

Combining additive manufacturing with microfluidics: an emerging method for developing novel organs-on-chips

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Additive manufacturing (AM) or 3D printing is an ideal technology for building flexible, complex, monolithic devices. Organs-on-chips (OOCs) are biomimetic microsystems that recapitulate the crucial structures and functions of human organs. Organ-level activities, mechanics and physiological response can be stimulated and investigated in OOCs. Convergence of AM technology along with OOCs offers a more efficient route for creating complex organ or tissue structures with precise 3D cell patterning, biomaterial heterogeneity and specific functionalities. Here, we focus on the recent advances in the field, specifically in the fabrication modalities, materials and characterization methods, which are commonly employed for OOCs based on 3D bioprinting. We also discuss the most significant potential applications from integrating 3D bioprinting with OOCs, aiming to provide future strategies for more efficient, automated, modularly integrated, and customizable OOCs.

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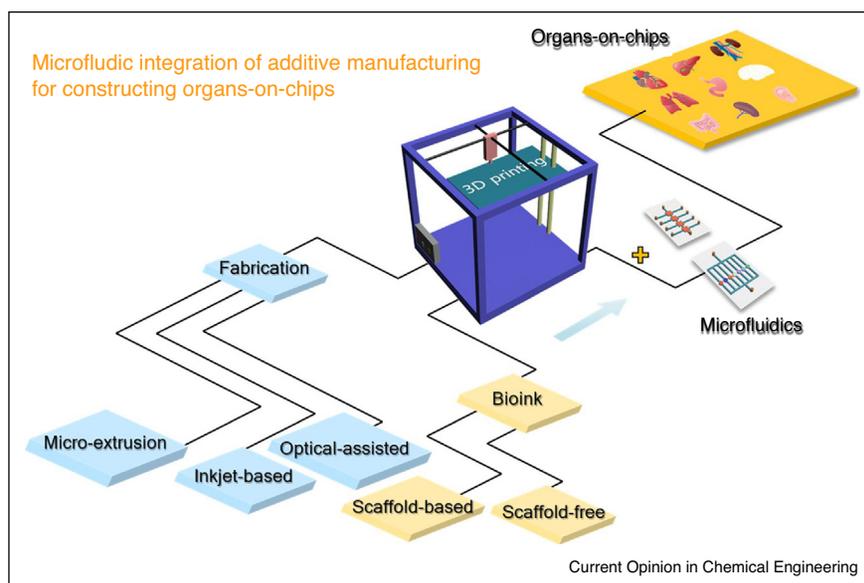
Introduction

Additive manufacturing (AM), also known as three-dimensional (3D) printing, spurs innovations in

fundamental research, engineering and education. Particularly, AM technology offers unprecedented digital platforms using computer-aided design and manufacturing tools to reconstitute biological architectures (e.g. tissue or organ) [1,2,3]. Organs-on-chips (OOCs) are the hierarchal organ-level microfluidic platforms for reconstituting complex structures and closely mimicking physiological functionality of real organs [4]. OOCs are potential alternatives for mammal models which will be eliminated by U.S. EPA before 2035 [5], and have been employed in the studies of tissue development, organ physiology, disease etiology, drug discovery and toxicity assays [6]. As a typical microengineered platform, early stage OOCs have been constructed predominately by using standard microfabrication techniques, which are often not adjustable to biological functionalities. Moreover, advanced OOCs should recapitulate multicellular architectures, mechanical features, tissue-tissue interfaces, and physicochemical microenvironments of the human body in a single platform. To achieve this, biocompatible materials, arranged cells, and supporting scaffold are often needed to be simultaneously patterned in precise geometries rather than additional biofunctionalization treatment after microfabrication.

3D bioprinting, a promising strategy that is derived from AM for building OOCs, is able to precisely control the spatial distribution and depositing sequence of cells, biomolecules and biomaterials layer by layer [7]. Then, living tissues or organs with heterogeneous structures and multiple materials are manufactured following a bottom-up order. Compared with conventional AM technologies, 3D bioprinting is more challenging because the choices of cell types, cell growth and differentiation factors, tissue construction and functionalities should be systematically considered. In the past years, there have been an increased number of efforts that integrate 3D bioprinting with OOCs [8]. Merits of such cooperation include the following: integration of large-scale microfluidics with micro-physiological structure, production of precise 3D cellular architectures, programmed flow control for stable microenvironment maintenance, mimicking functionality and extending cell viability, detectable generation of tissue/organ-level structures, and potential for building tissue-tissue interfaces. Schematic of main concept of integrating AM with microfluidics for constructing OOCs is illustrated in [Figure 1](#).

Figure 1



Concept of integrating AM with microfluidic for OOCs.

Herein, this mini review discusses principles, concepts, and approaches pertaining to 3D bioprinting. Also, the most significant advances in the past few years, potential and future perspectives of this integrated technology are discussed. A brief timeline and milestones of the OOCs and bioprinting development are illustrated in Figure 2 referred to Refs. [1,6,9,10].

Bioprinting modalities

In accordance with the working principle, technologies used for deposition and patterning of cells and relevant materials are mainly classified as micro-extrusion, inkjet-based and optical-assisted printing. Schematic of the principles and typical examples are illustrated in Figure 3.

Micro-extrusion bioprinting

Micro-extrusion bioprinting (MEB) deposits cells with biomaterials, usually in filament forms, onto a substrate, with predefined positions through a head or needle by physical forces. The forces are pneumatic-driven, mechanical-driven and solenoid-driven [11,12]. Materials can be continuously patterned onto the substrate layer-by-layer and solidified to have a sufficient mechanical integrity. The front layer serves as a supporting foundation for the next. Inks with viscosities ranging from 30 to 60×10^7 mPa/s are suitable for MEB and an increased ink viscosity strengthens the rigidity of fabricated structures. The most important strength of the technology is the ability to deposit cells with high densities, which is crucial for engineered tissues and OOCs. The universal applicability and cost-effectiveness of MEB made it the first and foremost method to use. While, cell viability by this

method is found to be relatively low (40–80%), resulting from the shear stresses in viscous fluids during dispensing procedure [1,2]. Recently, a tissue-organ printer based on MEB method has been used for stable, human-scale tissue constructs of any shape, providing a more realistic platform for OOCs development [13].

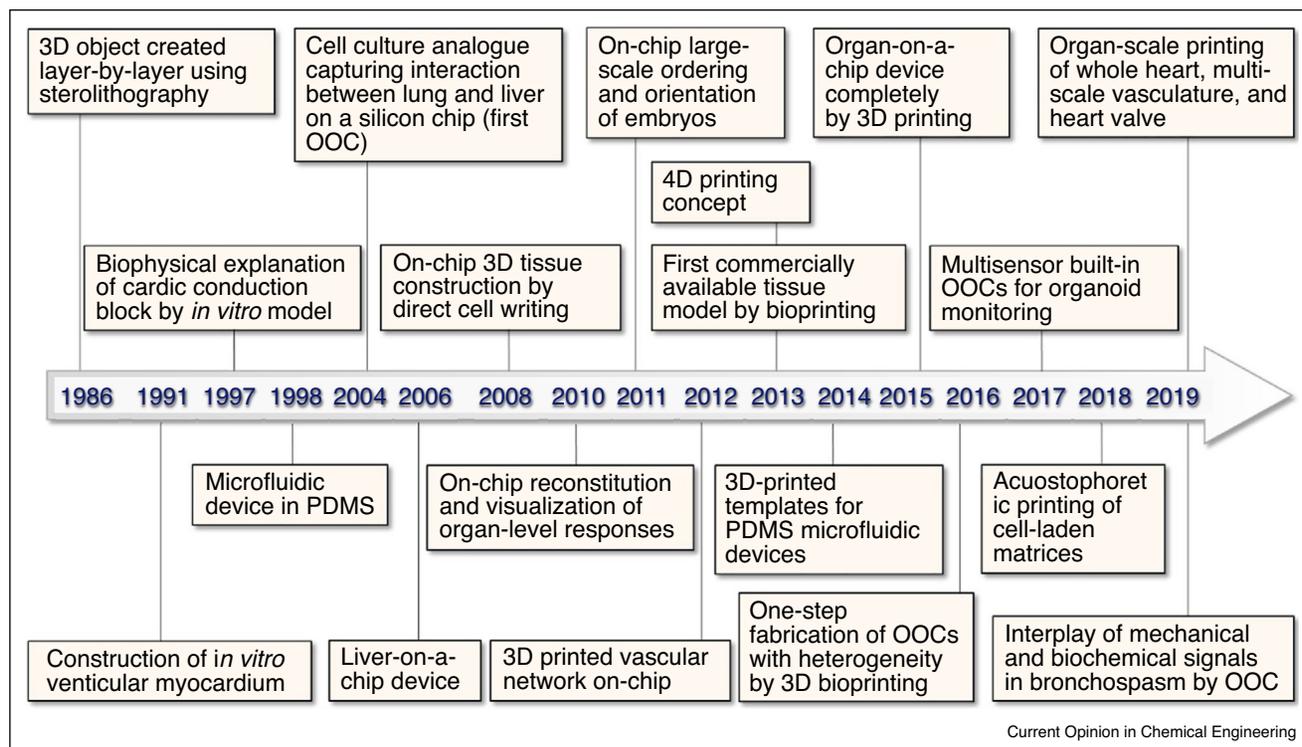
Inkjet-based bioprinting

Inkjet-based bioprinting (IBB) method fabricates 3D structures by delivering controlled volumes, usually in the form of droplets, which contain cells and cell-laden materials to substrates. Pressure pulses that spray droplets from a nozzle are usually generated by thermal, piezoelectric or acoustic methods [14]. IBB provides many advantages such as high throughput, precision, speed, reproducibility and wide availability. Main challenges of the IBB method lie within the formation of the droplets. Specifically, thermal-based methods induce localized thermal and mechanical stress that impact on cells and materials. Acoustic methods are prone to physical damages of the cell membrane since the working frequencies is in a range of 15–25 kHz by piezoelectric heads [15]. Also, bio-inks viscosity should be controlled between 3.5–12.0 mPa/s to minimize clogging of nozzles. Studies quote cell viabilities in excess of 85% by this printing modality. Recently, cell sedimentation in a microfluidic network has been demonstrated by the inkjet-based 3D-patterning method [16].

Optical-assisted bioprinting

Laser-assisted bioprinting (LAB) is a method based on the principles of laser-induced forward transfer. A LAB

Figure 2



A brief timeline and milestones of the development of OOCs and bioprinting.

system often includes a pulsed laser beam, a focusing system, a ‘ribbon’ containing a laser absorption layer, a biological layer, and a receiving substrate. Focused laser pulses on the absorption layer of the ribbon result in a high-pressure bubble that propels cell-containing materials toward the receiving substrate. Inks with viscosities ranging from 1 to 300 mPa/s are generally used. LAB studies have reported the highest cell viability of above 90% and the highest printed resolution compared to the two above modalities. While, preparation of ribbons is labor intensive and may become onerous once different cell types are involved. Also, laser power, biological layer thickness, and the length between the ribbon and the receiving substrate should be optimized before printing. Recently, a LAB-based technique has been reported for cell-laden microbeads patterning, which enables the growth and formation of self-contained, self-aggregating cells [17]. Besides, on the basis of the mechanism of photopolymerization, stereolithography (SLA), digital micro-mirror device-based stereolithography, two-photon lithography have also been used for bioprinting [10], particularly for functional microfluidic systems, with potential outlooks in OOCs [18*,19].

Other methods such as cell electrospinning [20] and surface tension-assisted manufacturing [21] have also been used in 3D bioprinting. Commercial bioprinters

have been developed for printing cells and biomolecules, such as EnvisionTEC 3D Bioplotter, Organovo NovoGen (the first 3D bioprinting company), and regenHU BioFactory™.

Bioprintable materials

Considering the inclusion or exclusion of exogenous elements, materials used in 3D bioprinting can be categorized into two types: scaffold-based and scaffold-free. The former bioink can be prepared by hydrogel, decellularized extracellular matrix (dECM) and microcarriers. The latter one is formed by cell pellet, tissue spheroids and tissue strands techniques.

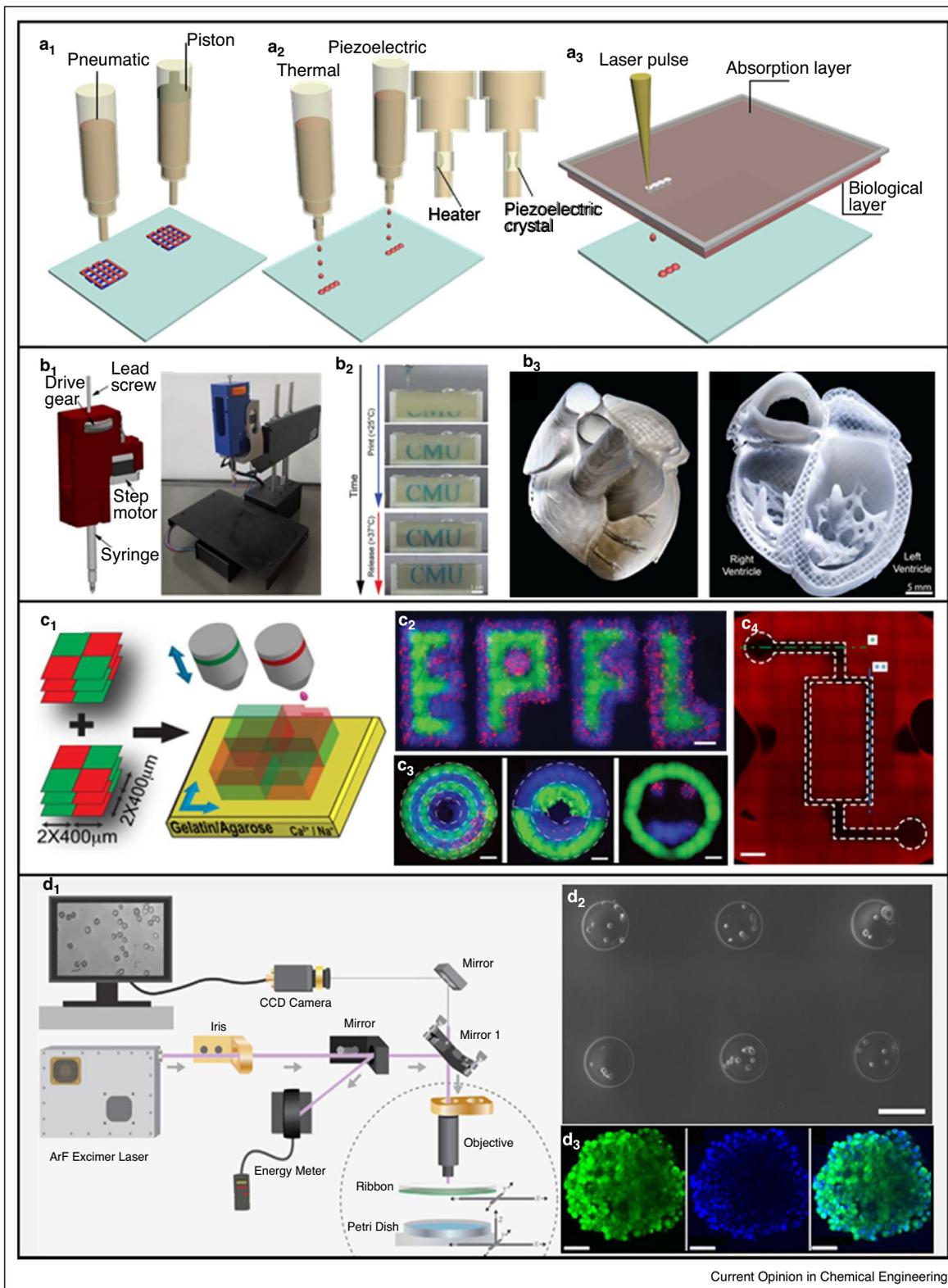
Scaffold-based bioink

Materials in scaffold-based bioinks serve multiple purposes: interacting with cells, providing vehicles for cell loading and building scaffolds for tissue formation.

As a cross-linked polymeric substance, hydrogel can absorb and maintain large amount of water to build a favorable environment for living cells.

Naturally derived (e.g. gelatin, fibrin, collagen, chitosan, and alginate) and synthetic hydrogels (e.g. polyethylene glycol and Pluronic©) have been widely used [22]. In cell-laden hydrogels, biologically active components

Figure 3



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Schematics of 3D-cell printing methods with different working principles: (a₁) micro-extrusion, (a₂) inkjet-based and (a₃) laser-assisted printing. **Micro-extrusion based printing** [12]. (b₁) Rendering of the assembled syringe pump extruder and a printer. (b₂) Time-lapse sequence of 3D bioprinting of a university logo. (b₃) Printed collagen heart and the cross-sectional view of the collagen heart. **Inkjet-based printing** [16]. (c₁) Schematic representation of 3D checkerboard composed of two patterns. Patterns of a university logo (c₂), concentric circles, partial circles

including growth factors, other extracellular matrix (ECM)-associated proteins are usually encapsulated for enhancing cell adhesion, cell proliferation or differentiation [23^{*}]. Solidification of printed hydrogels is realized through thermal, photo cross-linking, or ionic/chemical cross-linking processes. Recently, hydrogel bioinks have been doped with nanomaterials for improving robustness and cell differentiation [24^{*}].

In parallel, decellularized extracellular matrix (dECM) bioinks have been developed by laboratories and companies (Alloderm[®], SurgiSIS[®] and Synergraft[®]) for 3D bioprinting. dECM is isolated from organs/tissues to simulate the complex ECM microenvironment of human tissues [25]. In addition, microcarriers produced by synthetic (e.g. dextran) or natural (e.g. cellulose, gelatin, and collagen) materials are also used as reinforcements in bioinks by providing skeletons for cell growth [26].

Scaffold-free bioink

Multicellular aggregate can be directly used as a hospitable bioink since the evolution of organ is based on the cellular self-assembly mechanisms [1]. Techniques such as cell pellet, tissue spheroids and tissue strands have been utilized for preparing scaffold-free bioinks. Of these, cell pellet employs centrifugal or gravitational forces to concentrate cells at the bottom of conical tube, followed by cell transfer to a micropipette or other molds [27]; tissue spheroids are prepared by organizing cells into spherical-shaped aggregates which can serve as building blocks for tissue engineering [28]; tissue strands are formed by injecting and packing cells in a heavy density into hollow alginate tubes, which are used as filaments for 3D bioprinter [29]. The resulting cell aggregates can be directly used for downstream self-assembling without the need for sophisticated systems while improving the inter-cellular interactions.

Features including printability, biocompatibility, mechanical properties, material biomimicry, degradation kinetics and byproducts are essential to the real application of bioinks referred to [30].

Recent advances in OOCs by 3D bioprinting

Advanced bioprinting technologies and innovative biomaterials have boosted the development of OOCs systems. In the past years, there have been increasingly successful utilization in on-chip recapitulation of key structures/units and functions of tissues/organs [1,2^{**}].

Bioprinted liver-on-a-chip

Liver is the major organ for drug metabolism in the body. A one-step fabrication process based on 3D bioprinting has been employed for precise patterning of heterotypic cell types and biomaterials in a microfluidic platform, in which the liver function was reproduced [31]. A microfluidic biliary system equipped on a 3D liver-on-a-chip that is capable of liver cells co-culture and microenvironment modeling has been fabricated using native liver ECM components [32]. Also, a customer-tailored and tunable microfluidic channel fabrication method combining with cell-laden hydrogel constructs has been presented. Complex 3D-printer modifications and bioinks synthesis, processing hurdles can be avoided by this approach [33].

Bioprinted heart-on-a-chip

Heart is the vital organ for pumping blood through the vasculature to nourish tissues and organs. Endothelial cells within hydrogels have been printed and seeded with cardiomyocytes to generate aligned myocardium followed by embedding into a microfluidic bioreactor for cardiovascular toxicity assessment [34]. Also, instrumented cardiac microphysiological devices have been fabricated by using 3D printing of six materials. Drug responses and contractile development of human stem cell-derived laminar cardiac tissues have been studied in this device [35]. Most recently, formation of cardiac synthetic mini tissues derived from hiPSCs in a microfluidic chip has been achieved. Efficiency of regenerative cell transplantations has been improved [36].

Bioprinted kidney-on-a-chip

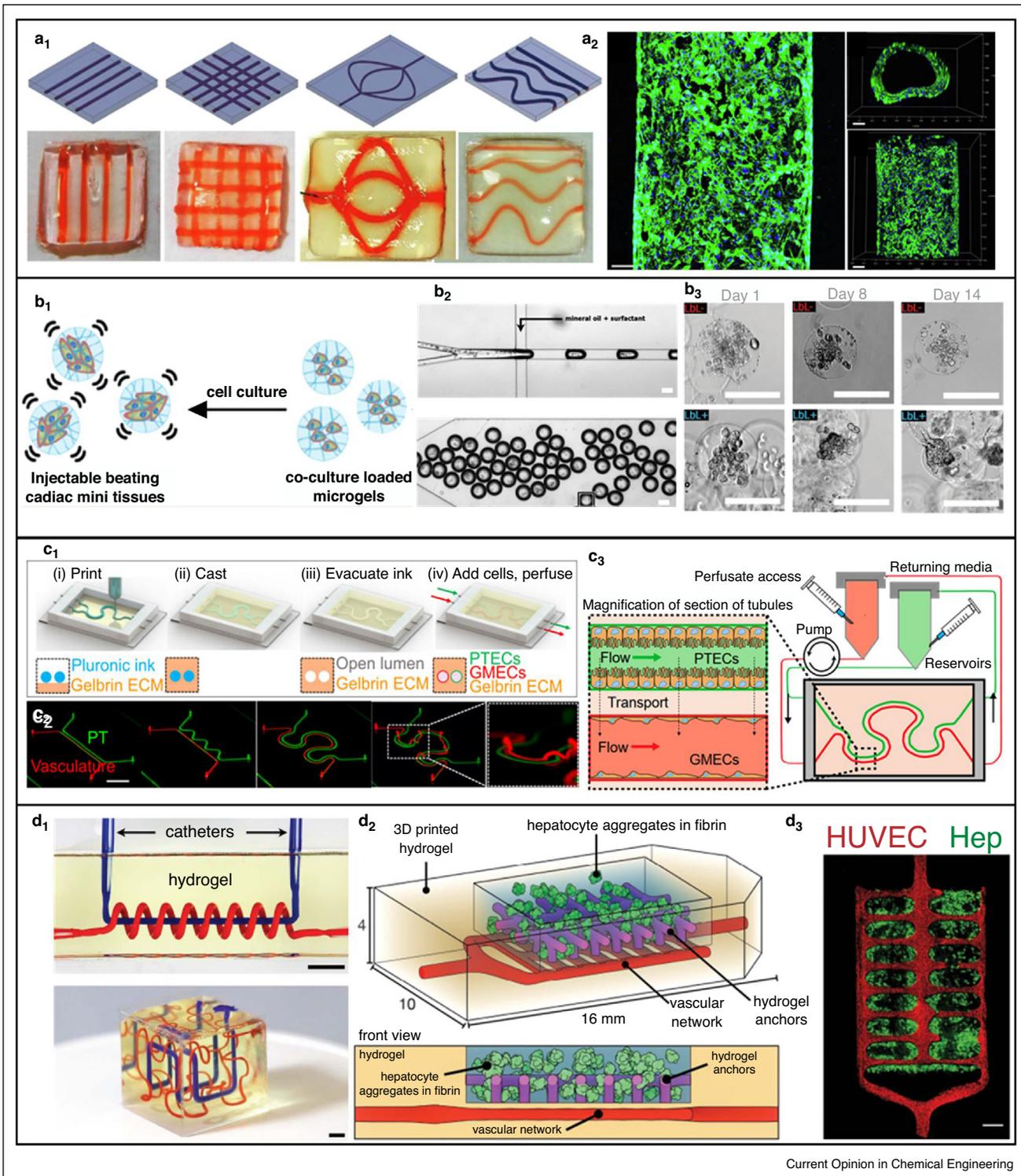
Kidney functions as a filter for blood, removing waste and reabsorbing useful substances, such as glucose. 3D human renal proximal tubules that are fully embedded within an ECM and housed in perfusable tissue chips have been printed. The on-chip proximal tubules have enhanced epithelial morphology and functional properties comparing with 2D samples [37]. Also, using a commercial bioprinter, a human proximal tubule model has been constructed, which can be used to predict clinical outcomes [38]. Most recently, 3D vascularized proximal tubule models consisting of adjacent conduits lining with confluent epithelium and endothelium have been embedded in a closed-loop perfusion microfluidic system to investigate renal reabsorption [39].

Bioprinted vasculature-on-a-chip

Vasculature provides essential supports for cell survival in a human body. A 3D micromolding technique using

(Figure 3 Legend Continued) pattern and 'Smiley face' (c₃) obtained by printing Fluo-ink (green) and Acri-ink (blue) containing Tomato NIH 3T3 fibroblasts (red). (g and h: scale bar 200 μm). (c₄) A bioprinting hydrogel-based microfluidic chip. Scale bar: 1 mm. **Laser-assisted printing** [17]. (d₁) Schematic of laser direct-write. (d₂) Fabricated microbeads and laden cells. Scale bar: 200 μm. (d₃) Confocal microscopy images of MDA-MB-231 3D aggregate. Scale bar: 100 μm. Reproduced with permissions from the American Association for the Advancement of Science [12] and Elsevier [17].

Figure 4



3D bioprinting integrated with microfluidic OOCs. **Liver-on-a-chip** [33]. **(a₁)** Digital designs and corresponding devices **(a₂)** Fluorescent images of the hUVECs cultured within channels (green showing F-actin and blue showing nuclei). Scale bar: 100 μm. **Heart-on-a-chip** [36]. **(b₁)** Principle of on-chip functionalized microtissue by coculture of hiPSC-CMs and normal human cardiac fibroblasts. **(b₂)** Microfluidic chip for cell-laden droplet generation (above) followed by transforming to microgels (below). Scale bar: 100 μm. **(b₃)** Micrographs of cultured hiPSC-CM/NHCF-Vs at days 1, 8, and 14. **Kidney-on-a-chip** [39]. **(c₁)** Schematic of 3D vascularized proximal tubule fabrication process. **(c₂)** Fabricated on-chip vascularized proximal tubule. Scale bar: 10 mm. **(c₃)** Integration of 3D vascularized proximal tubule tissue with a closed-loop perfusion for measuring renal reabsorption. **Vasculature-on-a-chip** [42**]. **(d₁)** Adaptations of mathematical space-filling curves to entangled vessel topologies of axial vessel

bioprinted agarose template fibers has successfully been employed to fabricate hydrogel-based functional and perfusable microchannel networks for vascularization study [40]. Cell-laden thick vascularized tissues integrated with parenchyma, stroma, and endothelium have been printed and perfused on-chip, establishing a foundation for tissue generation research [41]. Recently, multivascular networks and functional intravascular topologies within biocompatible or photopolymerizable hydrogels have been employed for investigating the oxygenation and flow of human red blood cells during tidal ventilation and distension of a proximate airway [42**].

Besides the above-mentioned, organs such as brain [43], skin [44], bone [45], gut [46], muscle [47] and tumor [48] have also been recapitulated *in vitro* by integrating microfluidics with 3D bioprinting. Examples of microfluidic integration of bioprinting for constructing novel organs-on-chips are illustrated in Figure 4. Albeit mostly being proof-of-concept, the potential of this technique for the development of various tissue/organ models has been verified.

Characterization

Characterization of 3D bioprinted OOCs is crucial to evaluate their development and functions. Biochemical and biomechanical analyses as well as viability are the most commonly used methods. However, novel characterization approaches and techniques are still required for 3D printed OOCs [15,49].

Cell viability is an essential parameter for the development of OOCs and is the most basic parameter that needs to be characterized. Mitochondrial activity by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay or its derivatives is commonly used to determine the viability.

Biochemical studies based on genomic and proteomic methods are employed for the evaluation of OOCs by providing genetic (DNA, or RNA) and protein expressions information. Genes or proteins expression levels in different stages of organ model development, drug metabolism and disease progression can be characterized on-chip. Cell detaching procedures are often performed on-chip before biochemical genomic and proteomic assays.

Biomechanical study of OOCs is important since the stiffness of the fabricated structure (e.g. tissue interface) affects the cell geometry, differentiation, and the tissue-level or organ-level physiology. Also, mechanical and physicochemical traits of the cell-laden hydrogel

structures should be examined before manufacturing. Compression and tensile tests cooperated with mathematical models have been used to assess the mechanical properties of on-chip models.

Conclusions and future directions

Convergence of 3D bioprinting with OOCs offers automated and high-throughput platforms for many applications such as toxicity evaluation, drug discovery and development. While, current bioprinted methods for tissue/organ study are still in the early developmental stage. Many issues including printing resolution, cytotoxicity and scaffold material should be better resolved. In brief, from a view of printing resolution, the extrusion-based printing, which has been the most widely accepted is still not yet compatible for all design when the on-chip structures become more sophisticated and heterogeneous. SLA has a higher resolution, but the cell viability is inevitably affected during laser or UV light exposing. Novel bioprinting processes and bioinks are under continuing development for these highly fruitful areas.

A recent strategy by adding ‘time’ to 3D printing (termed as 4D printing) based on smart materials has enabled the fabrication of structures with changeable shapes or functionalities [50]. Under external stimuli, these smart structures can be actuated to better simulate-specific functions of organs. In parallel, integration of embedded physical, biochemical and optical sensors with OOCs can record real-time cell behavior and environmental parameters. All these innovations will extend the applications of bioprinting integrated OOCs in fundamental research and clinical settings.

Conflict of interest statement

Nothing declared.

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(Figure 4 Legend Continued) and helix (up) and interpenetrating Hilbert curves (down) within hydrogels. Scale bar: 3 mm. **(d₂)** Hepatic hydrogel carriers created by seeding endothelial cells (HUVECs) in the vascular network after printing. **(d₃)** Confocal microscopy images of hepatocyte aggregates (Hep) in fibrin gel entrapped by hydrogel anchors. Scale bar: 1 mm. Reproduced with permissions from Elsevier [33], the American Chemical Society [36], the National Academy of Sciences of the United States of America [39] and the American Association for the Advancement of Science [42**].

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