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Review

Hydrogel microfabrication technology toward three dimensional tissue engineering

Fumiki Yanagawa, Shinji Sugiura*, Toshiyuki Kanamori

Drug Assay Device Research Group, Biotechnology Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology (AIST), Central 5th, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, Japan

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ABSTRACT

The development of biologically relevant three-dimensional (3D) tissue constructs is essential for the alternative methods of organ transplantation in regenerative medicine, as well as the development of improved drug discovery assays. Recent technological advances in hydrogel microfabrication, such as micromolding, 3D bioprinting, photolithography, and stereolithography, have led to the production of 3D tissue constructs that exhibit biological functions with precise 3D microstructures. Furthermore, microfluidics technology has enabled the development of the perfusion culture of 3D tissue constructs with vascular networks. In this review, we present these hydrogel microfabrication technologies for the *in vitro* reconstruction and cultivation of 3D tissues. Additionally, we discuss current challenges and future perspectives of 3D tissue engineering.

Contents

* Corresponding author.
E-mail address: shinji.sugiura@aist.go.jp (S. Sugiura).
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1. Introduction

A multicellular three-dimensional (3D) cell culture model in a collagen hydrogel prior to implantation was constructed in the 1990s [1], with the aim of repairing vascular tissues using hydrogels with encapsulated cells. Over the past several decades, *in vitro* tissue model reconstruction in tissue engineering relied on hydrogels to mimic native tissue, owing to the biocompatibility of hydrogels, their ability to encapsulate bioactive molecules and cells, and the efficient mass transfer by diffusion [2]. The hydrogels composed of natural materials, including collagen, alginate, gelatin, hyaluronic acid, chitosan, and fibrin, are useful for the investigations of cell–cell and cell–extracellular matrix (ECM) interactions as well [3,4]. Although these hydrogels provide a microenvironment that chemically mimics cell–cell and cell–ECM interactions, they may lack an appropriate mechanical strength. In order to improve the mechanical properties of hydrogels, synthetic polymers, including poly(vinyl alcohol) (PVA), poly(ethylene glycol) (PEG), and poly(lactic-co-glycolic acid) (PLGA), have been widely used [2,3]. Numerous strategies have been developed in order to alter the biochemical and mechanical properties of the hydrogels. For example, ECM proteins (*e.g.*, collagen, fibronectin, and laminin) and/or their functional peptide sequences, may be chemically incorporated into hydrogels to prompt the cells to adhere to the surface of a hydrogel [5]. The mechanical strength of hydrogels is often adjusted by controlling the cross-linking density.

A key requirement for the replication of functional organs and tissues is a comprehensive knowledge of the organization and composition of their components, based on the *in vivo* model, and the desirable 3D microstructure in the reconstructed tissue. Recent advances in the field of tissue engineering have been based on the precise 3D microfabrication technologies, such as micromolding, 3D bioprinting, photolithography, and stereolithography [6]. These technologies allow the fabrication of precise 3D architectures at the micron scale. Additionally, microfl-uidics technology has been used for the fabrication of building blocks for 3D tissue engineering, while the medical imaging technologies are attractive systems for the design of 3D tissue constructs, and they include X-ray computed tomography (CT), and magnetic resonance imaging (MRI). The architectural parameters can be designed by the application of computer-aided design (CAD), using the captured 3D image of the normal tissue. Furthermore, microfluidics technologies [7] offer an attractive platform for the enhancement of the biological functions of 3D tissues. The combination of the existing biomaterial [8], microfabrication, and microfluidics approaches has an excellent potential for the reconstruction of large organ models in the future. Here, we provide an overview of these microfabrication and microfluidics technologies using hydrogels, in 3D tissue model engineering.

2. Hydrogel microfabrication in tissue engineering

Hydrogel microfabrication technologies in tissue engineering have been extensively reviewed [9]. These technologies include micromolding, 3D bioprinting [10,11], photolithography [12], and microfluidics [13,14]. Here, we focus on hydrogel microfabrication, and highlight the abundance of recent studies in the field of tissue engineering. These approaches provide different advantages or disadvantages in the selection of material, complexity of the 3D architecture, resolution, damage to the cells, and fabrication speed, and we have taken into consideration these properties and compared them.

2.1. Micromolding

Various micromolding approaches for the fabrication of 3D tissue constructs have been reported. Most of the other microfabrication technologies are limited by the selection of suitable materials for each fabrication process, and this suitability depends on their physicochemical properties. The micromolding approach allows this limitation to be overcome, while offering the advantages of short processing time and easy-to-use procedures. In this technique, elastomers, such as polydimethylsiloxane (PDMS) and poly(methyl methacrylate) (PMMA), have been employed as templates for the creation of tissue constructs. Although alginate and poly L-lactic acid based polymers are often used as sacrificial hydrogels for the fabrication of complex structures [15], there are no technical limitations for the use of other materials as templates,

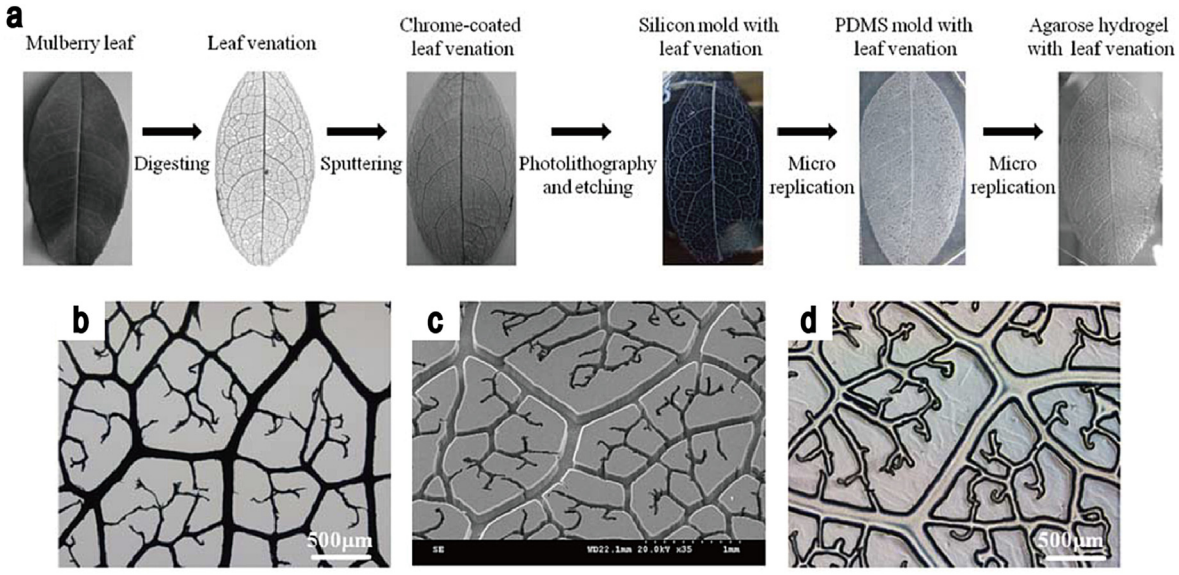

Fig. 1. The production of nature-inspired perfusable microfluidic network in the hydrogels, using micromolding technique. (a–c) Fabrication of agarose gel micromold using leaves. (d) The fabricated 3D perfusable structure in the hydrogel.
Source: He et al. [18], copyright (2013) with permission from John Wiley & Sons, Inc.

and numerous materials, including sugar [16] and gelatin [17], have also been used to create microvascular networks in hydrogels.

In a recent report [18], He et al. demonstrated the use of a sputtered natural leaf as a replica mold for the fabrication of a microvascular network in agarose hydrogels (Fig. 1). The layer-by-layer process allowed the fabrication of complex 3D structures. Minimizing the processing time for the fabrication of the desired structures presents a key challenge in the micromolding approach. A rapid micromolding, powered by an electrochemical cellular detachment, was performed by Seto et al. [19], who demonstrated that a microvascular 3D capillary-like structure can be created using this procedure, while providing the homogeneous cell adhesion inside the capillary structure [20]. Providing a perfusable platform for cell culturing inside the capillary structures, in order to enhance their biological functions present in normal tissues represents an additional challenge. In order to provide this platform, PEG diacrylate-based hydrogels with capillary structures were fabricated in a PDMS device by Cuchiara et al. [21,22]. These techniques allowed for the fabrication of perfusable hydrogel networks independent of overall scaffold geometry. Additional examples of a microfluidic approach for the development of a perfusion culture are outlined in the subsequent section on microfluidics.

2.2. 3D bioprinting

3D bioprinting technologies have been applied for the fabrication of 3D tissue constructs, using different biomaterials, and they have a high potential of precise deposition of materials to a desired location, enabling the production of a well-defined 3D architecture. These techniques allow the rapid prototyping of complex 3D tissue constructs containing cells. Additionally, they provide the possibility of using the direct copies of patients' architectural parameters, obtained by different scanning systems, such as X-ray CT [23,24], and MRI, and reproducing a precise biomimetic 3D-engineered tissue. In the cases of injury and disease, when a direct copy of structural parameters cannot be obtained from the tissues of the patients, the application of CAD [25,26] can be useful for the reproduction of 3D tissues and organs [10].

The conventional 3D bioprinting approaches in tissue engineering are classified into three major groups: (i) inkjet, (ii) microextrusion, and (iii) laser-assisted bioprinting (LAB). In most cases, the most important factor is the selection of a suitable material for each approach [27]. The printability depends not only on the physicochemical properties of pregel solutions (*e.g.*, viscosity) but on the gelation process as well. Recently, several reviews have provided an overview of these 3D bioprinting approaches [28,29], and here, we discuss recent advancements in 3D bioprinting hydrogel microfabrication.

2.2.1. Inkjet bioprinting

Inkjet bioprinters have recently been customized to print biocompatible materials with increased resolution and speed. The two approaches most commonly used to eject bioink onto a substrate are the thermal- and piezoelectric-nozzle approaches. Even though the advantages of inkjet bioprinting are high printing speed and low cost, the printing process usually requires: (i) a quick crosslinking reaction for gelation, (ii) the removal of nozzle clogging, (iii) the removal of cavitation bubbles. Alginate is a material commonly used in inkjet bioprinting, owing to its quick cross-linking through an ionic reaction. A PEG based polymer is used as well, as it has high biocompatibility and can be tailored to specific needs by adjusting its physical and chemical properties [30]. Although PEG based hydrogels can provide higher mechanical strength compared with the natural hydrogels, the cellular response to the PEG-based hydrogels (*e.g.*, adhesion) is very limited, and, in order to address this issue, gelatin [31–33] and hyaluronan [32,34] are used for the generation of 3D tissue constructs. Furthermore, an advantage of inkjet bioprinting is the possibility of printing multiple cell types and materials. Recently, Xu et al. demonstrated a novel method, fabricating complex and heterogeneous 3D constructs, while using multiple cell types, including stem cells, muscle cells, and endothelial cells [35].

The limiting factors in inkjet bioprinting are ink viscosity, due to excessive force required to eject droplets [36,37], and the potential of cell damage during the printing process [38]. During the thermal nozzle printing, the heat generated to eject droplets causes cell damage, whereas during piezoelectric-nozzle printing, even though there is no heating, the high pressure required to eject bioink droplets from the nozzle may cause some damage to the cells.

2.2.2. Microextrusion bioprinting

As an alternative approach to inkjet bioprinting, microextrusion bioprinting is often used to fabricate biomimetic 3D tissue constructs. The three typical techniques for the dispersion of the biomaterials onto a substrate, widely used in microextrusion bioprinting, are: (i) pneumatic-, (ii) piston-, and (iii) screw-dispensers.

Piston-dispenser is used most commonly, as it is suitable for the deposition of highly viscous materials onto the substrate. Most of the existing studies using this dispenser reported that 3D tissue constructs were printed using alginate [24,33,39] and agarose [40,41], and the gelation processes were ionic and thermal cross-linking. One of the reasons for the popularity of the microextrusion bioprinting is the compatibility of the dispensing system with the various cross-linking mechanisms. A robust hydrogel is required to maintain high resolution of 3D structures after printing, and the classical approach has been to increase the polymer concentrations and cross-linking density. Additionally, materials can be dispensed through small diameter nozzles under high pressure [42]. However, the dispensing pressure can affect cell viability during the printing process. Therefore, Cohen et al. [43] evaluated a relationship between the resolution of printed constructs and the mixing process of alginate and cross-linkers, and found that an increased mixing procedure before printing affects the resolution of printed materials without causing cell damage.

Generally, highly viscous materials are preferred for 3D bioprinting than less viscous ones, because the 3D shape needs to be maintained during the cross-linking reactions following the printing process. The main advantage of microextrusion bioprinting is that it enables the use of highly viscous materials, such as gelatin- and collagen-based materials. This type of bioprinting has been applied to combined gelation processes, including ionic/thermal [33], ionic/chemical [44] and thermal/chemical [45] processes, providing a biomimetic microenvironment with a high resolution for cell growth after the printing. Recent microextrusion techniques tend to be performed with photo-crosslinking reactions using photosensitive polymers, such as PEG diacrylate [40] and gelatin methacrylate [46,47]. In a recent study, Hong et al. [48] generated highly stretchable and tough hydrogels containing mesenchymal stem cells (MSCs) in the alginate and PEG-based hybrid hydrogels, combining ionic- and photo-crosslinking reactions (Fig. 2A). Pescosolido et al. adopted the semi-interpenetrating network in photopolymerization in order to optimize the rheological properties of hydrogels [49], and produced 3D printed constructs with hyaluronic acid and hydroxymethacrylate. In the most recent example, production of an *in vitro* 3D brain model was demonstrated by Lozano et al. [50], where layered 3D tissue constructs were fabricated, and biological function of these models were enhanced with peptide-modified gellan gum, in order for this model to resemble a cortical network with primary neural and glial cells (Fig. 2B). The axon

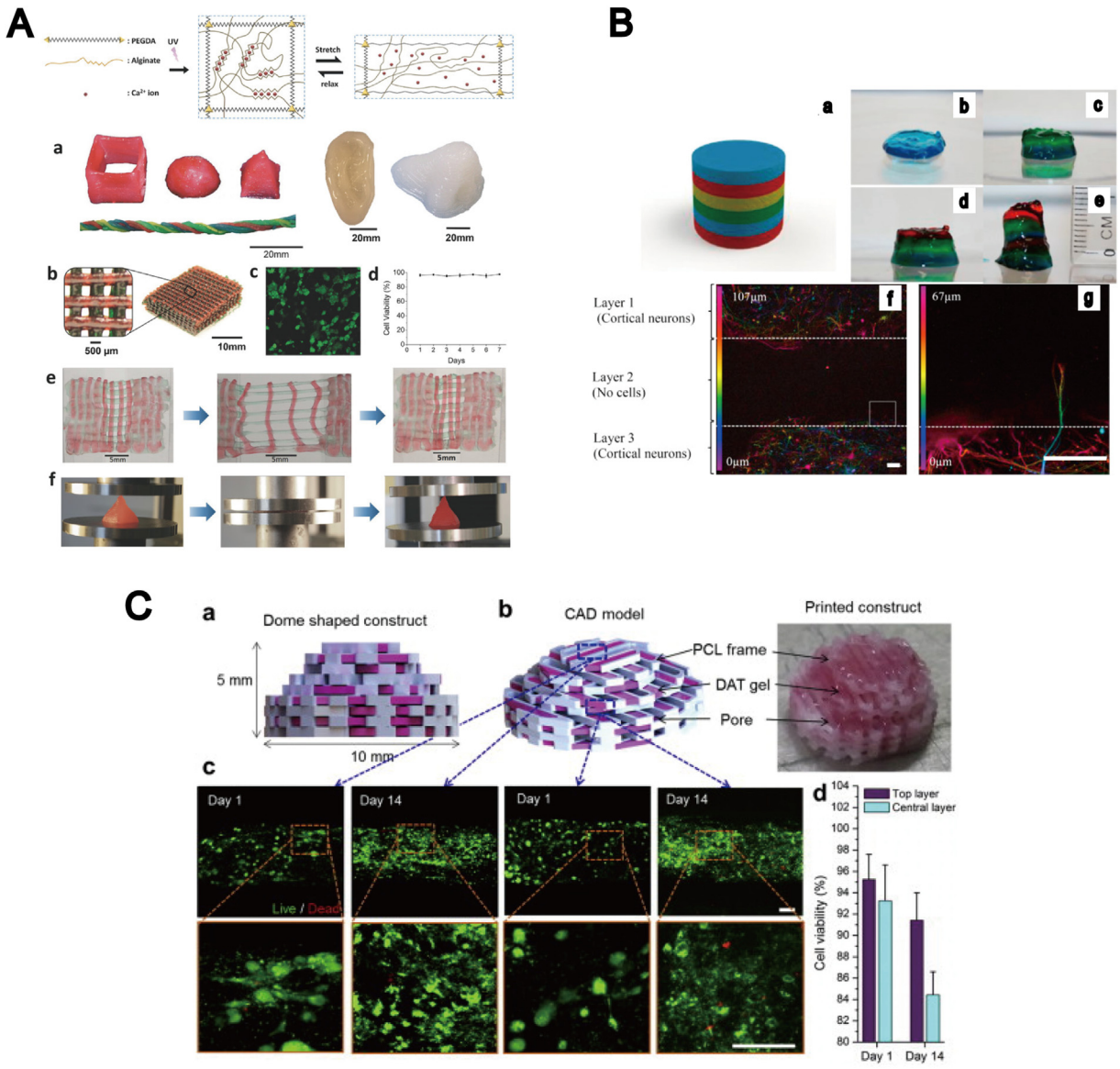

Fig. 2. 3D tissue constructs fabricated by microextrusion bioprinting. **(A)** Microextrusion bioprinting of tough and highly stretchable hydrogels composed of PEG-alginate-nanoclay polymer through ionic/photo crosslinking. (a) 3D structure printed using the hydrogel. (b) A 3D-printed mesh geometry with the hydrogels. (c–d) Cell viability test in a hydrogel, following the printing. (e) A printed bilayer mesh structure. (f) Compression test. Source: Hong et al. [48], copyright (2015) with permission from John Wiley & Sons, Inc. **(B)** Bioengineered layered 3D brain-like structures, produced using peptide-modified gellan gum substrates. (a–e) Printed brain-like layered 3D structure. (f–g) Axon elongation into adjacent hydrogels layer. Source: Lozano et al. [50], copyright (2015) with permission from Elsevier Ltd. **(C)** Microextrusion printing of biomimetic 3D tissue from CAD format. (a–b) CAD format model. (c) Confocal images of cell viability (green: live; red: dead) in printed tissue construct after 2 weeks, in top and central layers. (d) Cell viability in top and central layers. Source: Pati et al. [51], copyright (2015) with permission from Elsevier Ltd.

elongation into adjacent hydrogel layers was observed without significant cell damage during the printing and gelation process. Another recent example, together with the application of CAD format, was reported by Pati et al. [51], who fabricated adipose 3D tissue constructs using human matrix bioink, encapsulating human adipose tissue-derived mesenchymal stem cells (Fig. 2C). Although these approaches can be used for the production of simple 3D architecture and porous 3D structures with cells, they may have some limitations in the fabrication of complex 3D architectures. Additionally, the clogging of bioink in the nozzle may present a potential problem related with this approach.

2.2.3. *Laser-assisted bioprinting (LAB)*

LAB has been used to avoid the previously mentioned issues in the microextrusion bioprinting, since the laser-assisted approach is nozzle-free and can avoid clogging. The laser-induced forward transfer technique, which allows the printing with both inorganic and organic ink at micrometer resolution, was developed by Bohandy et al. [52]. The LAB designed for bioink printing was also reported in 2004 [53]. A typical LAB is composed of three components: (i) a pulsed laser beam, (ii) a ribbon that prints the scaffold, (iii) a substrate that collects the printed materials [54]. Concentrated laser beam pulses on the absorbing layer of the ribbon cause bioink to be propelled by a high-pressure gas towards the collector side. Therefore, LAB enables the generation of the desired geometry at a high resolution without cell damage [55]. Another advantage of LAB is that it allows the cell deposition on the substrate at high densities. Guillemot et al. developed a high-throughput laser printer for tissue engineering [56], and demonstrated the fabrication of microscale cell patterning using alginate hydrogels at high

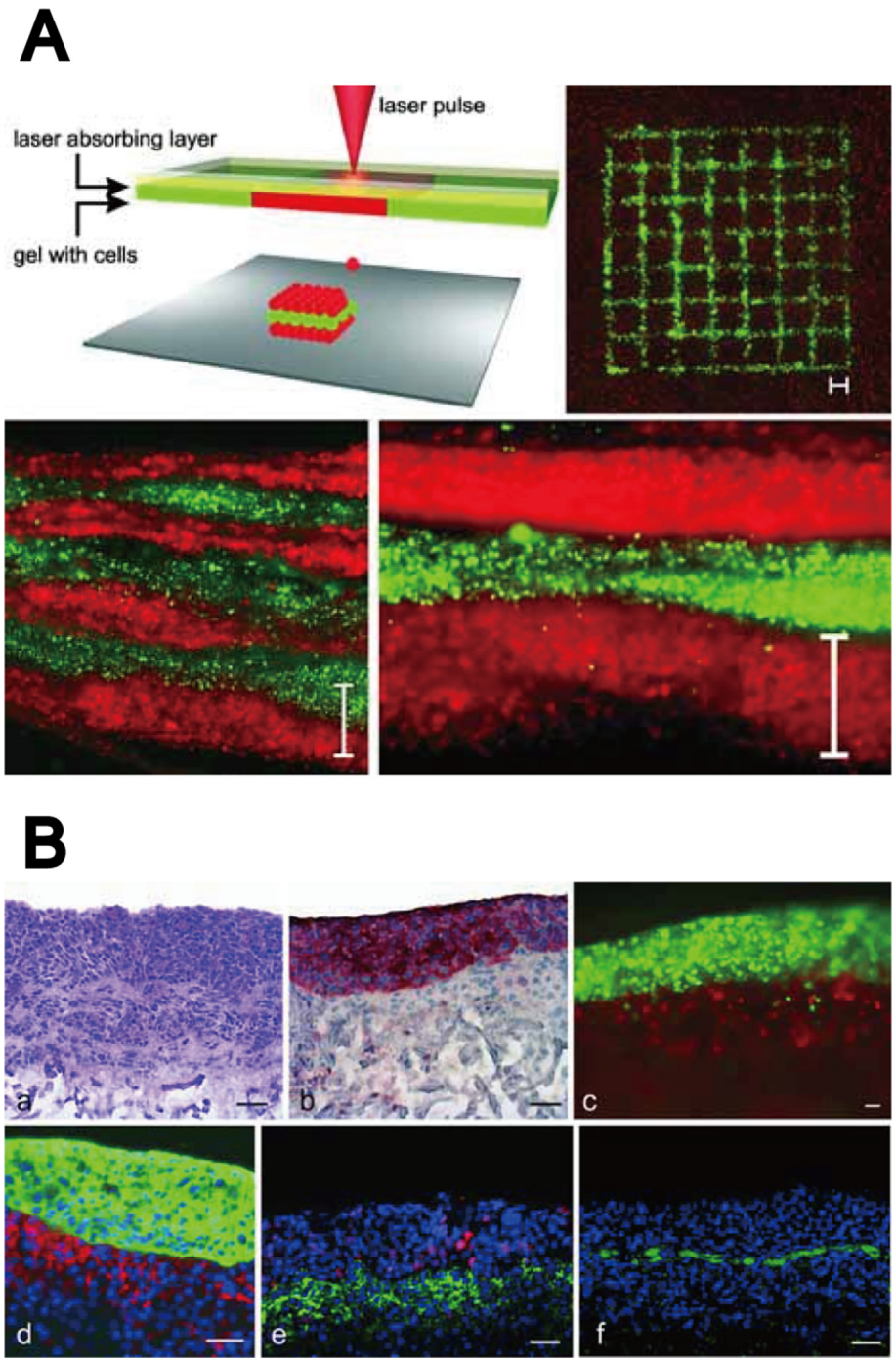

Fig. 3. 3D tissue constructs fabricated by LAB. **(A)** Skin tissue generation using LAB. **(B)** 3D skin tissue model printed using the layer-by-layer approach: Murine fibroblasts (10–20 layers) and human keratinocytes (10–20 layers) encapsulated in collagen hydrogel.
Source: Koch et al. [58], copyright (2012) with permission from John Wiley and Sons, Inc.

cell density [54]. An alginate solution is commonly used as a bioink to print the engineered tissue, owing to the rapid gelation during the printing process. Although the typical concentration range is 1–2%, a higher concentration of alginate is required to fabricate 3D engineered tissue at millimeter scale. Yan et al. succeeded in the fabrication of long tubes and annular structures using 2–8% alginate [57]. More recent LAB approaches tend to focus on the use of natural hydrogels that provide improved biomimetic microenvironment, for better cell growth. In a recent study by Koch et al. [58], a skin tissue model was constructed, demonstrating the fabrication of 3D thick-tissue constructs with collagen hydrogels, including fibroblasts and keratinocytes, based on a layer-by-layer approach (Fig. 3A and B). These 3D tissue constructs exhibited cell–cell interactions, such as gap junction.

2.3. Photolithography

Conventional types of photolithography used for tissue engineering are generally photomask-based photolithography and maskless photolithography, which can be either digital light

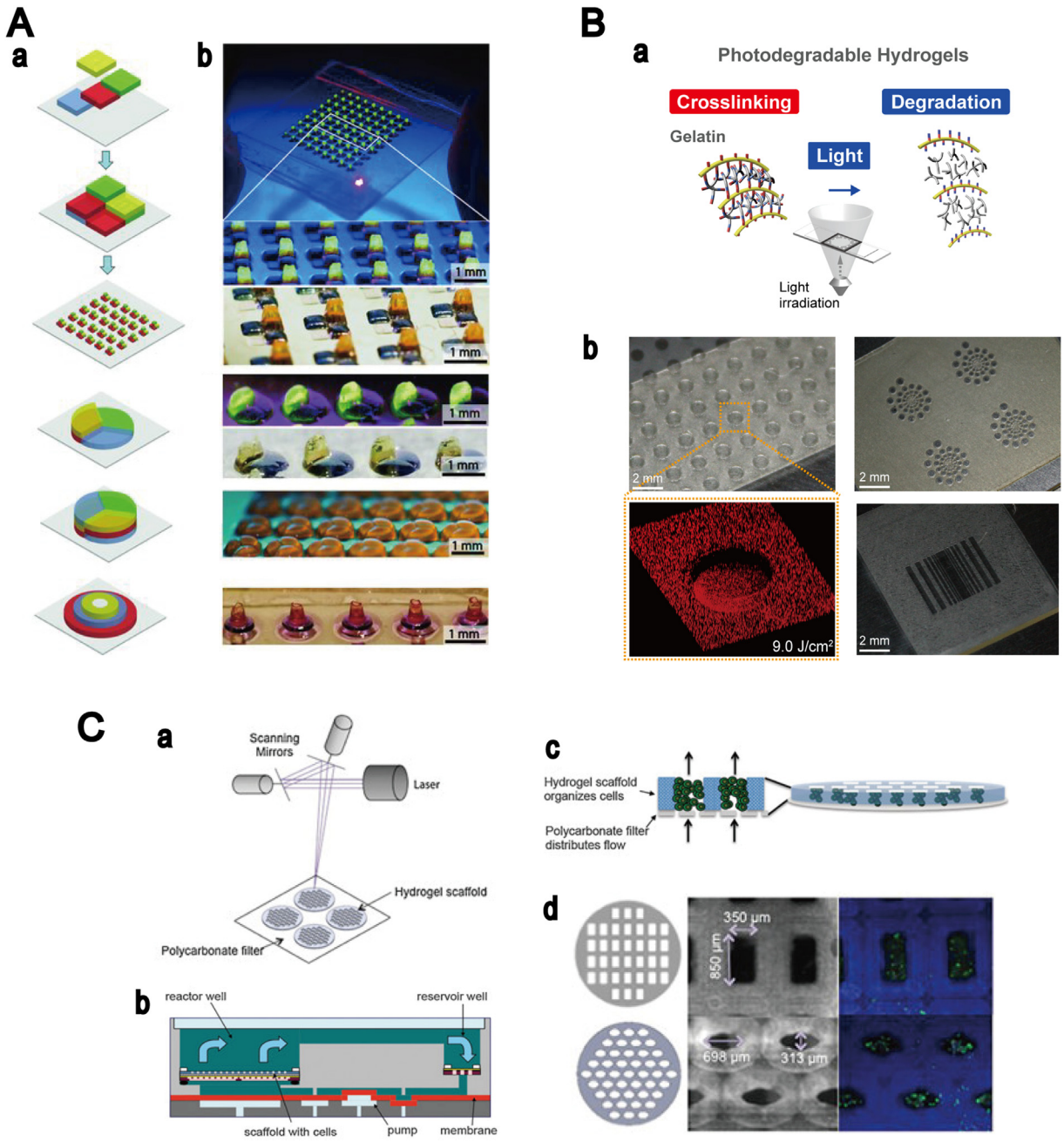

Fig. 4. 3D tissue constructs created using photolithography. **(A)** Multilayer digitally specified hydrogels with spatially heterogeneous 3D structures. (a) The process of fabrication of 3D structured hydrogels using multilayer photolithography. (b) Microfabricated array of multilayer digitally specified 3D tissue prototypes. Source: Gurkan et al. [61], copyright (2013) with permission from John Wiley and Sons, Inc. **(B)** 3D hydrogels patterning using gelatin based photodegradable hydrogels. (a) Micropatterning procedure. (b) Micropatterned photodegradable hydrogels. Source: Yanagawa et al. [75], copyright (2014) with permission from John Wiley and Sons, Inc. **(C)** Photopatterning of hydrogel scaffolds for perfusion culture. (a) Hydrogel scaffold fabrication. (b–c) Design of perfused bioreactor. (d) Cell viability in fabricated 3D scaffold. Green: live; red: dead cells. Source: Neiman et al. [80], copyright (2015) with permission from John Wiley and Sons, Inc.

projection stereolithography or laser-based stereolithography [59]. These approaches have different characteristics: cost issues, cell damage levels, resolution, and fabrication speed. Here, we compare these approaches taking into consideration their features.

2.3.1. Photomask-based photolithography

Several studies on 2D hydrogel micropatterning by photolithography have reported the regeneration of biomimetic tissue constructs. The early investigations in tissue engineering relied on photomask-based photolithography because of the simple and low-cost preparation process. A key engineering challenge in photomask-based photolithography is building thick 3D constructs [60]. In recent studies, three groups reported the production of 3D hydrogel constructs with a millimeter-scale thickness, by photomask-based photolithography. Gurkan et al. employed a multilayer photolithography system, used in semiconductor technology development, to fabricate 3D digitally specified hydrogels with multiple cell types (Fig. 4A) [61]. A different approach was used by Hammoudi et al. [62], who succeeded in culturing cells in thick hydrogels, in order to understand stem cell interactions with

injured tissue. An alternative approach was reported by Occhetta et al. [63], demonstrating the production of 3D cell-laden microgels through photo-mold patterning. Although photomask-based photolithography is a very simple and low-cost process for the fabrication of 2D patterned hydrogels, this approach requires a substantial amount of photomasks for the generation of 3D architectures.

2.3.2. Maskless photolithography (stereolithography)

Stereolithography has been used for the production of many well-defined scaffolds for implantation [64]. These techniques offer a lot of potential for the fabrication of 3D structures using non-biological materials, such as resin. Two different methods, digital light projection and laser-based stereolithography, combined with CAD format [65], enable the generation of 3D tissue or organs based on the geometrical information obtained from patient databases, with feature sizes ranging from micro- to millimeters. An important challenge in stereolithography is finding a suitable photosensitive materials for the fabrication of 3D tissue containing cells. Cell encapsulation in hydrogels offers several advantages compared with the seeding of cells on hydrogels.

2.3.2.1. Digital light projection stereolithography. The cell encapsulation in biocompatible hydrogels was first shown by Dhariwala et al. in 2004 [66], who succeeded in the fabrication of engineered hydrogels with polyethylene oxide and PEG dimethacrylate. Early studies in tissue engineering using the digital light projection relied on PEG based hydrogels [67] to fabricate a 3D tissue with fibroblasts, hepatocytes, and MSCs [68], due to the strong mechanical properties of this material and high resolution. However, PEG based hydrogels are inert to cell adhesion, and therefore, their application is limited [69]. In order to improve the characteristics of the materials used, Arg–Gly–Asp (RGD) peptide has been used additionally in hydrogels, to provide cell adhesion on PEG based hydrogels. Another approach uses natural polymers, such as gelatin and polysaccharide, for these purposes. These natural polymers require chemical modification for the introduction of photosensitive moiety, because the materials are not photo-reactive. Recently, Gauvin et al. reported the production of well-designed 3D cell-laden hydrogels with gelatin methacrylate, using the digital light projection approach and layer-by-layer process [70]. A similar approach was employed by Zhang et al., using the CAD format [71], who succeeded in fabricating well-defined 3D architecture with human umbilical vein endothelial cells (HUVECs) and mouse embryonic fibroblast cells, using the layer-by-layer process.

Recent investigations have been devoted to the studies of the regulation of the growth of different types of cells, including fibroblasts, endothelial, smooth muscle, and MSCs cells, by the mechanical properties of hydrogels. Additionally, the studies of stereolithography moved to the use of photodegradable hydrogels based on PEG [72,73] and gelatin [74,75]. These hydrogels were used in the studies of cell behaviors, on and in the hydrogels, in contrast to the fabrication of tissue constructs. Providing a platform for the control of the mechanical properties of hydrogels is crucial for the formation of an appropriate microenvironment, aimed at obtaining the desired cellular functions. The dynamic modification of the elasticity of the hydrogels has been a focus of many studies recently. Photodegradable hydrogels offer several advantages as compared to photopolymerized hydrogels, such as the spatiotemporally tunable physicochemical properties, controlled by light. 3D hydrogel patterning on photodegradable hydrogels was first reported by the Anseth group [72], where the fabrication of microstructures in photodegradable hydrogels, and cell adhesion, migration, and spreading in the photodegradable hydrogels were demonstrated and investigated [76]. Yanagawa et al. reported the use of photodegradable hydrogel [75,77] in the combination with the digital light projection, and they were able to produce micropatterned structure with HUVECs, using gelatin based photodegradable hydrogels (Fig. 4B), and showed the elasticity pattering of hydrogels by light irradiation [78].

2.3.2.2. Laser-based stereolithography. Conventional laser-based stereolithography uses an ultraviolet laser and photosensitive materials, and this approach was used in 3D hydrogel production by Zorlutuna et al. [79], producing multifunctional polymer hydrogels that recapitulate cell–cell interactions between skeletal muscle myoblast cells and primary hippocampus neuron cells. The production of perfusable structure in these hydrogels is required for the long-term culturing of 3D structured tissue, even though the research of stereolithography in tissue engineering has a tendency of focusing on static cultures. Neiman et al. [80] recently produced an open channel structure in 3D hydrogels for the perfusion culture (Fig. 4C).

An alternative laser-based stereolithography uses two- or multi-photon laser system, in order to fabricate 3D hydrogels using photosensitive materials [81]. The advantage of these approaches is that they are able to produce 3D tissue constructs with micro- or nanometer-scale precisions [82–84]. Compared with short-wavelength lasers, such as an ultraviolet laser, both two- and multi-photon laser systems provide a mild processing environment, which does not cause photochemical damage to the cells. Although a few researchers reported the use of two- and multi-photon laser systems for the production of well-defined 3D hydrogels with cells, most investigations demonstrated a significant cell damage, caused by the photoinitiator during the gelation process [85]. In response to this, a photoinitiator-free multi-photon method was recently developed by Applegate et al. [86], who showed 3D multiscale micropatterning on the silk hydrogels by a multi-photon laser without significant cell damage. Although two- and multi-photon laser-based stereolithographic approaches are attractive options for the production of 3D tissue constructs, many additional issues need to be investigated and resolved, including the toxicity of the initiator and the possibility of high speed fabrication of large tissue constructs [85].

3. Microfluidics

Many researchers use microfluidics technologies for the development of new drug testing platforms [7,13], and they offer a new opportunity as attractive platforms for the fabrication of functional 3D tissue constructs on a micrometer scale. The main microfluidics technologies are building-block and microfiber approaches, and both are predominately affected by the viscosity of materials used, as well as the various factors described above in Section 2.2. An advantage of microfluidics technologies is the ability to control the flow of fluids, which allows the control of the size and shape of fabricated structures. Additionally, these technologies provide a potential platform for culture medium perfusion through the vascular network in the 3D tissue constructs. The different properties of microfluidics technologies, enabling the fabrication of engineered 3D tissue constructs and the development of perfusion cultures in the following sections.

3.1. Microfluidics for microfabrication

3.1.1. Building-block microfabrication

Microfluidics technologies are often used for the fabrication of tissue building blocks containing cells, known as cell-laden microgels, in order to construct complex 3D tissue structures. Various factors, such as the viscosity of the materials, fluid flow

rate, and substrate wettability, must be considered during the creation of these building blocks [87]. An emulsion-based approach is commonly used to fabricate microsphere-shaped microgels as building blocks, using PEG based polymers [88,89], agarose [90], and alginate [91–94]. This approach can be divided into methods based on flow-focusing and T-junction microchannels. Comprehensive reviews of microfluidics technologies for the fabrication of microsphere-shaped microgels exist [95], while we focus on the research in the field of 3D tissue engineering.

Manipulating the fabricated cell-laden microgels to construct thick 3D tissue structures represents a major challenge in microfluidics technologies for tissue engineering. Matsunaga et al. [96] reported a novel method for rapid assembly of well-designed tissue on millimeter scale. The cell-laden collagen microspheres produced using the microfluidics technologies were stacked, in order to construct thick tissue structures. Although the emulsion-based approach enables precise control of microsphere size, the possible fabrication shapes are limited. To address this issue, Doyle et al. developed flow lithography, combining photolithography with microfluidics technologies [97,98]. Flow lithography requires the use of photosensitive materials and patterned light projection, as described previously, in Section 2.3. The application of flow lithography in the tissue engineering was reported by Panda et al. [99], who demonstrated the fabrication of well-designed architectural microgels containing cells. In order to construct 3D tissue structures from the microgels, Chung et al. developed a railed track microfluidics channel that uses flow to guide and assemble the cell-laden microgels inside the microfluidic device [100]. While this approach allows high-throughput fabrication, the ability to create microgels with cells is restricted, due to the higher concentration of monomer and the presence of photoinitiator.

3.1.2. *Microfiber-based microfabrication*

Microfibers in tissue engineering have been widely used for the fabrication of 3D complex fiber geometry [101]. Several approaches have been described, such as electrospinning [102–104], wet

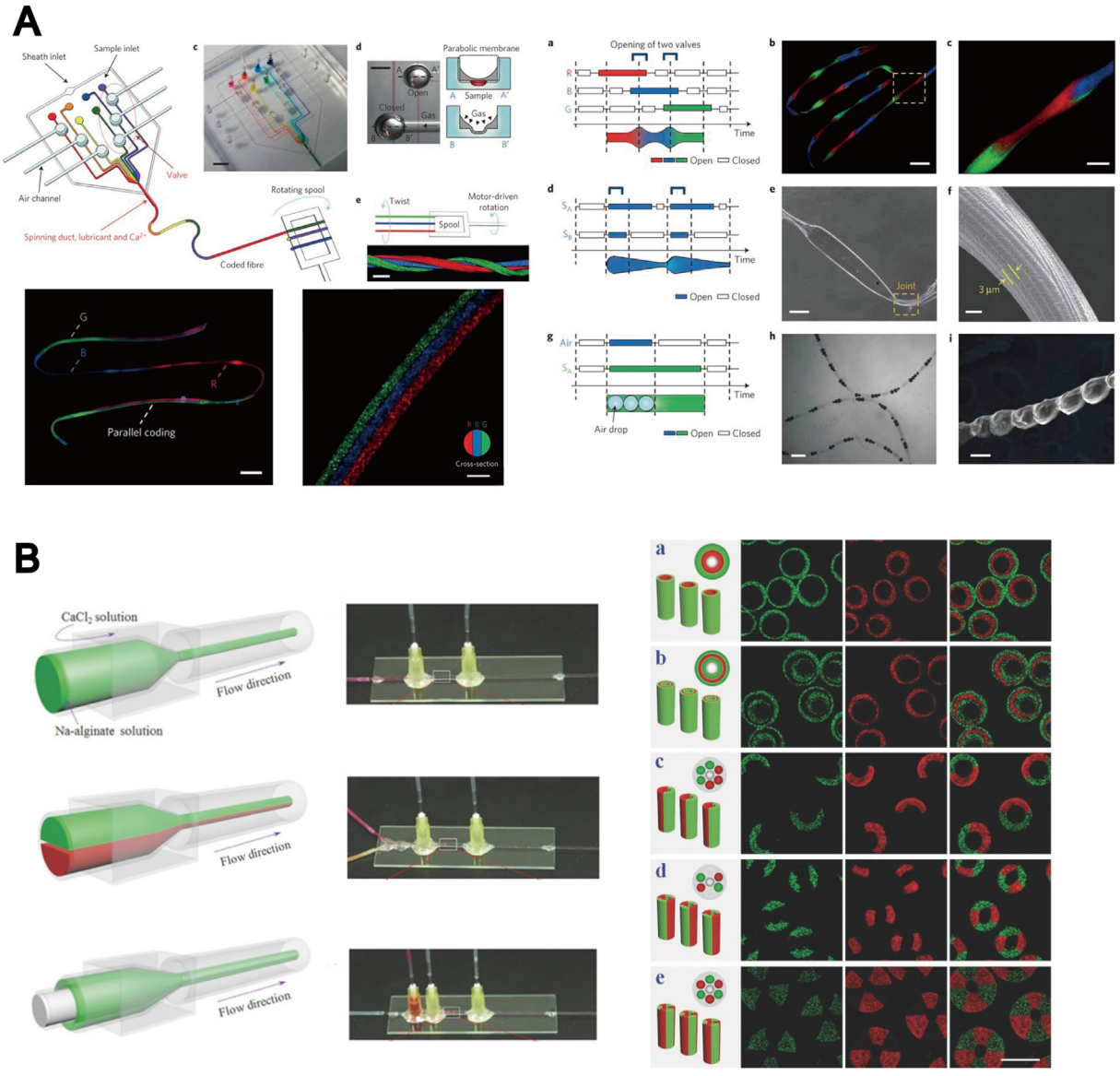

Fig. 5. Microfluidic procedure. **(A)** Microfluidic devices for the fabrication of spatially tunable microfibers. Source: Kang et al. [117], copyright (2011) with permission from Nature Publishing Group. **(B)** Microfluidic injection channels, creating different hollow microfibers. Source: Cheng et al. [119], copyright (2014) with permission from John Wiley and Sons, Inc.

spinning [105,106], and melt spinning [107]. Single fibers with a simple, homogeneous chemical structure have been produced using these methods. Additionally, microfiber encapsulated cells were produced with a microfluidics-based approach known as microfluidics fiber spinning [108]. A main advantage of this process is the reduced damage to cells during the process of cell encapsulation within microfibers. This approach allows the precise control of the diameter, which is predominately controlled by fluid flow rate in addition to the building block microfabrication described previously. Microfluidics based microfibers have been developed with various hydrogels [109], including PLGA [110], alginate [111,112] mixed with PLL [113], chitosan [114], and collagen [115]. Most of the existing approaches use alginate [116], due to the rapid gelation based on the ionic crosslinking.

Recently, the advancements in microfluidics fiber spinning allowed a precise design of 3D fiber geometry with multiple layers. Kang et al. [117] developed microfibers with tunable morphological and chemical properties, using digital and programmable flow control system (Fig. 5A). A similar method was developed by Yamada et al. [118], who fabricated an anisotropic microfiber structure with primary rat hepatocyte and feeder cells, mimicking a hepatic micro-organoid. Cheng et al. [119] reported a multiple-laminar-flow microfluidics method for the fabrication of multi-component 3D microfibers using alginate (Fig. 5B). This approach permits the precise control of the morphology of cells encapsulated in the fibers.

3.2. Microfluidic scaffold for 3D perfusion culture

The microfluidics technologies have been used to generate 3D tissue constructs with perfusable microvascular networks [120,121], because these networks play a vital role in the

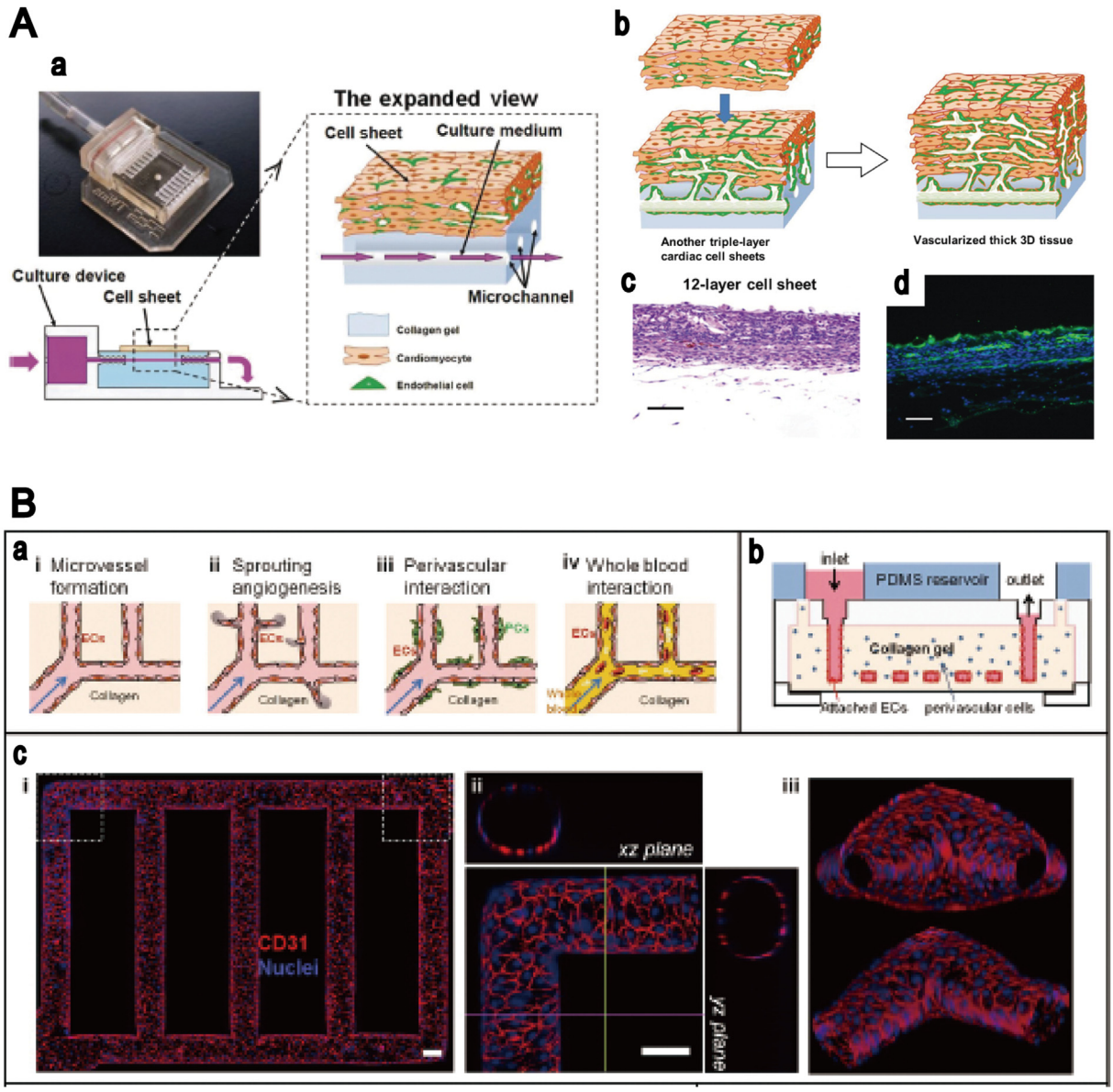

Fig. 6. Microfluidic devices for the culturing of cells in perfusable hydrogels. **(A)** *In vitro* development of vascularized tissue using perfusable collagen hydrogel. (a) Culturing device and cell system with medium perfusion. (b) The process of fabrication of vascularized 3D tissue based on a layer-by-layer technique. (c) Twelve-layer cell sheet on collagen hydrogel. (d) Six-layer cell sheet of cardiac muscle. Source: Sakaguchi et al. [134], copyright (2013), with permission from Nature Publishing Group. **(B)** Microvascular network in a collagen hydrogel, fabricated by micromolding. (a) The processes of fabrication and microvascularization. (b) Design of microfluidic device. (c) Confocal images of an established microvascular structure. Source: Zheng et al. [123], copyright (2012), with permission from the National Academy of Sciences.

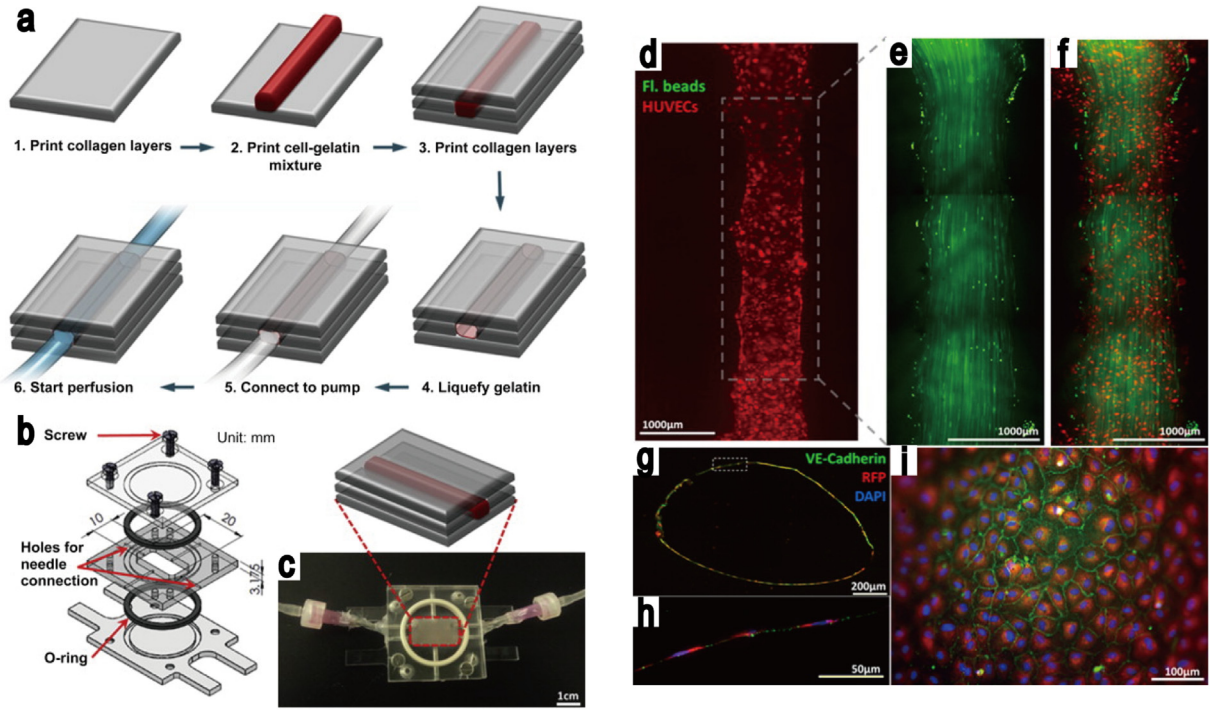

Fig. 7. The fabrication of perfused functional vascular channels, using 3D bioprinting technology. (a) The schematics of the vascular channel construction procedure using cell gelatin mixture. (b–c) Custom-designed flow chamber. (d) Fluorescent images of printed vascular channel with perfusion, after five days of culture. (e–f) The visualization of fluorescent bead motion with flow. (g–i) Vascular channel images, following five days of cell culture, with flow. Blue: DAPI nuclei staining; Red: RFP-transfected HUVECs; Green: VE-cadherin.
Source: Lee et al. [136], copyright (2014), with permission from Elsevier Ltd.

distribution of oxygen and nutrients within the engineered thick tissue [122]. The introduction of a flow medium into hydrogels is a necessary process, in order to maintain the viability of fabricated tissue constructs. Therefore, combining microfluidic platforms and 3D microfabrication systems, such as micromolding [123], bioprinting [124] and photolithography [80], is an important task during the generation of microfluidic perfusion cultures.

The typical materials that represent a suitable microenvironment for endothelial cell growth in microvascular networks are based on naturally derived polymers, such as (i) collagen [125–127], (ii) fibrin [128–131] and (iii) gelatin [132]. Cell culturing in collagen hydrogels with fluid flow was reported by the Tien group [133], who were successful in obtaining cultured HUVECs in a cylindrical channel. They demonstrated that the lifespan of microvascular cells engineered in collagen gel depends on the flow rate of the medium used. Recently, perfusable hydrogels were combined with microfluidics technologies, and a multi-layered bioreactor was developed by Sakaguchi et al. [134], who produced thick tissue with a microchannel in a collagen hydrogel (Fig. 6A). Although a removable substrate, for example, needles and steel, is often used for the fabrication of 3D tubular structures in hydrogels, the technique is limited to the generation of simple structures, such as single and straight channels. In order to improve the applicability of this model, alginate hydrogels have been used for complex channel production inside hydrogels. Golden and Tien used sacrificial hydrogels to create a perfusable microchannel in the collagen hydrogels [17], while a similar method was developed recently by Baker et al. [135], where the effect of angiogenetic growth factors on angiogenesis were investigated in patterned collagen hydrogels. An alternative approach has been reported by Zheng et al. [123], where the perfusion culture was obtained in 3D patterned hydrogels that were fabricated using the micromolding technique combined with a microfluidics system. The endothelial cells were cultured for up to two weeks in the microvascular networks developed in the collagen hydrogels, in order to study the angiogenesis and thrombosis (Fig. 6B). The combination of micromolding and 3D bioprinting systems was also developed by Lee et al. [136], who used the alginate layer as a sacrificial layer, creating a single channel in the collagen hydrogels, and obtained the HUVEC culture in collagen hydrogels with fluid flow (Fig. 7).

The inability of this methodology to generate well-defined 3D vascular structures with enhanced biological functions, for example a barrier function, similar to what occurs in *in vivo* models, is their prominent feature. More relevant tissue models, developed with stem cells [137] and with the aim of performing drug testing and investigation of disease mechanisms using them, require not only key structural functionality but also biofunctional features, in order to investigate molecular responses to receptors and transporters. These areas require further research in the future in order to appreciate their full potential.

4. Limitations and future challenges

The goal of 3D tissue engineering is not only the fabrication of whole-organ structures, but also the generation of functional engineered organs and tissues, in order to restore the sites of injury [13,138]. Although the hydrogels are useful for fabricating and maintaining 3D structure, the engineered tissue for transplantation therapy should synchronize with the tissues of the recipient following the transplantation. Therefore the ideal transplantation scaffolds should be hydrolytically or enzymatically degradable. A number of studies have focused on the development of biodegradable hydrogels [139,140], designed to degrade by hydrolysis [141], reduction [142], enzymatic reaction [143,144], or a

combination. Since the hydrogel degradation can, in practice, be tuned by chemical moieties of the hydrogels, the degradation rate and profile are controllable as well.

The future challenge for 3D tissue engineering in the field of regenerative therapy is the development of 3D tissue constructs without hydrogels. One of the recently developed hydrogel-free approaches is the use of a decellularized extracellular matrix (dECM) as a native scaffold [145]. This approach was first reported by Cho et al. [146], who fabricated the complex channel structure with dECM, using 3D printing technology. Despite this success, numerous issues remain, with the most significant being the potential removal of the various types of molecules in the ECM during the decellularization process. Therefore, the mechanical properties of fabricated tissues remain less strong as compared to those of the native tissues. Another example of hydrogel-free approaches is the use of cellular aggregates, such as spheroids. Hydrogel-free tubular tissues have been created by the 3D printing of spheroids into needle array [147]. These approaches may overcome the limitations of the hydrogels, but their potential is still being investigated. The understanding and methodology developed in the hydrogel microfabrication can contribute to the advancements in hydrogel-free technologies.

5. Concluding remarks

With recent advancements in 3D tissue engineering, hydrogel microfabrication technologies, such as micromolding, 3D bioprinting, photolithography, and stereolithography, are providing a more realistic approach to drug discovery and the development of alternative methods of organ transplantation. The production of well-defined architectures that mimic natural tissues and organs, without causing a significant cell damage, is one of the key challenges during the fabrication process. Additionally, combining the hydrogel microfabrication technology with cell culture platform, such as microfluidics devices, to provide nutrients and oxygen to the cells within the hydrogels, has not been investigated thoroughly to date. The progress in the fabrication of hydrogels and the development of methodology for cell cultivation will offer long-term improvement of the biological functions in 3D tissue constructs.

Conflict of interest

All authors declare no conflicts of interest.

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