually decrease over time by tuning their degradability through incorporating hydrolytically or enzymatically degradable blocks into the hydrogel networks [46, 90]. Recently, a photodegradable PEG-based hydrogel was developed by decreasing crosslinking density within minutes under light exposure, which allowed 3D modulation of hydrogel stiffness temporally and spatially in the presence of cells [91]. Directed cell spreading was observed along the degradation gradient, and the largest cell spreading area was observed in the most degraded regions.

Many biological processes are more related to stiffening of CMM rather than softening, and thus a variety of new hydrogels have been developed *in vitro* to mimic the stiffening process of CMM. Guvendiren *et al* [87] developed a light-induced stiffening MeHA hydrogel system (∼3–30 kPa) to investigate the short-term (minutes to hours) and long-term (days to weeks) responses of hMSCs to a dynamic stiffening microenvironment. Gillette *et al* [26] developed a collagen–alginate composite hydrogel, in which collagen was first gelled as a stable structural element, and alginate was gelled subsequently with the introduction of divalent cations to alter the mechanical performance of the composite hydrogels (figure 2(*B*)). However, light-induced stiffening of MeHA hydrogels is irreversible. In addition, the stiffening process changes rapidly, which does not match the time scale of microenvironmental stiffening during maturation of the myocardium and fibrosis of the heart muscle (which may take up to several weeks) [87, 88]. Furthermore, the use of

divalent cations may affect cell behaviors by interfering with signaling pathways.

The change in hydrogel stiffness often results in alternation of hydrogel permeability, which can in turn affect cell behavior in hydrogels due to aberrant local metabolite concentrations (e.g. integrin ligand in collagen) [92, 93]. To overcome this limitation, several PEG-based hydrogel systems have been developed to decouple the effects of hydrogel stiffness and permeability [94, 95], as well as hydrogel stiffness and biochemical cues [96], on cell responses. Cha *et al* [95] developed methacrylic alginate (MA) crosslinked PEGDA hydrogels with a stiffness changing over one order of magnitude by tuning the concentration of MA and methacrylic groups, whereas the swelling ratio associated with the permeability of hydrogels changed minimally because of multiple hydroxyl groups of MA (figure 2(*C*)). The same group later reported PEGDA–PEG monoacrylate (PEGMA) composite hydrogels with tunable stiffness by changing the PEGMA concentration and almost constant swelling ratio [94]. Compared to pure PEGDA hydrogels, the composite hydrogels enhanced biphasic viability dependency and an expression of vascular endothelial growth factor in encapsulated fibroblasts. PEG-based IPN hydrogels [96] and PEG-fibrinogen [97] were also designed to decouple the effects of stiffness and biochemical cues on cells. These composite hydrogel systems may help better understand the effects of stiffness on cells in a 3D microenvironment by decoupling different cues (e.g. mechanical, physical, biochemical cues).

3.2. Control of stress/strain in 3D hydrogel-based constructs

The simplest way to control stress/strain in cell-laden hydrogels is to apply mechanical constraint, in which the boundaries of the hydrogel constructs are partially or entirely constrained to resist deformation induced by active contraction of the encapsulated cells. The compliance between local matrix deformation and cell contraction forces may lead to directed cell migration, alignment and differentiation [98]. For example, corneal fibroblasts encapsulated in uniaxially constrained collagen aligned in parallel to the long axis of the construct [99]. In addition, constrained constructs showed more collagen fibril density than unconstrained constructs. ECs in constrained collagen formed larger and thinner-walled lumens than those in free-floating collagen [100]. In another example, fibroblast-laden collagen microtissues were constrained by two micro-cantilevers (figure 3), which were used to measure forces during the remodeling process [74]. It was shown that both cellular contractility (figures 3(*A*)–(*E*) and protein deposition (figures 3(*F*)–(*I*) increased with increasing stiffness of collagen and micro-cantilevers. This method has been recently extended to engineering cardiac microtissues for high-throughput drug-screening applications [101].

Hydrogels are sensitive to changes in environment and can undergo a dramatic volume change, resulting in stress/strain to the encapsulated cells. For example, fibronectin-immobilized temperature-responsive poly(N-isopropyl acrylamide) (PNIPAAm) hydrogels swell when the environmental temperature changes, leading to equibiaxial

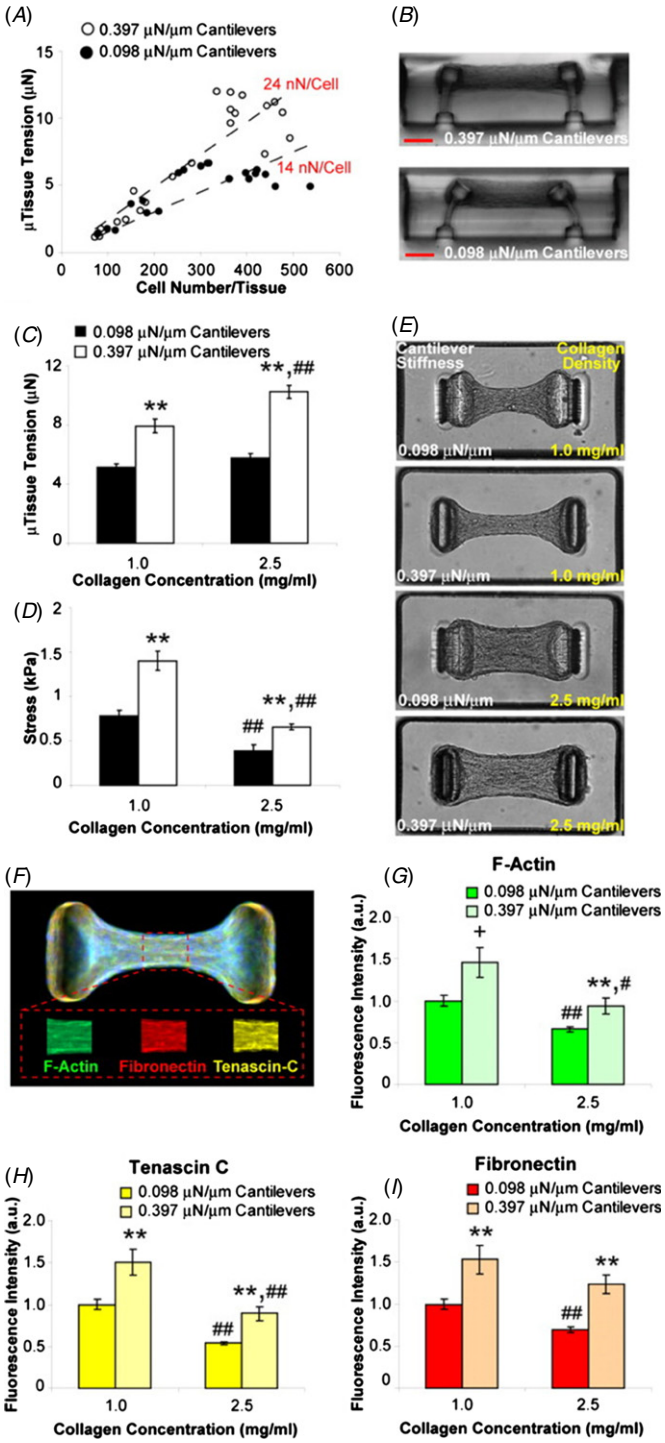

Figure 3. Boundary and matrix mechanics regulate contractility and protein deposition of NIH 3T3 fibroblasts in collagen. Reprinted with permission from [74]. Copyright 2009 National Academy of Sciences, USA. (*A*) Microtissue tension increased with number of cells per tissue, depending on the stiffness of microcantilevers. (*B*) Cross-section view of microtissues tethered to rigid (0.397 μN μm^{-1}) or flexible (0.098 μN μm^{-1}) cantilevers. (*C*) Microtissue tension increased with collagen concentration both on rigid and flexible cantilevers. (*D*) The average midpoint stress in microtissues decreased with collagen concentration. (*E*) Top view of collagen microtissues. (*F*) Immunofluorescence staining of fibrillar actin, fibronectin and tenascin C within microtissues. (*G*)–(*I*) Fluorescence intensity of fibrillar actin, fibronectin and tenascin C levels in microtissues. Scale bars: 100 μm.

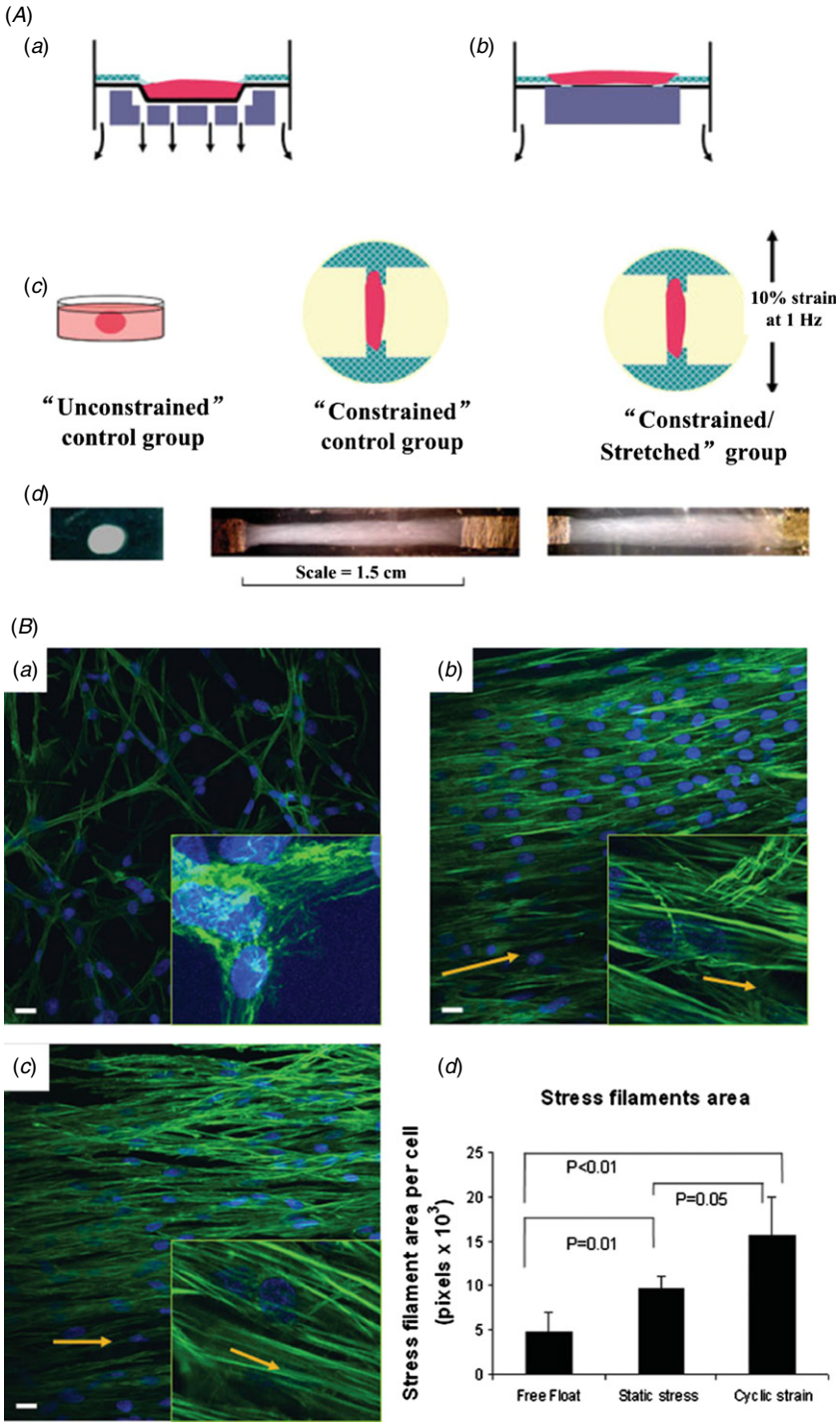

Figure 4. Mechanical stretching for bone-marrow-derived progenitor cells (BMPCs) in fibrin hydrogels. Reprinted with permission from [108]. Copyright 2006 Wiley Periodicals, Inc. (*A*) Schematic illustration (*a*)–(*b*) and top view (*d*) of Flexcell^{TM} Tissue-Train^{TM} plates for stretching of 3D constructs; (*c*) is the representation of experimental groups. (*B*) F-actin filaments (green) within 'Unconstrained' control group are randomly organized (*a*), while those within 'Constrained' control group (*b*) and 'Constrained/Stretched' group (*c*) are aligned parallel to the direction of stress or strain. (*d*) F-actin filament area per cell increases in 'Constrained' control group and even more in 'Constrained/Stretched' group. Insets = × 100; Scale bars = 10 μm.

stretching of encapsulated cells [73]. This mechanical stimulation facilitated formation of filopodia-like structures at peripheral regions and paxillin-containing fibrous structures in cytoplasm. In addition, DNA hydrogels that can temporally swell/deswell through controlled delivery of DNA have also been developed [42, 43]. However, the DNA mediated crosslinking process is dynamically slow (from 4 h to 1 day), and it may not be biocompatible due to the use of negatively charged DNA [43, 45, 102].

The type, magnitude, frequency and duration of mechanical loading in hydrogels all affect the response of encapsulated cells. However, it is challenging for methods based on scaffold constraint and environment condition change to control these parameters. External stretch, compression

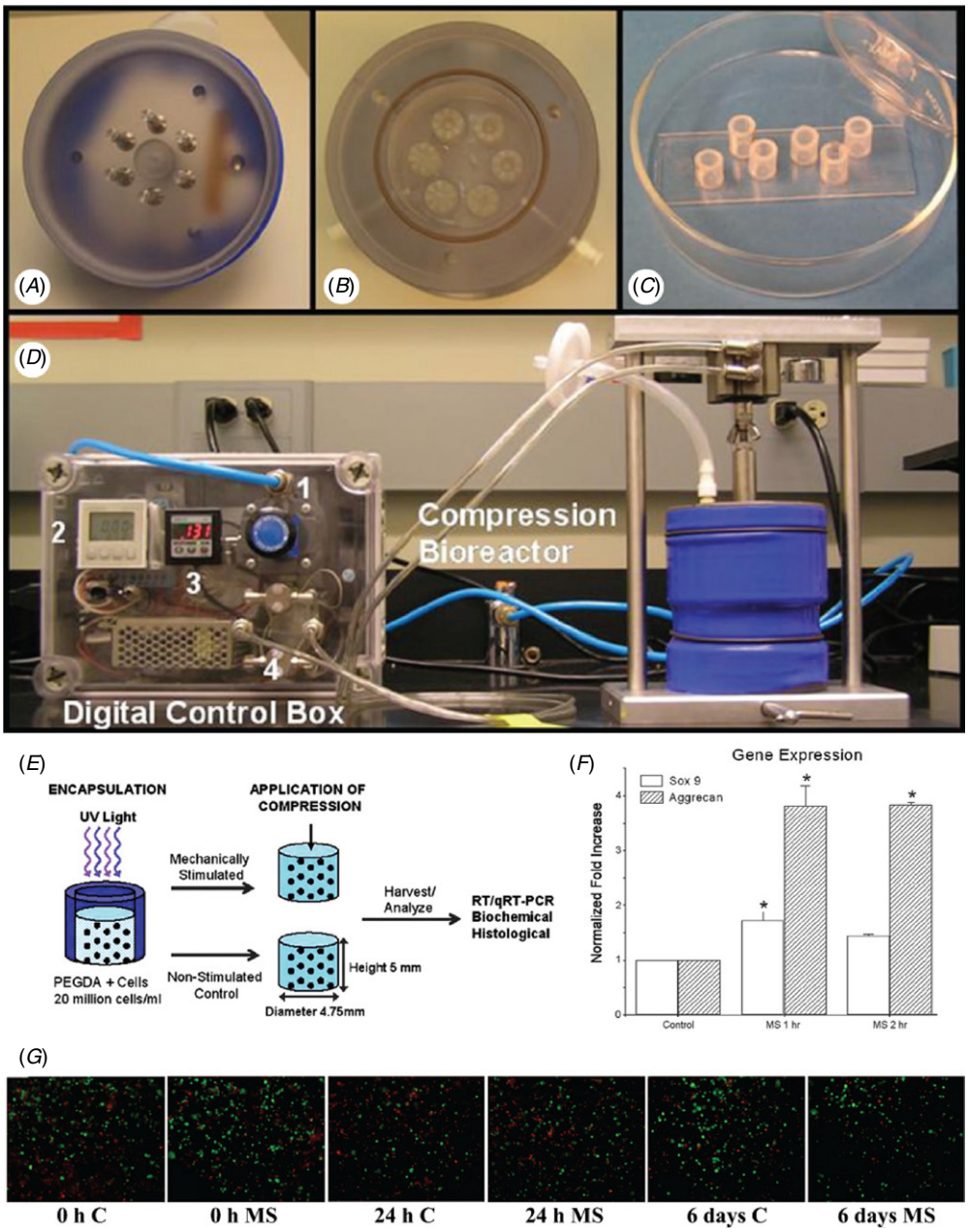

Figure 5. Mechanical compression for MSC in PEGDA hydrogels. Reprinted with permission from [62]. Copyright 2007 AlphaMed Press. Interior (*A*) and base (*B*) of a stimulation chamber. (*C*) Silicon tubing molds for fabricating cell-laden hydrogels. (*D*) Assembled compression bioreactor composed of stimulation chamber, metal housing, piston and control box. (*E*) Schematic diagram of cell encapsulation, mechanical stimulation, and result observation. (*F*) Sox-9 and aggrecan gene expression of MSCs increased under mechanical stimulation. (*G*) Live-Dead staining of encapsulated cells (10 ×). Abbreviations: C, control; h, hour; MS, mechanically stimulated; RT/qRT-PCR, real-time quantitative reverse transcription–polymerase chain reaction.

and shear stress have been explored to overcome these limitations with controllable loading parameters. Traditional stretch devices use elastic membranes on which cell sheets or 3D tissue constructs are anchored. Stretching of cells is obtained when the membranes are stretched through pneumatic deformation, a clamping mechanism or bending [50, 103]. Most of the membrane stretching devices can only provide uniform strain over the membrane center, and are limited to small (up to several square centimeters) and thin tissue constructs. With the development of commercial stretching apparatuses, such as Flexcell, Bose ElectroForce, and bioreactors developed by Tissue Growth Technologies, a variety of stretch parameters (e.g. stretching mode, strain magnitude, loading frequency, insertion of rest periods) have been investigated and found to significantly affect cell behaviors [104–107]. Nieponice *et al* [108] utilized Flexcell^{TM} Tissue-Train^{TM} plates to investigate the effects of cyclic stretch (10% strain, 1 Hz) on the morphology and differentiation of marrow-derived progenitor cells (BMPCs) in fibrin hydrogels (figure 4(*A*)). It was found that cyclic stretch induced the morphological and phenotypical changes of BMPCs toward SMCs (figure 4(*B*)). However, there are

limited systematic studies on 3D hydrogel tissue constructs, partially due to the technical challenges to apply stretch force on cell-laden hydrogels under a designed mode. The application of clamp, pin, suture and pressure to stretch hydrogels may lead to slip, undesired compression and local tearing of hydrogels [50, 109, 110].

Compared to mechanical stretch, compression and shear stress are relatively easier to apply to 3D cell-laden hydrogels. To study the effect of compression on cells, cell-laden hydrogels are usually fabricated into cylindrical samples and vertically placed in a chamber filled with a medium, with a computer-controlled force loading and transducer on the upper surface of the samples. This method has been used to enhance matrix production and to control stem-cell differentiation *in vitro* [53, 111]. Terraciano *et al* [62] designed a customized bioreactor system to apply unconfined and dynamic compressive loading to MSC-laden PEGDA hydrogels (figure 5). MSCs showed enhanced chondrogenic gene expression under mechanical compression. Bian *et al* [112] developed a bioreactor with unconfined axial compressive loading or sliding contact loading applied on chondrocyte-laden agarose hydrogel disks. The stiffness of the constructs increased significantly upon culturing under mechanical loading (both compressive and sliding contact) compared to that of the free-floating control. Tien's group [52, 68] developed a molding method to form endothelialized microfluidic channels in collage hydrogels to mimic human microvessels. Fluid was perfused through microvessels to apply mechanical stimulation on ECs. It was found that the barrier function and stability of microvessels increased with increasing shear stress and transmural pressure, respectively.

4. Conclusions and future perspectives

One advantage of engineering CMM with hydrogels is their optical clarity, which allows observation of cell behaviors using microscopy-based methods such as phase contrast, fluorescence and laser confocal microscopes. With these methods, cell morphology and function, including cell spreading, migration, proliferation, protein secretion and gene expression, in a 3D microenvironment can be tracked in real-time. In addition, cells actively probe changes in CMM and feed back to remodel ECM. Thus, it is of great interest to study cell-hydrogel interactions and quantify cell and hydrogel mechanical properties. Micro-indentation and atomic force microscopy can be employed to probe the local surface properties of hydrogels. Rheometry has been routinely used to test bulk hydrogel properties. The fluorescence resonance energy transfer method has been utilized to investigate cell-hydrogel interactions and quantify hydrogel degradation by encapsulated cells. Particle tracking methods have been developed to measure stress and strain fields within hydrogels. However, most of these methods are limited to 2D applications or lack microscale resolution. Recently, microrheology is combined with spatiotemporal image correlation spectroscopy to capture local deformation of fibrin hydrogels induced by cells and to characterize local hydrogel mechanical properties in 3D [113]. The 3D particle trafficking method is developed to measure the contraction forces of cells, and 3D stress and strain distribution in hydrogels [114–116]. The feasibility and universality of these methods for application in a variety of 3D cell–hydrogel systems need to be further investigated. Moreover, associated software tools should also be developed to acquire, manage and analyze enormous experimental data [117].

It is essential to spatiotemporally control 3D CMM in tissue development, tissue engineering and pathology studies due to the heterogeneity, diversity and dynamic nature of CMM and to study their important effects on cell behavior. Although a variety of hydrogels and associated technologies have been developed, several issues still need to be addressed:

(1) Hydrogels are inherently viscoelastic, similar to natural cells and tissues. The mechanical stimulation of stiffness and strain/stress on cells encapsulated in hydrogels may also be affected by hydrogel viscoelasticity. However, little has been done to investigate hydrogel viscoelasticity.
(2) Novel biocompatible hydrogels still need to be designed so as to control their physical and biochemical properties. The development of click chemistry and composite hydrogel systems may provide solutions.
(3) Most of the current methods to engineer a cell mechanical microenvironment are either 2D or lack of 3D controllability, especially for stretch loading approaches. The development of 3D loading protocols may facilitate the study of development, regeneration and disease propagation.

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