Journal of Science: Advanced Materials and Devices 1 (2016) 1–17

Contents lists available at ScienceDirect

Journal of Science: Advanced Materials and Devices

journal homepage: www.elsevier.com/locate/jsamd

Review article

Three-dimensional printing of biological matters

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ARTICLE INFO

Article history:
Received 3 April 2016
Accepted 6 April 2016
Available online 19 April 2016

Keywords:
3D bio-printing
3D positioning system
Bio-ink
Hydrogel
3D scaffolds
Organ construction

ABSTRACT

Three-dimensional (3D) printing of human tissues and organ has been an exciting research topic in the past three decades. However, existing technological and biological challenges still require a significant amount of research. The present review highlights these challenges and discusses their potential solutions such as mapping and converting a human organ onto a 3D virtual design, synchronizing the virtual design with the printing hardware. Moreover, the paper discusses in details recent advances in formulating bio-inks and challenges in tissue construction with or without scaffold. Next, the paper reviews fusion processes effecting vascular cells and tissues. Finally, the paper deliberates the feasibility of organ printing with state-of-the-art technologies.

1. Introduction

The invention of the printing press changed the course of human history. The disruptive technology of printing text and images impacted society globally, acting as media for education, religion, politics, language, and culture [1]. Since then, a number of innovations further enhanced the printing technologies. For example, the introduction of dot matrix printers revolutionized the consumer market, where a computer linked to a printer as its peripheral device allowed desktop publishing and on-demand printing, reducing cost and time. The advent of the Internet introduced further an advancement, which allows documents to be available anywhere and printed just by the click of the mouse. Personalised printing made education, scientific research and arts more accessible to the broad population. Table 1 lists the major milestones in the history of printing technology. Although Charles Hull first introduced in the late 1980 three-dimensional (3D) printing through the so-called stereo lithography technology, its significance only started to materialise at the turn of the 21st century [2,3]. This versatile printing technology allows the fabrication of a wide range of 3D objects, from electric components to biological implants, through layer-by-layer patterning with ultraviolet (UV) exposure of photoresist films [4].

A 3D printer can also dispense biological materials making bio-printing possible. Generally, bio-printing can be achieved with layer-by-layer positioning of biomaterials as well as living cells. The precise spatial control of the functional materials allows for the fabrication of 3D tissue structures such as skin, cartilage, tendon, cardiac muscle, and bone. The process starts with the selection of the corresponding cells for the tissue [5]. Next, a viable bio-ink material is prepared from a suitable cell carrier and media. Finally, the cells are printed for subsequent culture into the required dimensions. The several approaches of 3D bio-printing are biomimicry, autonomous self-assembly and mini-tissue building blocks [6]. In contrast to conventional 3D printing, 3D bio-printing is more complex in terms of the selection of materials, cell types, growth/differentiation factors, and sensitivity of the living cells construction.

A typical 3D bio-printing process consists of the pre-processing, processing and post-processing stages. Pre-processing consists of the formation of an organ blueprint from a clinical bio-imaging system (i.e. MRI) and the conversion of this information into a direct instruction software of the standard template library (STL) for the printing hardware, which includes but is not limited to a series of integrated tools such as automated robotic tools, 3D positioning systems with printing head, ink reservoir, nozzle

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Peer review under responsibility of Vietnam National University, Hanoi.

http://dx.doi.org/10.1016/j.jsamd.2016.04.001

Table 1
Major milestones of the history of printing technology.

Milestone	Year (CE)	Details
Book printing	200	Woodblock printing used in China.
	1040	Letters rearranged for each page in movable typing.
	1440	Printing press introduced by Johannes Gutenberg.
	1884	Introduction of hot metal type setting.
	1907	George C. Beidler invented the Photostat machine.
Desktop printing	1968	Dot matrix printing invented by Digital Equipment Corporation.
	1970	Inkjet printing produced by Epson, Hewlett-Packard, Cannon.
	1979	Laser printer developed by HP for desktop.
3D printing	1984	3D printing invented by Charles Hull called stereo-lithography.
	1991	Word's first fused deposition modelling (FDM) invented by stratasys that uses plastic and an extruder to make 3D model.
	1992	Selective laser sintering machine (SLS) invented by DTM using power with laser to print the 3D model.
	2000	3D ink jet printer and multi-colour printer produced. Following year, desktop 3D printer introduced.
	2009	Commercial 3D printer available to market.

systems, video cameras, fiberoptic light sources, temperature controllers, piezo electric humidifiers, and integrated controlling software.

The processing stage is the actual printing session of the bio-ink using the bio-printers. The processing stage includes bio-ink preparation, clinical cell sorters (e.g. Celution, Cytori therapeutics), cell propagation bioreactors (e.g. Aastrom Bioscience), and cell differentiators to construct the desired biological structures.

The post-processing stage comprises the necessary procedures to transform the printed construct into a functional tissue engineered organ, suitable for surgical implantations. The post-processing stage may also include perfusion bioreactors, cell encapsulators and a set of bio-monitoring systems [7]. Each of these auxiliary machines has their own important roles for scaling up bio-printing. For example, cell encapsulators and bioreactors are essential to restrict undesirable fusion processes after the construction. Mironov et al. proposed a bio-reactor that is believed to maintain fragile tissue construct with sufficient time for post processing of tissue fusion, maturation and remodelling [8].

2. Technological considerations

The main technological challenges of 3D bio-printing are (i) the 3D positioning process, (ii) the formulation of a bio-ink and (iii) the dispensing system.

2.1. Three-dimensional positioning

Precise positioning of the print head plays a crucial role for the additive layer-by-layer construction of a 3D object. The positioning system is sometimes referred to as the bio-assembly tool (BAT) that utilizes computer aided design/manufacturing (CAD/CAM) software to precisely deposit various 3D heterogeneous cells [9]. BAT generally consists of multiple printing heads that can travel in a XY plane and adding through the Z axis for the printed layer [10]. A number of sensors are necessary to detect the thickness of each printed layer, and to adjust the print head for the next layer. Control software allows for the synchronization of these printing heads in the 3D space. The software may also consist of a number of text files or scripts for organizing the movement of the BAT and controlling the speed, air pressure as well as temperature. The 3D platform should be able to stop at various points during the printing process to change the bio-ink if necessary. Fig. 1 illustrates a typical 3D positioning system incorporating a print head and a printing bed.

For mapping a human organ, an X-ray, magnetic resonance imaging (MRI) or computed tomography (CT) scan can be converted to a bio-computer aided design (Bio-CAD) [11,12]. Surgical navigation software such as Stryker (Kalamazoo, United States), MedCAD (Dallas, United states) are some of the commercially available Bio-CAD packages. The Bio-CAD software visualizes 3D anatomic structures, differentiates heterogeneous tissue types, measures and differentiates vascular and nerve tissues and generates the desired computational tissue model [13]. A specialized software such as Rhinoceros 4.0 (real time simulation integrated with MATLAB/Simulink) can modify this bio-CAD design in extremely detailed slices with contour boundary paths that then can be synchronized with the 3D positioning system [13–16]. The software consists of a console and a master. The console analyses the 3D model, renders it onto a series of commands to be sent to the positioning stage. The master controls the positioning coordinates of the print head.

Surface mapping observes the printing status of each layer and decides the time to begin the construction of the next layer. The waiting time may vary from material to material, depending on its concentration and its thickness. For instance, Song et al. utilized a prototype system consisting of stepper motors for each X, Y, and Z axis movement and another axis for dispensing materials with a syringe. The positioning system had a precision of approximately 0.05 μm along the X and Y axis and of 0.125 μm in the Z axis. The optimum speed for depositing the material is typically between 1 and 10 mm/s. The software transferred the CAD model to a layered process path in Extensible Markup Language (XML) that directly controls the positioning system [17].

One of the most common problems of additive printing is the accumulation of errors that is associated with the printing height. This problem poses a big challenge to the construction of a large number of layers [18]. The accumulative errors eventually lead to an unsuccessful attempt for the 3D construct. However, for better

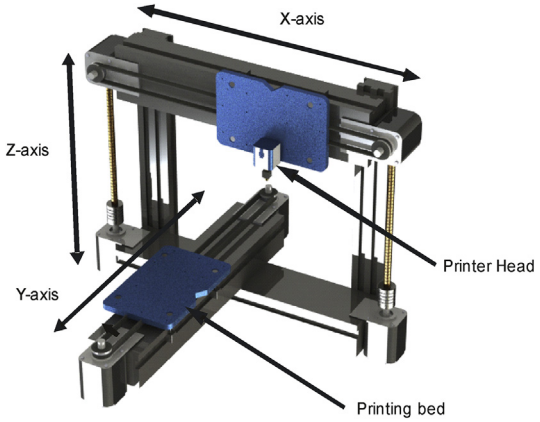

Fig. 1. Schematic of a 3D positioning system incorporating a print head and printing bed system.

observation and mitigating these errors, each print head could have individual controllable video cameras attached. Furthermore, fiberoptic light sources will illuminate and cure the constructed layer. A controlled heaters and piezoelectric humidifiers can prevent the polymerization in each head. Biomaterials such as collagen and pluronic-F127 can be easily constructed for a finite number of layers but will eventually lose shape due to swelling or dissolution [19,20]. Specialized techniques incorporating other bio-degradable materials may solve this problem.

Surface mapping feedback (SMF) is an algorithm-based geometric feedback software that can find errors between the printed layers. The software compares the measurement of the constructed cell with the virtual CAD model. Accounting for the errors detected by a displacement sensor, the deposited parameter can then be adjusted for in subsequent layers [21].

The BAT reported by Smith et al. has a resolution of around 5 μm, a linear speed between 10 μm/s to 50 mm/s and a deposition rate between 12 nl/s to 1 ml/s [10]. Smith's group developed a script to construct a five-layer artery branch of a pig heart using bovine aortic endothelial cells (BAECs) suspended in type 1 collagen. Cohen et al. improved upon a custom built robotic platform for solid-free fabrication of alginate hydrogel and calcium sulphate to construct pre-seeded living implants of arbitrary geometries [19]. The robotic platform has X–Y axes with a maximum transverse speed of 50 mm/s. The Z-stage served as a building surface with a positioning precision of 25 μm. Keating et al. used a 6 axis robotic arm (KUKA KR5 sixx R850) that limits the deposition of support material by building a rotating platform for printing complex structures [22]. The first 3 axes are used to position the robotic arm and the last 3 axes move the platform. The robotic arm used KUKA robot language and Python scripts to control the movement of the axes.

2.2. Bio-ink

Bio-ink developments are one of the most challenging issues in the 3D bio-printing process. Generally, the ink must fulfil the biological, physical and mechanical requirements of the printing process. Firstly, from a biological aspect, the ink should be biocompatible whilst allowing cell adhesion and proliferation. Physically, the ink requires a viscosity low enough to dispense from the print head. Finally, the paramount mechanical requirement is to provide sufficient strength and stiffness to maintain structural integrity of the ink after printing. Bio-inks are composed of living cells (typically 10,000–30,000 cells per a 10–20 μL droplet) suspended in a medium or pre-gel solution by polymer cross linkers (such as thrombin, $CaCl_2$, gelatin, fibrinogen, NaCl) that are activated by photo or thermal processes. For instance, poly (L-lactic acid) and poly (D, L- Lactic acid) can be dissolved in dioxane, with bone morphogenic protein grounded into particles and suspended in deionized water which can be used for making bone scaffold material [23].

Bio-inks without living cells are generally used to form scaffold support for later cell culture and growth. Typical scaffold materials include hydrogels such as agarose, alginate, chitosan, fibrin, gelatin, poly(ethylene glycol)-PEG hydrogels, poloxamers and poly(2-hydroxyethyl methacrylate)-pHEMA [24–28]. Besides forming the scaffold, these materials also help to culture functionalize cells. For example, agarose is a natural polymer that forms a gel at room temperature. Low melting point at 37° revert the gel into a solution allowing it to be washed away [29,30]. Alginate is a linear copolymer found in the walls of brown algae. Crosslinking with $CaCl_2$ at high concentration and low temperature, alginate can rabidly form a gel with high viscosity [29]. Chitosan is another linear polysaccharide obtained from shrimp and crustacean shells. Crosslinking with NaOH allows chitosan to rapidly form a gel matrix [29]. Collagen is a natural protein found in the body, as one of the materials in cartilage and bone tissues [29,31]. Fibrin is a protein produced in human body after the injury. Scaffolds with fibrin can help to repair bone cavities, neurons, heart valves in the human body [31,32]. Gelatin is a protein that helps to strengthen bones, joints, fingernails and hair qualities [33]. Poly(ethylene glycol) (PEG) hydrogels provide excellent biocompatibility, because this material can attach to most proteins, cells and antibodies [29]. Most common PEG hydrogels used for scaffold materials are polyethylene (glycol) diacrylate (PEGDA), poly (ethylene glycol) methacrylate/dimethacrylate (PEGDMA), poly (D, L)–lactic acid-co-glycolic acid. These hydrogels exhibit different transitional temperature. Poloxamer is a copolymer soluble in aqueous, polar and non-polar organic solvents [29]. The most common poloxamer for 3D printing is pluronic F127. This material is liquid at 4–5 °C and becomes a gel at room temperature (>16 °C). Poly(2-hydroxyethyl methacrylate)-pHEMA is a transparent polymer forming hydrogel in water. Oxygen to diffuse through the layer, makes them a good selection for bio-scaffolds [34].

Due to the ability to rapidly form a gel, the above hydrogels are suitable candidates for scaffold supports in later cell cultures. Fig. 2 shows the schematic presentations of the bio-ink for hard and soft bio materials. The next two sub-sections will discuss their formulations.

2.2.1. Bio-ink for hard materials

Bone marrow stromal cells (BMSCs), calcium phosphate (CaP), tri-calcium phosphate (TCP), poly(lactic acid) (PLA), poly-glycolic acid (PGA), poly-caprolactone (PCL) have been used to formulate bio-ink for hard materials [35,38–42]. BMSCs is a source of surrounding tissues with capability to migrate extensively in bone, cartilage and fat. This material also results in muscle degeneration. CaP has chemical similarity, biocompatibility and mechanical strength of bone, offering a huge potential for its construction, and repair. Over 70% of the bone is formed with CaP minerals. Another unique property of CaP is the ability to absorb different chemical species onto their surfaces [43]. Different compositions of CaP provide beneficiaries for the formulation of the bone grafts and its surroundings. TCP is one of the major components of bone mineral. The crystalline polymorphs of alpha/beta TCP provides improved compressive strength and better osteo conductivity. Hydroxyapatite (HA) is another form of CaP that efficiently purifies and separates proteins, enzymes, nucleic acids, growth factors and other macromolecules surrounding the bones [44]. Tetra calcium phosphate (TTCP) formed at temperature above 1300 °C is used for self-setting CaP bone cements [45]. Biphasic calcium phosphate (BCP) is a mixture of HA, Ca and beta-TCP. This material is used in orthopaedic and dental applications for forming micro porous structures with higher compressive strength, and better osteo conductivity [46]. PLA, PGA and PCL are the most common synthetic biodegradable polymers for bone fixations and cartilage repairs because of their excellent biocompatibilities, biodegradability's and mechanical strength [47]. These synthetic polymers accelerate the bone repair process without any sign of inflammation or foreign body reactions [48].

Bio-ink used for hard biomaterials were utilized predominately to construct strong connective tissue (i.e. bone). However, before forming a bio-ink, essential parameters such as powder packing density, flow ability, wettability, drop volume needs to be optimized [40]. Moreover, the printed bio-material should serve as ample support for the embedded cells, e.g. stiff enough to allow fiber arrangement whilst sustaining the force for handling and implantation.

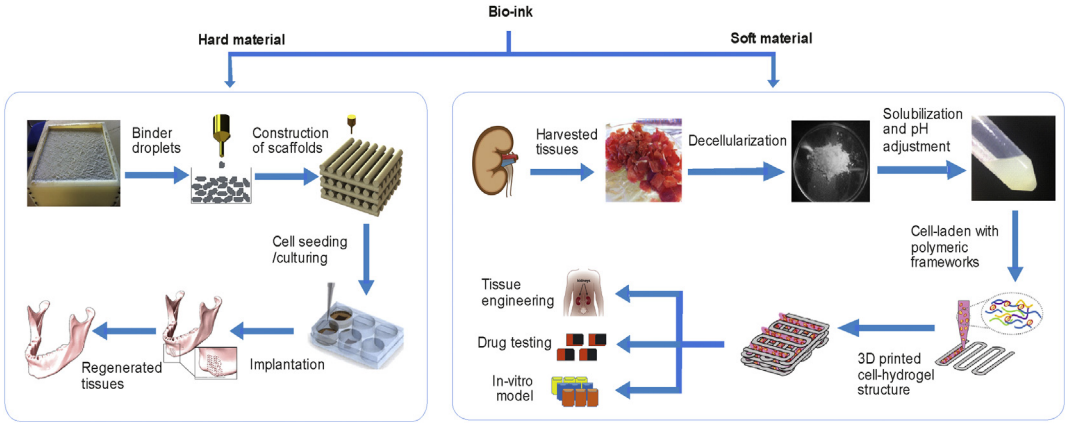

Fig. 2. Bio-inks for hard and soft materials (rearranged and redrawn from [35–37]).

Zhou et al. prepared a bio-ink material for hard tissue constructions (natural bone) with CaP (hydroxyapatite, HA and beta tricalcium phosphate, β-TCP) blended in calcium sulphate ($CaSO_4$) at different ratios [35]. Bergmann et al. fabricated a bone scaffolds by utilizing β-TCP as a bone cement mixing with bio-active glasses (45S5 Henchglass) [49]. Different combination of orthophosphoric acid (H_3PO_4), pyrophosphoric acid ($H_7P_2O_7$), isopropanol solution mixed with the processed powder, formed the predesigned scaffold structures. Inzana et al. implanted a 3D printed bone graft for tissue engineering applications in a mouse model [50], and subsequently proposed a number of steps to achieve a composite material of α-TCP and HA from CaP powder solutions. Their acidic binder solutions were prepared by dissolving collagen into phosphoric acid and the two solutions produces dicalcium phosphate dehydrate (DCPD) that was printed through a thermal ink jet printer.

Incorporating collagen in to CaP improved the overall bone strength, the osteo conductive and the osteo inductive characteristics, as well as the cellular attachments, viabilities, and proliferation of the cells. To observe the cell viability on the scaffolds, C3H/10T1/2 cells were seeded onto the printed constructs, which showed excellent biocompatibility and growth up to 72 h [50]. Kao et al. formulated a number of bio-ink materials as functionalized 3D printed scaffolds from poly(lactic acid) (PLA) [51]. However, the hydrophobic nature of PLA resulted in less cell recognition. So subsequently, polydopamine (PDA) surface coating was required to improve cell adhesion, proliferation and differentiation. Human adipose-derived stem cells (hADSCs) seeded on various fabricated PDA coated PLA scaffolds displayed improved cell adhesion and extracellular matrix (ECM) secretion. In conjunction with collagen, Shim et al. encapsulated recombinant human bone morphogenetic protein-2 (rhBMP-2) cells within collagen and gelatin solutions and dispensed them into a hollow cylindrical type PCL/PLGA scaffolds [52]. The combination of PCL/PLGA/collagen/rhBMP-2 showed a better bone healing capability over PCL/PLGA/gelatin/rhBMP-2 in a rabbit model. The 20-mm bone defects partially regenerated through newly formed bone tissue, fused with the rabbits native tissue after eight weeks post injury. Moreover, sufficient incorporation of oxygen and nutrients are imperative for hard tissue such as bone, in order to functionalize the printed structures and to facilitate vascularization into the host tissue [53,54].

2.2.2. *Bio-ink for soft materials*

Collagen, fibrin and decellularized adipose tissue (DAT) were used as ECM for soft materials bio-ink. Human mesenchymal stem cells (hMSCs), SMCs, HeLa, hepatocarcinoma (HepG2), fibroblasts, ovary cells, keratinocytes, neural cells, BMSCs, chondrocytes, epithelial cells, ADSC, ovary cells, hepatocytes cells have all been integrated into soft bio-materials [24,55–60]. Cui et al. developed a bio-ink for repairing defects in bone-cartilage plugs by combining human articular chondrocytes and PEG/DMA with a photo-initiator [61]. The printed construct produced excellent viabilities of almost 89.2%. Li et al. developed a bio-ink materials for constructing vascular channels using a combination of gelatin/alginate/chitosan/fibrinogen hydrogels as the supporting materials and rat primary hepatocytes (ADSCs) cells cross linked with thrombin, $CaCl_2$, $Na_5P_3O_{10}$ and glutardialdehyde [62]. A combination of these hydrogels and cross linkers can enhance the integrity of the vascular channels for more than two weeks. Human livers can be repaired or fabricated by seeding this ADSCs that performed liver like metabolic functions.

Each of the cells used in bio-ink need a different preparation process, so that they can retain their natural extracellular environment. For example, for forming a bio-ink with adipose tissue, decellularization is first needed. To decellularize the adipose tissue and achieve a high concentrated solution for printing, a number of steps were initiated to completely remove the cell's nuclei from the tissue for extrusion through the printing nozzles [63]. Decellularized extracellular matrix (dECM) was one of the best options for bio-ink material, as these cells can naturally obtain the microenvironment similar to their parent tissues. However, the challenge of formulating the bio-ink is to minimize the cellular material while keeping ECM loss and damage to a minimum. Pati et al. successful decellularized adipose (adECM), cartilage (cdECM) and cardiac muscle (hdECM) tissues utilizing physical, chemical and enzymatic processes with 3D open porous structures. The decellularization efficiency was quantified through DNA analysis, showing a 98% reduction of cellular contents [37]. Furthermore, the authors successfully printed these soft material structures up to a thickness of 10 layers. Song et al. used a hyaluronic acid-HA (an extra cellular matrix protein) based hydrogel as the bio-ink. To form the gel, HA was cross linked with poly(ethylene glycol) which can be used at a later date as the base material for bio-printing [17]. De Maria et al. trialled human skin fibroblast at concentrations of 100,000 cells/ml in the bio-ink, that is supported by Eagle's minimum essential medium (EMEM). In this case, 360 drops or 50 μl (about 5000 cells) were dispensed in a predesigned well, and the well was filled with 450 μl EMEM to avoid the impact of the droplets with the rigid substrate [64].

Hydrogel materials pose excellent bio compatibility, biodegradability and tuneable mechanical properties, albeit their high water content. Hydrogel materials are reported as an encapsulator for viable cells, as they can keep cells alive without affecting

cell–cell interaction and to support the cell constructions. For example, Duan et al. implemented a 3D bio-printing system to fabricate an aortic valve conduits [65]. Aortic root sinus smooth muscle cells (SMC) and aortic valve leaflet interstitial cells (VIC) were separately encapsulated in an alginate/gelatin hydrogel solution. These encapsulated cells were still viable within the hydrogel encapsulator over a seven day culture (81.4 ± 3.4% for SMC and 83.2 ± 4% for VIC). Lozano et al. constructed a 3D brain like structures with bio-ink materials consisting of primary cortical neurons encapsulated by gellan gum arginine-glycine-aspartate (RGD-GG) which is a modified bio-polymer hydrogel [66]. To stabilize the pH of the bio-ink, NaOH was added afterwards. The study of Lozano et al. suggested that the gellan gum (GG) is a good encapsulation material for neuronal cells with low cost, high gelling efficiency, and improved bio-compatibility [67]. Moreover, GG modified with RGD increases cell adhesion and proliferation. Chung et al. utilized three different concentrations of sodium alginate solutions in phosphate buffered saline (PBS) separately blended with gelatin solutions [68]. The solution was ionically cross-linked with $CaCl_2$ and equilibrated in dulbecco's modified eagle medium (DMEM)/fetal bovine serum (FBS) culture medium. Primary myoblast (BL6) cells were cultured with appropriate media (Hams F10, FBS, penicilin) and combined with the solution as an encapsulator. The prepared hydrogel-based bio-ink showed excellent cell culture viability support and cell proliferative facilitation for primary muscle growth. Lee et al. fabricated a hybrid scaffold material consisting of an acrylate trimethylene carbonate (TMC)/trimethylolpropane (TMP) and alginate hydrogel solutions to encapsulate chondrocyte cells. The seeded cells and the scaffolds structures remained stable up to four weeks upon implanting into a mouse model [69,70].

Miniature tissue spheroids can be incorporated into a bio-ink, allowing uniform geometry that is necessary for cell–cell interactions [71,72]. Tissue spheroids are sphere shaped groups of cells formed by spontaneous assembly within cellular suspensions. Uniform sized tissue spheroids are essential for bio-printing large tissues and organs. As tissue spheroids are formed by aggregation of cells, they possess maximum possible cell density within each spheroid. The average diameter of the tissue spheroids ranges from 100 to 300 μm [73]. Spheroids intrinsic capacity of being fused over time, makes them an ideal candidate for forming bio-ink materials [74].

Norotte et al. used Chinese hamster ovary (CHO) cells, human umbilical vein smooth muscle cells (HUVSMCs), human skin fibroblasts (HSFs) cells cultured in various ratios to form a desired cell spheroids as a bio-ink materials to construct vascular tubes [73]. The spheroids fused within 5–7 days resulting the final structure. However, a large quantity of spheroids for constructing longer structure is time consuming and a long fusion time could lead to a non-uniform hollow structures. Almost 4000 spheroids of 300 μm were needed to construct a simple 10 cm long and 1.5 mm diameter tube. Therefore, to form a large structure, rapid deposition process and fast fusion of spheroids are necessary. This research group also developed a bio-ink with similar cells (multicellular cylinder as a bio-ink) dispensing continuously to form a cylindrical shapes. The multicellular cylinders fused faster than the spheroids structure, and needed only 2 –to 4 days to form the final shapes. However, the outer diameter of 900 μm (dispended with 300–500-μm diameter micropipette) limits the cell viabilities. A smaller micropipette could construct a narrower tube resulting in more viable cells.

Recently Raja et al. exploited the floating liquid marble platform to generate spheroids of olfactory ensheathing cells (OEC) [75]. 5000 cells per 10 μL of marble generated numerous uniformed spheroids (around 30 spheroids per marble) with an average diameter of 90–120 μm. The OEC spheroids showed extensive cell–cell interactions indicating robust growth and healthy behaviour over time. The floating marble on appropriate culturing medium provided sufficient nutrients for the cell spheroids to survive. The group is expecting to utilize these OEC spheroids as a 3D printable bio-ink material to analyse spinal cord injury system in *in-vivo* applications. It is possible to formulate enormous amount of spheroids within a short period of time.

Furthermore, cells must be encapsulated in a non-adhesive and lubricated hydrogel such as hyaluronan to prevent preliminary tissue fusion inside the cellular suspension reservoir. Tan et al. formed tissue spheroids by mixing ECs and SMCs (1:1 ratio) seeded into non-adhesive agarose hydrogel moulds [76]. Approximately 840 uniformed cell spheroids with an average uniform diameter of 300 μm were prepared and printed. The cells can further be encapsulated within other hydrogel material such as alginate, collagen and cross linked with Ca^{2+} solutions to restrict their aggregation and sedimentation. However, these encapsulating approaches are not suitable for all cell types, as some cell types require a specific arrangement according to their phenotypic functions [24]. For instance, Ferris et al. tested a consistent printing output of cells without allowing for settling and aggregation, over an extended time periods [77]. Ferris et al. formed a micro gel with biopolymer gellam gum combined with DMEM, and/or poloxamer 188 surfactant in different concentrations. C2C12, PC12 and L929 cells were separately maintained in DMEM, FBS and mixed with the microgel solution to form the bio-ink. The printed construct on collagen hydrogel makes the cells hydrated and viable without settling and aggregated for a long time period. Table 2 presents the bio-ink materials with appropriate media/cross linkers conducted by various groups.

It is important to find out the nature of the extruded bio-inks. For example, if the bio-ink is acidic in nature, it must first be adjusted to the physiological pH before encapsulation with cells, whilst maintaining the desired temperature [37]. Rutz et al. proposed a versatile method with various hydrogels that can tune the mechanical, physical, chemical and biological properties of the bio-ink [78]. Investigations were conducted to validate these formulations for cell viabilities after printing with live/dead assays in PEGX-gelatin and PEGX-fibrinogen.

2.3. Modification of the print head

Depending on the deposition technique of the print head and the bio-ink, bio-printers are categorized in three types: (i) ink jet, (ii) laser jet and (iii) extrusion.

An ink-jet printer consists of an ink chamber with a number of nozzles. A short current pulse passes through an integrated heating element creating a bubble forcing the ink out of the nozzles [84]. A piezoelectric actuator can also be used for this purpose. A voltage pulse induces a charge on the piezoelectric material and ejects droplets out of the nozzle [85]. The ink-jet technique offers advantages such as low cost and minimal contamination of the cells due to the non-contact deposition technique. However, heat, mechanical stress and vibration could adversely affect the cell viability, clog the nozzle and make it harder to construct a multi-layer 3D structure [86].

Laser jet is the next deposition technique that utilizes the energy of a laser pulse to create the actuation bubble ejecting the cells onto a substrate [55]. This technology can work with a high-viscosity bio-inks such as hydrogel consisting of alginate and collagen and provides a high degree of precision. However, the relatively long printing time and the heat generated from the laser lead to a higher rate of damaged cells [87].

Table 2
Bio-ink materials with appropriate media/cross linkers.

Printed objects	Printing technique	Bio-ink formation				Media	Implants	Ref.
		Scaffold	Encapsulator	Cells/Protein	Cross linker			
Hard tissues	Thermal	CaP:$CaSO_4$, HA:$CaSO_4$ and, β-TCP:$CaSO_4$	–	–	Water based binders	–	*In-vitro*	[35]
	–	β-TCP, bio-active glass (45S5 Hench glass)	–	–	H_3PO_4, $H_7P_2O_7$	–	*In-vitro*	[49]
	Thermal	CaP solutions with α-TCP and HA	–	C3H/10T1/2	Collagen, Acidic binder (phosphoric acid)	–	*In-vitro*/ *In-vivo*	[50]
	–	PLA coated with PDA	–	hADSCs	–	DMEM, FBS, penicillin, streptomycin	*In-vitro*	[51]
	Extrusion	PCL,PLGA	Collagen, gelatin	hTMSCs, rhBMP-2	–	DMEM, FBS	*In-vitro*/ *In-vivo*	[52]
	Thermal	Chondrogenic progenitor plugs (bio-paper)	PEGDMA	Human articular chondrocytes	Photo initiator	DMEM, Human serum, penicillin, streptomycin, glutamine	*In-vitro*	[61]
	Laser	Titanium powder	–	Human osteogenic sarcoma (MG63)	Silica sol	–	*In-vitro*	[79]
Soft tissues	Extrusion	–	Agarose rods	CHO, HUVSMCs, HSFs, PASMCs	–	DMEM, FBS, antibiotics (penicillin, streptomycin, gentamicin), Geneticin, Hams F12, glutamine, gelatin	*In-vitro*	[73]
	Extrusion	Gelatin, alginate, chitosan, fibrinogen	Gelatin, alginate, chitosan, fibrinogen	Hepatocytes, ADSCs	Thrombin, $CaCl_2$, $Na_5P_3O_{10}$ and glutaraldehyde	DMEM, FBS, penicillin, streptomycin, aprotinin,	*In-vitro*	[62]
	Extrusion	PCL	DAT	hASCs	–	DMEM, FBS, penicillin, streptomycin	*In-vivo*	[63]
	Extrusion	PCL	adECM, cdECM, hdECM	hASCs, hTMSCs	–	DMEM, αMEM, FBS, antibiotics (penicillin, streptomycin)	*In-vitro*	[37]
	Extrusion	–	RGD-GG	Primary cortical neural cells	DMEM or $CaCl_2$	Collagenase, FBS, neurobasal media, glutamine, penicillin/ streptomycin	*In-vitro*	[66]
	Extrusion	–	Sodium alginate, gelatin	Primary myoblast (BL6)	$CaCl_2$	DMEM, FBS, penicillin, streptomycin, Hams F10, glutamine	*In-vitro*	[68]
	Ink jet	–	Sodium alginate	ECs and SMCs	$CaCl_2$, gelatin	EGM-2 (Endothelial growth medium)	*In-vitro*	[76]
	Piezo-Ink-jet	Collagen bio-paper	Gellam gum	C2C12, PC12 and L929	Poloxamer 188 (P188) and/ or fluorinate	DMEM, FBS, HS (horse serum)	*In-vitro*	[77]
	Extrusion	–	Gelatin, fibrinogen, 4 arm PEG amine	HDFs, HUVECs	PEGX-gelatin, PEGX-fibrinogen, EDC {N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide}, NHS (N-Hydroxysuccinimide), thrombin	PBS (phosphate-buffered saline) or DMEM, FBS, antibiotics (penicillin, streptomycin)	*In-vitro*	[78]
	Thermal	Sodium alginate-collagen composite	–	hAFSCs, dSMCs, bECs	$CaCl_2$	MEM, DMEM, EBM-2, clonetics, FBS, glutamine, penicillin/streptomycin,	*In-vitro*/ *In-vivo*	[80]
	Extrusion	Gelatin methacrylamide	Hydrogel solutions (Bovine type B gelatin)	HepG2	1-{4-(2-Hydroxyethoxy)-phenyl}-2methyl-1-propane-1-one, and 2,2′-Azobis{2-methyl-N-(2-hydroxyethyl) propionamide}	DMEM, FBS, penicillin and streptomycin	*In-vitro*	[81]
	–	–	–	HAFs and HUVECs	–	DMEM, penicillin/ streptomycin, EGM-2	*In-vitro*	[82]
	Extrusion	PCL, alginate solution	Sodium alginate	Chondrocytes, osteoblast	$CaCl_2$, NaCl solutions	DMEM/FBS/penicillin and streptomycin.	*In-vitro*	[83]

Extrusion technique is another deposition technology that utilizes a pneumatic dispensing system for delivering the cells. This technology suits a wide range of bio-ink viscosities and allows continuous deposition, fast printing time and better structural integrity [87]. Even though extrusion process is considered to be the most adopted technique to date, the technology also faces several limitations such as limited material selection due to rapid cell encapsulation and increased shear stress resulting in more cell injuries [86].

Fig. 3 illustrates the above-mentioned printing technologies. Each of the printing technologies has their own advantages and limitations. A suitable technology and the corresponding print head must be considered based on the cell characteristic, resolution, desired accuracy, number of deposition layers, structure of the constructed tissue, printable size and overall printing time before experimentation ensues.

A print head generally consists of a dispenser control unit, a number of sensors, a set of reservoirs, biocompatible nozzles, and supplementary components such as filter, hose tubes, camera and curing light. The print head needs to be biocompatible allowing for non-toxic delivery of bio-ink without exposing the cells to elevated temperatures and pressures. Conventional print heads have fixed

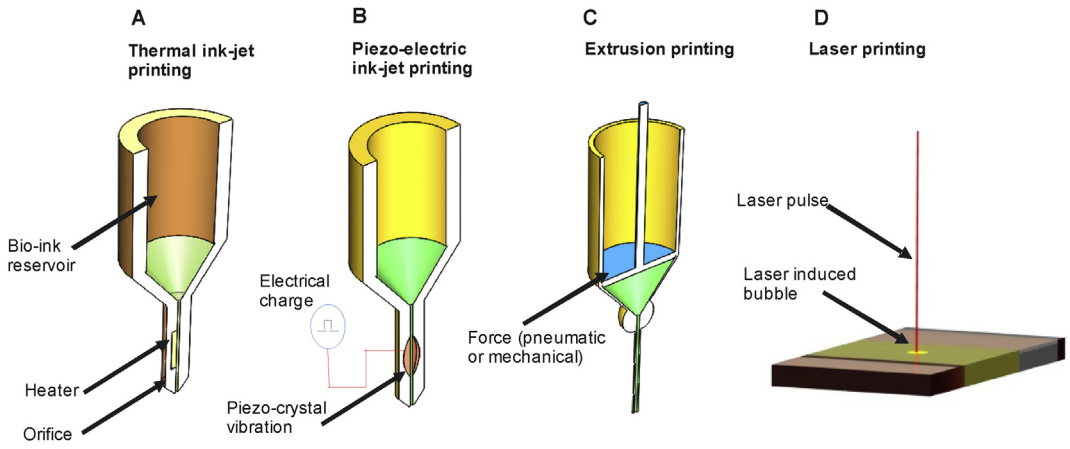

Fig. 3. Schematic diagram of (A) thermal ink-jet printers, (B) piezo-electric ink-jet printers, (C) extrusion printers, and (D) Laser printers.

structural parameters and operational characteristics. Only a small number of selective materials can be dispensed from these print heads. For the purpose of bio-printing, the head needs to be modified allowing multi-nozzle capabilities to dispense different polymers, hydrogels or the combination of both, simultaneously [8]. To date, researchers have always customized and modified the print head according to their specific needs [88,89]. Thus, the print-head operation may vary from continuous flow to extrusion modes to drop on demand (DOD) modes [64]. Print head clogging, reduced cell viability, and DNA damage of cells are a few among many challenges in designing and modifying a print head.

De Maria et al. modified a piezo-electric ink jet print head. The flow was controlled by an electronic board equipped with a micro-controller (ATmega328P) [64]. Ang et al. utilized a print head consisting of a robotic dispensing system and a pneumatic dispenser to deliver chitosan at a variety of viscosities [90]. Moreover, the authors used Teflon lined nozzle to prevent adhesion and accumulation of cells around the nozzle tip. Pati et al. utilized six printing heads and six holders to dispense cells and hydrogels simultaneously. Each of the print heads operated at a different temperatures depending on the properties of the materials [63]. Norotte et al. used two print heads to simultaneously deposit scaffolds in the form of gels and multicellular mixture [73]. Coatney et al. used three print heads to construct blood vessels and cardiac tissues. Coatney et al. utilized the first two print heads to dispense cardiac and endothelial cells. The third print head dispenses collagen to ensure support for the cell structure during the printing [91].

Dispensing bio-ink through a modified print head has to consider the shear rate the cell will endure during the extrusion. The average shear rate is the ratio between the speed of a droplet (ms^{-1}) and its radius (m). Previous reports suggested that the allowable shear rate for cell survival should be below $5 \times 10^5\ s^{-1}$ [92]. Therefore, the expected shear rate has to be determined before the printing process, and correlated with the viscosity of the cells. A high shear force will damage the cells and thus reducing their viability in the printed construction [37]. For instance, in syringe based bio-printing, dispensed cells will endure higher shear force with small nozzle diameters. The movements of the print head could expose the constructed cells to either compressive or tensile forces. Chang et al. examined the effect of pressures and varying nozzle sizes on viability, recovery, and functional behaviour of HepG2 liver cells encapsulated by alginate [93]. The report suggested that cell viability is proportional to nozzle diameter, and inversely proportional to the applied pressure.

Commercially available one or two reservoir systems have been reported incorporating a nozzle system with an average inner diameter of 200 μm−1600 μm. Reservoir material could be made of aluminium, stainless steel, polyethylene or polypropylene coated by bio compatible solutions [94]. Each reservoir can carry specific scaffold or cell materials. These reservoirs could have a number of sensors to synchronise the nozzles of the print head.

Selecting a right nozzle for printing biological cells is another crucial design consideration for a print head. Conventional nozzles/needle could be converted into biocompatible nozzles by coating bio-compatible silicone to increase the hydrophobicity of the inner and outer surfaces. The coating prevents ink adhesion within the nozzle/needle [64]. Nozzle size also affects the printing speed. Song et al. showed that printing speed linearly increases with reduced needle diameters [17]. However, a small needle diameter would result in a smaller printed pattern. So the right reservoir and nozzle has to be selected depending on the characteristics of the cells and the constructed tissues. The nozzle can be controlled to dispense bio-ink droplets of different sizes.

Billiet et al. conducted an experiment with the nozzle shapes (conical and cylindrical) on HepG2 cells. The results showed higher cell viabilities using conical shaped nozzle compared to cylindrical shape nozzles under low inlet pressures [81]. Moreover, cells printed with a bigger nozzle diameter maintained a higher cell survival rates of around 97% then smaller diameters. Yan and his groups varied the process parameters such as applied pressure and nozzle size affecting the cell viabilities [95]. They conducted a computational fluid dynamic (CFD) analysis based on shear stress and exposure time in term of cell damage. Experiments were carried out on cells (Rat adrenal medulla endothelial cells-RAMEC) mixed with alginate solutions deposited on calcium chloride solutions with different pressure and nozzles sizes. The experimental data shows that cell damage increases with high pressure whereas larger nozzle diameter minimizes it. Moreover, exposure time also has an impact on cell viabilities. A combination of higher pressure, and longer exposure time could lead to a higher cell damage.

Jones et al. examined the effects of nozzle length on cell viabilities. The result suggested that the short nozzle length (8.9 mm) provides higher cell viabilities of almost 84% compared with the long nozzle length (24.4 mm) with a cell viabilities of 71% [96]. As long nozzle increases the dispensing time of cells subjected to face shear forces throughout the nozzles, viability of the cells dramatically reduces.

2.4. Computer aided design and manufacturing (CAD/CAM)

As mentioned in the earlier section, the information of the sliced layered design with individual cell types and sizes passes to the

control of the nozzle position [97]. The software (called synchronizer or advance programming interface (API) synchronizer), the motion control unit, and the reservoirs connected to the print head work in a real time. The software passes signals requesting material information from the sensor in the reservoirs. The designated sensor then sends back the present status of the material of an individual reservoir [15]. Subsequently, the controller sends a set of commands to the individual reservoir to dispense the bio-ink droplets considering the specific cell types, cell sizes and viscosities. After dispensing the droplets containing the cells, feedback information returns to the control unit. This unit has a microcontroller-based motion control software that directs the print head to a specific coordinate according to the pattern and changes the reservoir and supplementary component if different materials are needed [15]. Fig. 4 illustrates the representative working steps of a hypothetic human organ transferring into a printed model. Depending on the needs, more print heads associated with a set of reservoirs, nozzle systems, and sensors can be appended.

For printing, heads containing multiple nozzle systems and a set of microcontroller units synchronize the multiple nozzles with the positioning system. The control software might be integrated with the 3D positioning software or could work independently. However, the software must know the position and the type of material to be deposited. In this regard, both the dispensing software and the 3D positioning software need to be synchronized. Users should be able to configure each nozzle depending on their need. For example, Yan et al. designed a multi-nozzle deposition system based on extrusion printing for fabricating scaffolds of bone tissue structures [23]. Each of the nozzles played a different role for the construction and the maintenance of the cells. The first nozzle (screw pump) deposited a composite of poly (L-latic acid), tri calcium phosphate (TCP) to form bone tissue scaffolds. The second nozzle (solenoid) dispensed de-ionized water as a supportive material, and the third nozzle (ultra-sonic homogenizer) sprayed bone morphogenic protein (BMP) particles with de-ionized water to recruit stem cells from the surroundings.

The selection of the print head and the nozzle type depends on the property of the bio-ink. For example, extrusion type print head and nozzle with high dispensing pressure are suitable for bio-inks with high viscosity. For inks with medium viscosity, a screw pump design can be selected to dispense cells with high viability. For ink with low viscosity, a solenoid nozzle is preferable [23]. Saedan et al. developed two types of nozzle systems: piezoelectric nozzle for materials with low viscosity and low flow rates, and the solenoid nozzle for materials with relatively high viscosity [15]. Khalil et al. constructed a multiple nozzle system for up to 40 layers of hydrogel scaffold made of sodium alginate of various viscosities [98]. Each of these nozzles has a different deposition technique. For example, a current pulse activates solenoid nozzles. An applied voltage actuates a piezoelectric nozzle made of a glass capillary. Pneumatic syringe nozzles operate with a pressure pulse. A spray nozzle also operates with a pressure pulse. These nozzles are also capable of printing cells, growth factors and other scaffold materials.

To speed up the printing process, it is possible to use more than one automated arm with multiple print heads. Ozbolat et al. developed two independent and identical 3-axes bio-printers called multi-armed bio-printer (MABP), capable of printing multiple bio-inks simultaneously [99]. This deposition system operated with stepper motors and linear actuators. The dispensing nozzle is connected with a pneumatic fluid dispenser. The deposition rate of the bio-ink is controlled during the deposition process. Modified ink jet printers with piezoelectric pumps have been reported for assembling cells onto a 3D shape. The modified printers use individual cell spheroids to form the 3D scaffolds [88,89]. The modified ink jet printer works similar to the BAT system. They utilize a syringe and a needle tip capable of sterilizing separately. The print head can be modified to allow multiple nozzles to work at the same extrusion time to form cell patterns.

3. Recent applications of 3D bio-printing

The human body consists of more than 200 different and sophisticated cell types with their own biological, chemical, and physical properties [100]. The main aim of bio-printing is achieving printed functional cell and tissue systems towards organ printing. To achieve this aim, researchers need to investigate the viability and longevity of cells during and after the printing process. This section will elaborate recent attempts of printing cells, tissues and organs.

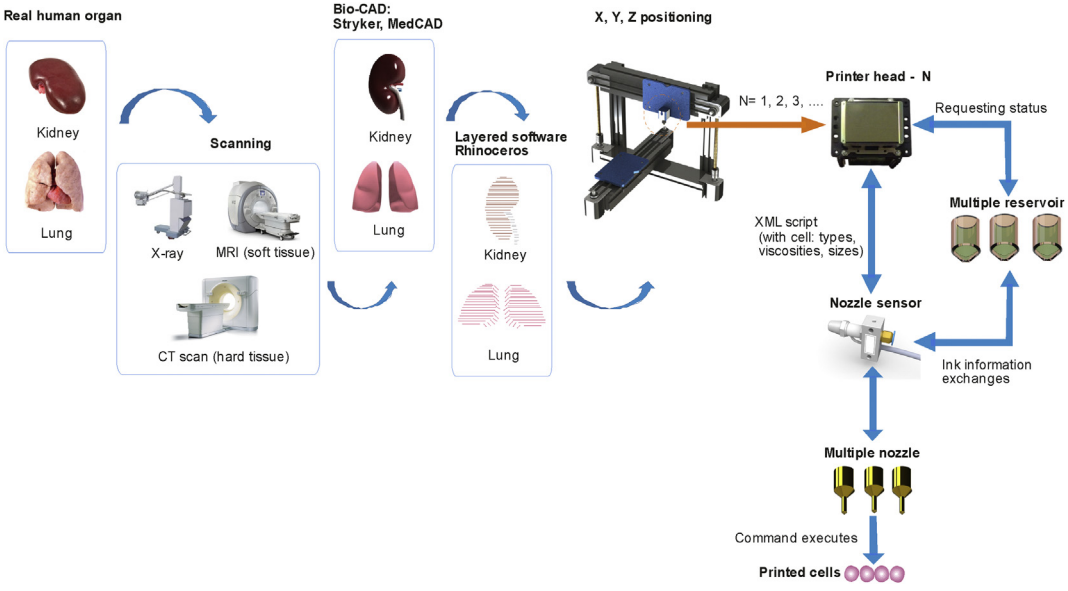

Fig. 4. Representative working steps of a human organ transferring into a printed model.

3.1. Simple construct of cells

The shape of the printed cell structures plays a significant role for its viability, legibility, and longevity. For example, dome shaped structures show better stress distribution over cubic structures [63]. The design should provide sufficient transportation of nutrition and oxygen within the tissue to keep the cell alive. Diffusion of nutrition, oxygen and protein has limited depth dependency of about a few hundred microns. To keep the cells and tissues alive, the printed structures should have ample vascular space. For this purpose, porosity between cells and cell layers is required to facilitate cell viability and proliferation.

It is also important to select the right scaffold to prevent the cell structures from collapsing and to support remodelling and repair. A scaffold is a three dimensional porous substrate, where cells are cultured to form living tissues. Generally, low-viscosity bio-inks are dispensed onto a more viscous bio-substrate to produce the scaffold. During the *in-vitro* experiments, desired cells are placed into the biomaterial scaffolds to provide structural and logistic templates for tissue formation. Later the whole construction is cultured in a bioreactor to promote continued cell growth prior to being implanted into the host body to further mature and integrate. However, as the constructed cells release their own ECM, the scaffold biomaterial should fully degrade to form tissue like structures that can subsequently integrate within the surrounding host tissue upon implantation [101,102].

Conventional scaffold manufacturing techniques are fiber bonding, solvent casting, particulate leaching, membrane lamination, and melt bonding [23]. To date, polycaprolactone (PCL) [103], modified PCL with calcium phosphate [104], glycerol with soy protein [105], PLC with alginate [83], collagen and gelatin [106] have been reported as potential candidates for scaffold materials. The major issues for forming a scaffold are balanced apoptosis, cell proliferation, cell attachment, cell density, cell differentiation and migration, as well as mechanical, biological and chemical transduction to guide the constructed cells [8,73]. Moreover, depending on the characteristics of the cells, the properties of the scaffolds should vary including scaffolds porosity, elasticity, stiffness, and anatomical shapes. For instance, a polycaprolactone (PCL) framework as a base has been reported for tissues printing. Pati et al. utilizes scaffold based PCL material to support Decellularized Adipose Tissue (DAT) encapsulated with human adipose tissue-derived mesenchymal stem cells (hASCs) as a bio-ink material to form adipose tissue construct [63]. The viability was evaluated in mice showing positive tissue infiltration, remodelling and formation in both top and middle layers between 1 and 14 days.

Shim et al. used PCL and two alginate solutions as a supporting framework to construct a 3D porous structures with chondrocytes and osteoblast cells utilizing a printer with six dispensing heads [83]. The cells were encapsulated in sodium alginate, diluted with DMEM and cross-linked by $CaCl_2$, NaCl solutions. The dispensed cells remained viable for at least seven days with a rate of 95.6 ± 1.8%. The PCL framework provides enhanced mechanical stability whereas the encapsulated alginate solution allows suitable environment for the cellular arrangements and prevent damage from the printing pressures.

Xu et al. prepared multiple cell types such as human amniotic fluid-derived stem cells (hAFSCs), canine smooth muscle cells (dSMCs), bovine aortic endothelial cells (bECS) separately mixed with calcium chloride ($CaCl_2$) cross linkers to print with a thermal inkjet printer [80]. The multiple cell types were delivered onto an alginate-collagen composite scaffold. The 3D pie shaped constructions survived and matured as functional tissues in mice over seven days with a cell viability of almost 90%. Schurman et al. utilized sodium alginate solution dispensed between polycaprolactone (PCL) strands crosslinked by $CaCl_2$ solution to create a viable hybrid construct [107]. Combination of alginate-PLC structures shows a better mechanical property then alginate alone, PCL alone structures. C20A4 cells (cultured in DMEM, supplemented with FBS, penicillin, and streptomycin) were embedded in sterilized alginate solution as a bio-ink material and deposited on the hybrid structures. The printed cell shows a high cell viability of almost 80% just after the printing.

Decellularized adipose tissue (DAT) and injectable DAT based micro carriers allow for the formation of adipo-inductive substrate for human adipose derived stem cells (ASCs). This adipo-inductive substrate can act as scaffolds for adipose generation [108]. Stable non-cross linked porous foam utilizing human DAT has been reported as scaffolds for tissue engineering mimicking biochemical and biomechanical properties of the native cell [109]. The paper suggested advantages of the DAT foam based scaffold over the DAT scaffold with higher angiogenic capacity, better cell migration and suitable degradation. Work has been conducted on direct cartilage repair using a 3D printed biomaterial scaffold. For instance, Cui et al. modified a thermal inkjet printer and utilized a combination of poly(ethylene glycol) dimethacrylate (PEGDMA) and human chondrocytes to repair osteochondral plugs for cartilage [61]. Significantly improved printing resolution was reported with cell viabilities of 89.2 ± 3.6% for simultaneous photo polymerization.

Hydrogels such as alginate, collagen, chitosan, fabrin and synthetic polymer such as pluronics, polyethylene glycol [86] has been used as a 3D scaffolds for cell culturing, monitoring cell–cell interaction, and cell control for both soft and hard tissue regenerations [110]. Their presence increases the cell seeding efficiency. Griffith et al. introduced two DNA-based hydrogels for forming a bio-degradable bio-ink, one consisting of polypeptide-DNA and another of double stranded DNA (dsDNA). The inks were extruded from a modified 3D printer [111]. Due to the biodegradability of the DNA bio-ink system, the rapid formation of a 3D constructs for temporary scaffolding in biomedical applications was achieved.

Lee et al. developed a 3D printing method to construct a larger fluidic vascular channel (lumen size of around 1 mm) allowing an adjacent capillary network through a natural maturation process [112]. Collagen hydrogel was used as a main scaffold material and gelatin as a sacrificial material to create the channels. Fibrinogen, thrombin, human umbilical vein endothelial cells (HUVECs), normal human lung fibroblasts (NHLFs) with a combination of growth factors and culture medium were mixed and deposited between the two vascular channels. HUVECs were seeded into the channel to create the cell lining. Flowing media through the channel shows robust interconnected vascular lumen up to few weeks. Hydrogel bio-paper (fibrin, matrigel, fibrinogen, polyethylene glycol tetra-acrylates) could also be used as a temporary supports for the deposited bio-ink material for large tissue and organ constructions [28]. Aria et al. uses a bio-paper with hydrogel consisting of $CaCl_2$, polyvinyl alcohol (PVA) and hyaluronan for supporting the alginate based bio-ink material [113]. Boland et al. utilizes a thermos-sensitive gel (N-isopropylacryamide-co-2-(N,N-dimethylamino)-ethyl acrylate) above 32 °C to serve as a bio-paper for 3D construction of cells [89]. This bio-paper could easily be removed after the fusion of the printed cell spheroids.

The stiffness of the framework is sometimes greater than the printed tissues and causes problems for future adjustment with the native cells [63]. The mechanical properties of a scaffold should also match with the native cells, and thus do not create any complications. Scaffold degradation, mechanical mismatch with native cells causing immunogenicity, toxicity, and host inflammatory response are the issues of using scaffold as printed tissue supports [73].

Moreover, the residual polymer from the scaffolds may disrupt the normal activities of the constructed cells.

Many research groups also focused on fabrication techniques for scaffold-free engineered tissues. In order to maintain a certain shape, integrity and composition, the printed cell construct must have a rapid tissue maturation process in the absence of solid scaffolds. Some of the common advantages of this approach is the absence of scaffold degradation, better intercellular communication due to similar host environment and more functional capability with host cells, high cell density, rapid tissue formation [114]. Scaffold-free vascular reconstruction *in-vitro* for smooth muscle cells and fibroblasts have been reported for layer-by-layer printing on agarose rods [73]. Tan et al. proposed and developed an alginate based fabrication process [76]. The group fabricated a ring shaped structures with micro droplets of alginate solution (tissue spheroids consists of ECs and SMCs encapsulated by the alginate) deposited onto an alginate hydrogel substrate. The analysis showed a sufficient amount of collagen-1 secretion from the construction promoting cell–cell adhesion, formation and maturations. Fig. 5 illustrates the different combination of scaffold-based and scaffold-free approaches for constructing 3D bio-structures. Both approaches need to maintain sufficient waiting time to stabilize each layer before constructing another new layer. Otherwise, the whole structures may deform or collapse.

The scaffold-free approach also faces a number of challenges. For instance, the fabrication process needs a large amount of spheroids that consume much time affecting the subsequent fusion process. Further problems are vascularization of thick tissue construct, and precise positioning of various multiple cell types [8]. The reports suggested thermoreversible, photosensitive moulding gels, stimuli sensitive polymers for scaffold free solutions that reduce the complexity to separate the gels, while a complicated vascular structure needs to be printed [73,115]. As both scaffold-based (indirect printing) and scaffold-free (direct printing) approaches have their own advantages and limitations, a hybrid method incorporating both approaches may solve the above challenges.

3.2. Tissue printing

One key construction process of cell structure is tissue fusion [116]. Tissue fusion is a process where multiple tissues merge together due to this surface tension forces and cell intergrowth. Tissue fusion relies on self-organizing properties of cells that in turn promote cell proliferation, cell–cell and cell-ECM interactions. Moreover, cell polarity is an important factor for the fusion process allowing mutual adhesiveness of different cells to merge together. Merging similar cell types is called homotypic cell fusion. Osteoclast – bone cells that maintain, repair and remodel bones – is an example of homotypic cell fusions [117]. Merging different cell types is called heterotypic cell fusion. Bone marrow derived dendritic cells (BMDCs) fused with neuron/glial cells of brain, or with myocyte cells of the heart are an example of this heterotypic cell fusion [118].

The printed cell structure may shrink or become shorter after a certain time due to the fusion phenomena. This shrinking of multiple cells could deform the whole printed structures. Sufficient scaffold supports (scaffold based approaches) around the fused cells or deposited hydrogel substrate (scaffold free approaches) can prevent the undesired deformation. The fusion process also helps to shape the structures while unwanted fusion stages are avoided. For example, Thompson et al. chopped embryonic avian heart tubes into myocardial rings, and then made them fuse and morph overnight onto a synchronized heart tube for supporting a tubular frameworks [119]. This process is due to the biological capacity allowing closely positioned soft tissue fragments to fuse over time [120]. Fig. 6 presents the formation of heterogeneous cell spheroids from individual cells. Cell spheroids can be used as a potential bio-ink material to construct multi-layer artery system. The printing process fuses and forms the final shapes. For a large volume of tissue and organ printing, a fast fusion process might be needed. Fast fusion can be achieved by reducing the distance between the cells (high resolution) through shaking in a way that the printed constructs do not deform [121,122].

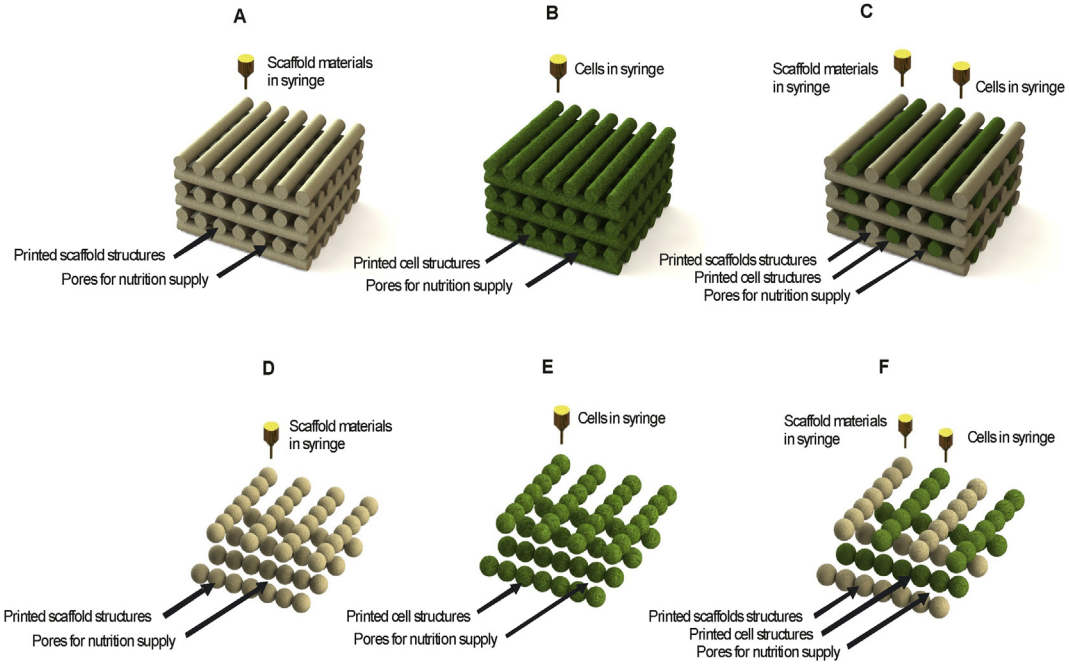

Fig. 5. Tissue constructions with pores (A) continuous deposition of scaffolds materials without cells; (B) with only cells; (C) Combination of cells and scaffold materials; and (D) drop on demand deposition of scaffolds materials without cells; (E) with only cells, (F) combination of cells and scaffold materials.

Vascular systems are one of the major tasks in bio-printing. The vascular system is a network of perfusable channel capable of delivering oxygen, nutrients and removing waste solutions to confirm the viability and functionality of the printed construct. A vascular system consists of a complex networks of blood vessels with various lengths and diameters. The diameter ranges from 20 μm to 2.5 cm from very fine capillaries to the aorta of the body [123]. However, the inner part of the whole vascular system is unique and is lined with a monolayer of flat endothelial cells (ECs). Without a vascular system, adequate perfusion of growth factors (such as proteins or hormones), oxygen and nutrition is not feasible leading to both normal and premature cell death [120]. Another essential prerequisite for constructing vascular cells are to have both defined inlet and outlet branches to pass on these growth factors, oxygen and nutrition.

Before printing a functional human organ such as lung or kidney, a blue print of the vascular system has to be designed. For printing the complex networks, developmental mechanism of the vascular system has to be understood in details. For example, vasculogenesis (forming new micro vessels from non-endothelial cells) [124,125], angiogenesis (forming new micro vessels from endothelial cells) [125], and arteriogenesis (remodelling small vessels into larger one) [126,127] are essential prerequisites to create the intra-organ hierarchical vascular branched of different diameters. Recent papers suggested that the printed vascular segment undergoes on a retraction stages which results almost a two folds reduction in its printed dimensions [128]. Therefore, the designed blue print should be as twice of its final sizes to achieve a viable vascular tree. However, the compaction and the retraction properties of tissues are different. A systematic research accounting the predicted construct after the printing is necessary.

Researchers considered all of these issues for vascular vessel printing. For example, Kucukgul et al. constructed an anatomically correct macro vascular aorta from a real human aorta model [129]. To avoid compaction and retraction of the model, a computer-aided algorithm was developed. The aorta was constructed utilizing primary mouse embryonic fibroblast cells supported by a thermos-responsive hydrogel named Novogel. The accuracy of the constructed cellular structures was around 91–95% with 97% for the support materials. Hockaday et al. fabricated an anatomically accurate, heterogeneous aortic valve of inner diameters ranging from 12 to 22 mm [130]. Porcine aortic valve interstitial cells (PAVIC) were seeded with the PEG-DA hydrogel to formulate the constructions. Alginate-gelatin solution was used to support the constructed geometry of the overhanging ostia and leaflets. The printed geometric accuracy (swelling affects) was quantified for each layers with the micro-CT scan and compared with the corresponding CAD STL files. The comparison indicates higher geometric precision of almost 93% that reduced somewhat as inner diameter of the valve decreases. The constructed valve swells outwards due to the surface tensions indicating for printing of tinner wall to match the target shapes. The printed aortic valve maintained near 100% of cell viably over 21 days.

Kelm et al. reported a scaffold-free concept to create blood vessels of small diameter utilizing the self-assembly of human artery-derived fibroblasts (HAFs) and human umbilical vein

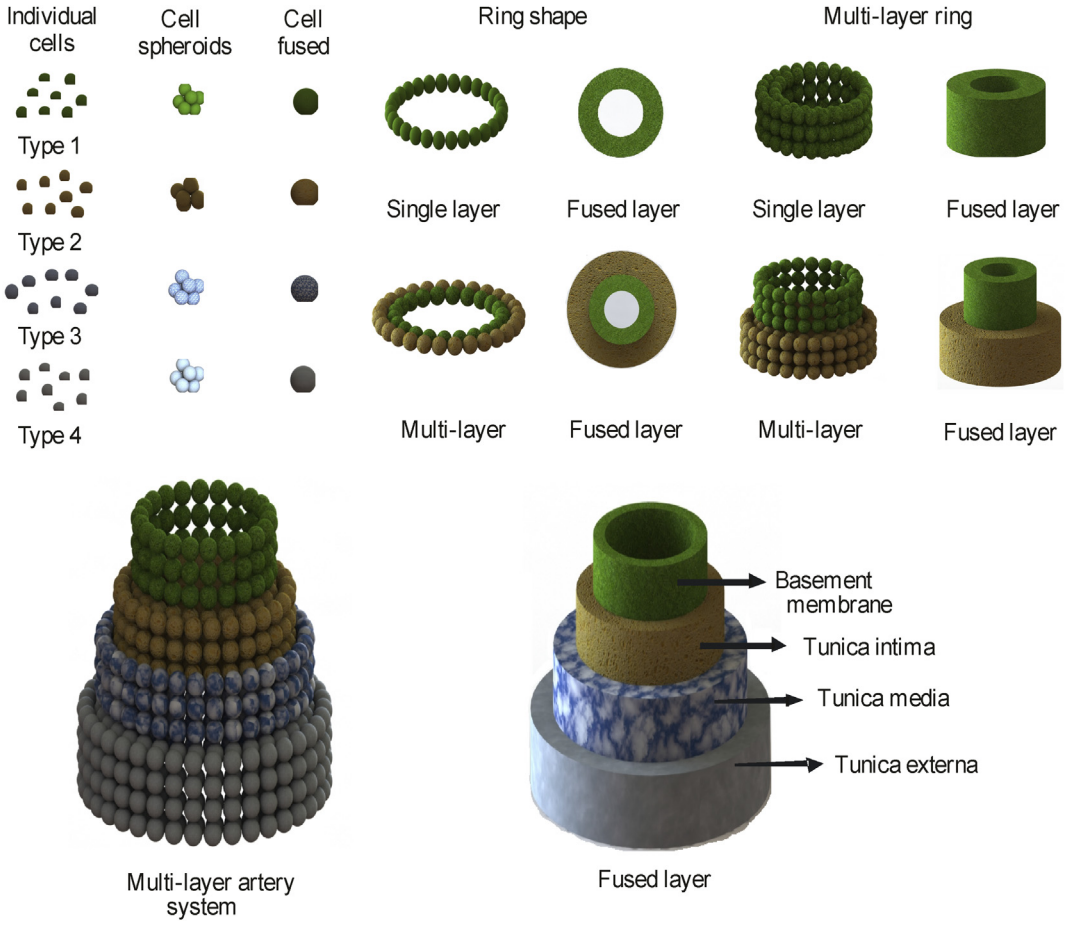

Fig. 6. Step by step construction of an artery wall for multi-cellular artery systems.

endothelial cells (HUVECs) under pulsatile flows. This approach required around 4000–5000 micro tissues to fabricate a vessel of 5-mm length, 3-mm diameter and 1-mm thickness of the wall [82]. Lee et al. developed a methodology to form a functional *in-vitro* vascular channel (up to 5 mm distance and 5 million cells/mL density) within thick collagen hydrogel scaffolds [131]. The biomaterial includes HUVECs of different densities in 5% CO_2 and endothelial cell growth medium-2 (EGM-2). Gelatin was used as a sacrificial material to create the fluidic channels. The process yields a high cell viability of almost 90% in only two weeks. Nishiyama et al. formed an alginate-based tubular structures into CaCl2 solution allowing an adjustable thickness from 30 to 40 μm and an inner diameter from 30 to 200 μm respectively by varying the micro gel solution [132]. Xu et al. used an inkjet bio-printer to construct a vertical vascular tube using hemi-branching points. They successfully formed a 5-mm long freestanding tube consisting of 210 layers of Ca-alginate droplets [133]. However, alginate is not a good selection for constructing a vascular network as it does not help cells to grow and bond. Moreover, biodegradability of this material has not yet been confirmed by *in-vivo* applications. Therefore, more research is needed for including alginate with other materials allowing native ECM like behaviours [134].

Norotte et al. reported a scaffold-free approach using agarose rods to construct a multi-layer vascular tube using human uterine smooth muscle cells (HUSMC) and human skin fibroblast (HSF) cells. These printed tubes are similar to vessels in microvasculature with diameters ranging from 0.9 to 2.5 mm. The fused constructions are sufficiently stable to handle and to transfer into a specifically designed bio-reactor for further maturation and for implantation [73]. Li et al. constructed a vertical hollow channel without scaffold support using various combinations of alginate/gelatin/chitosan/fibrinogen hydrogel as a printed materials [62].

A double-layer sturdy tube could also be made with HUVSMCs and human dermal fibroblast (HDFs) capable of transferring directly onto a bio-reactor for further maturation [135]. Miller et al. introduced rapid casting of a vascular channel based on carbohydrates with a mixture of glucose, sucrose, dextran forming self-supporting lattices. The diameter of the vascular channel ranges between 150 and 750 μm, coated and encapsulated by poly(lactid-co-glycolid) (PLGA) and living cells of fibrin/agarose/matrigel/poly(ethylene glycol) (PEG), respectively [136]. Engelhardt et al. conducted a free-form construction of a tubular system with diameters ranging from 10 μm to 100 μm with synthetic polymer-protein microstructures. Due to the hydrophobic nature of the material in an aqueous environment, the vascular network retains its shape and mechanical properties allowing a higher elasticity [137].

Sometimes, the printed cells cannot survive even with ample supply of proteins, oxygen and nutrition. A new technique needed to be developed to increase the life span of the printed cells. For example, Wu et al. utilized laser assisted printing technology to construct human umbilical vein endothelial cells (HUVECs) on a branch/stem structure. The printed structures fused and connected with each other within one day, but could not survive longer [138]. Introducing an extra layer of human umbilical vein smooth muscles cells (HUVSMCs) on HUVECs dramatically increases the longevity of the constructed blood vessels. It is likely that the HUVEC and HUVSMC have the symbiotic relationship allowing proliferative state and higher viabilities. The constructed branch remains intact after 9 days of deposition. Campos et al. fabricated high-aspect-ratio hollow tubes using a syringe-based deposition of agarose hydrogel encapsulating human mesenchymal stem cells (hMSCs) and human MG-63 cells [139]. The construction was submerged in a hydrophobic high-density fluid named perfluorotributylamine ($C_{12}F_{27}N$) promoting mechanical supports and higher cell proliferations. This fluid reduces the surface tension, increases the contact angel of each droplets (from 55° of air to almost 70° into the fluid). Fabrication of complex and large volume of vascular tree without a supporting scaffold is achievable with this approach. Moreover, the fluorocarbon allows sufficient oxygen and carbon dioxide diffusion that keeps the cell alive for a long printing time. The printed cells were viable up to 21 days from deposition. However, once printing is done, the fluid needs to be replaced with cell culture medium. This process could deform the complex printed structures. Moreover, printing speed and resolution need a major improvement.

3.3. Organ printing

One of the biggest issues of human organ transplantation is the limited number of donors compared to the number of patients. Sometimes, infection and rejection of the organ causes suffering and often death [140]. The ultimate aim of bio-printing technology is the rapid design and fabrication of operational human tissues and organs to replace those damaged, injured or lost. Moreover, the organ of a living body needs a network of vessels and capillaries to provide sufficient oxygen, cytokines, nutrients, as well as to remove the toxic waste from them.

For this purpose, all constructed cells need to be interconnected and placed close to the capillary network to receive enough oxygen and nutrients. For example, kidney vascular tree consists of 10–12 branches incorporating around 10,000 of segments. If the researchers can successfully design and fabricate functional, long and viable blood vessels, organ printing of for example lung or kidney will become close to the reality. As tissue engineering is still in its early stage, fabricating a whole operational organ needs to solve a number of current challenges such as printing speed, resolution, biocompatibility, cell viability, cytotoxicity, and gentle fusion.

Tissues utilize organizational capacity and chemical signals from cells to build a specific structure leading to the organ formation by copying the natural morphogenesis. For example, ECs will form tubular like structures on their own due to the genetically predestined form, if a suitable external environment is provided. Supplying sufficient media incorporating nutrients, oxygen, and proteins can create suitable environment. A bioreactor can provide this environment, the structural and functional maturation of the printed organ/tissues [141]. Iwsaki et al. developed a pulsatile bioreactor that regulates pressures, flow circulations, heart rates, concentration of carbon di-oxide and pH of an engineered *in-vitro* blood vessels. The group fabricated a three-layer robust and elastic artery system from polyglycolic acid (PGA) seeded with smooth muscle cells (SMCs), PCL seeded with SMCs, and PGA seeded with fibroblasts. The whole construct was wrapped around a silicon tubing [142]. After removing the supportive tube, the lumen was seeded with ECs and was mounted with the bioreactors. The result shows a similar appearance, strength and elasticity of a native artery. As the fabrication process was conventional, the results can be acknowledged to formulate a more complex 3D printed vascular systems and functional organs with similar pulsative bioreactors.

3D bio-printing has been utilized in urologic applications particularly for bladder replacements [143,144]. The process involves the collection of tissues from the bladder and cells proliferation outside the body. The fabricated bladder scaffold was then covered with the harvested cells that can be later implanted. Atala et al. successfully fabricated a whole human bladder of three distinctive layers with modified ink-jet bio-printing technologies [145,146]. The bladder scaffolds were fabricated from collagen or a composite of collagen and polyglycolic acid (PGA) [147,148]. The smooth muscle cells (SMCs) collected from individual patients were seeded on the exterior surface of the biodegradable bladder shaped

scaffolds. After settling of the exterior parts, inner surface were seeded by coating the urothelial cells. Finally the whole construction were wrapped with omental during the implantations for enhance vascularization of flaps and grafts. The printed bladder was transplanted on different patients with end-stage bladder disease requiring cystoplasty. The engineered bladder showed long term functionality with no major complications. Moreover, report suggested silk fibroin as a promising bio-material over collagen-PGA scaffolds that has been tested on mice bladder constructions [149]. However, before the clinical reality of the bladder reconstructions, a number of improved trials with more legitimate functional and durable steps are needed to be validated for these experimental approaches.

Fig. 7 shows the representative construction of a human lung system. A number of robotic arms each incorporating number of reservoir's with heterogeneous bio-ink are needed to print the whole functional lung systems. The multiple robotic arms build the desired trachea, bronchus, bronchi and bronchioles system with diverse tissue spheroids integral to the organ. The organ tissues can be created from vascularized organo-specific tissue spheroids. Post processing stages are essential to keep the organ fully functional before implanting in the host body. For printing a whole organ with multiple cell types, bio-plotting technology (printing the construct into a less viscous solution by utilizing buoyancy compensation principle) could perhaps be a good options to reduce the surface tension and the gravitational force of large printed structures [94,127]. The printed structure of this method has a smooth surface, which is not suitable for cell adhesions and cell–cell interactions. A surface treatment could solve these problems. Moreover, several challenges such as accuracy, resolution, limited range of scaffold materials, processing speeds, and cell encapsulation, all of which need to be addressed and improved in order to print a large structures.

High cell density obviously allows for quick tissue assembly and cell maturation, suitable for organ printing. For example, to fabricate a human kidney, over one million glomeruli and nephrons are needed which seems practically not feasible with current technologies [8]. Self-assembly and self-organisation, which are autonomous processes of cells to form from an initial state to a final pattern, could make this complex fabrication process more practical. An example of these two phenomena are the histogenesis and organogenesis of cells leading to a cell–cell, and cell-ECM interactions, which help to form the final shapes of the tissues [135]. Ultimately, appropriate and feasible approaches considering the self-assembly and self-organizing ability of human tissue spheroids could make organ printing possible [150].

Rezende et al. suggested the fabrication of a non-adhesive mould placed 1250 wells containing 5000 tissue spheroids of each in order to fabricate a human kidney. The process needs five robotic dispensing systems (each consists of 250 multi wells) to handle the whole printing process [74]. Although their suggestion remained an idea, it is not impossible to achieve this target with an advanced technology. Computer aided design (CAD) and a blue print of the particular organ are essential before initiating the printing attempts. Digitized image reconstruction, magnetic resonance imaging (MRI), computed tomography, and mathematical modelling using theoretical principle will enable the detailed 3D reconstruction of organs [120,151]. It is also essential to know in advance, the structural determinants of material properties at different stages of development for tissues and organs.

A more efficient approach is the separation of the complicated organ printing task into many simpler tasks that can be done independently at the same time. The result can later be eventually combined to produce a functioning organ ready for transplantation. For example, mapping a human organ, converting it to a suitable 3D design, slicing a 3D design into layer of 2D format for dispensing, modifying the coordinate system, synchronizing the dispenser with the software, could be done by engineering experts. Chemistry experts could provide ample nutrition to the printed construct using heterogeneous bio-ink. Medical experts can transplant the desired organ into an *in-vivo* subject.

For organ printing, researchers may consider technological challenges and solutions associated with the organ transplantation process. Organ transplantation can be done by decellularization and recellularization of cells. Decellularization is the process to isolate the extra cellular matrix (ECM) of a tissue from its cellular components. The decellularization process retains the structural, and functional characteristic of the original micro-vascular network and prepares the scaffold for tissue engineering. This scaffold product also maintains the protein, and growth factor of natural

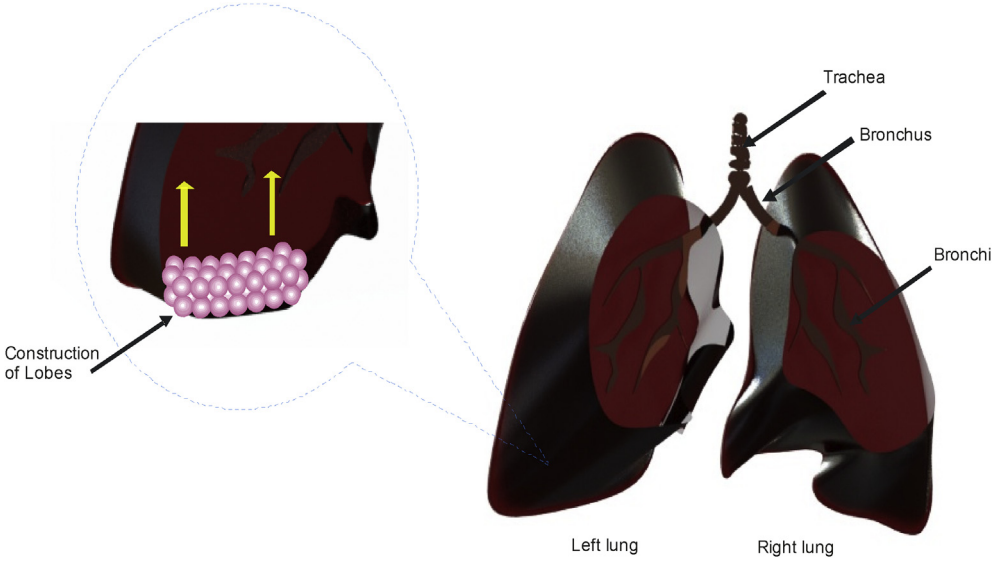

Fig. 7. Demonstration of organ printing from multi-layer complex bronchi system of a lung to whole printed lung. The printing process can be initiated from bottom-up setup incorporating multiple robotic dispensing systems with number of reservoir and nozzles. Sacrificial scaffolds are used to hold the structures if necessary.

tissues. Successful decullularization has been reported for various organs such as heart, liver, bladder, artery, skin and trachea [152–154]. Similar initiatives can be addressed for preparing functional bio-ink metrics allowing for printing the whole organ.

In contrast, recellularization allows the vascular network to be connected for the circulation, facilitation of rapid oxygen and nutrients with the host for cell viabilities, and cell functions [152]. During the recellularization process, functional organ can be reproduced by introducing progenitor or adult stem cells within the scaffolds. This knowledge may be useful after successful printing of a whole organ, maintaining its functional properties with bioreactors facilitating the transplantation. Furthermore, a detailed analytical approach of cell formations and cell interactions might allow to form a set of universal bio-ink material suitable for commercialization. For example, human induced pluripotent stem cells (hiPSCs), human embryonic stem cells (hESCs) have been reported to be present in varieties of tissues and organs. Combination of these cells with a suitable hydrogel solution could make a versatile bio-ink for constructing a number of engineered tissues and organs. These cells have excellent ability to self-renew for indefinite times, and pluripotency – the capability of forming any type of adult cells or tissues by mimicking the early stages of embryogenesis. Moreover, human pluripotent stem cell shows similar physiological reaction of a whole organ in a smaller scale. Utilizing this stem cell line for fabricating micro tissues and organs will promote more reliable drug testing platform and an end of animal testing.

Jones et al. used hESCs derived hepatocyte like cells with alginate hydrogel matrix as a bio-ink material to construct a circular structure of 40 layers inside of a multi well plates [96]. The constructed structures maintained their pluripotency and showed excellent viabilities, and proliferation for longer periods. Ouyang et al. used extrusion-based 3D printing to fabricate ESCs into a 3D cell-laden structures [155]. ESCs were mixed with a matrix material of gelatin/alginate hydrogel solution and printed into a layer-by-layer cubic porous structures. Cell viabilities were more than 90% promoting pluripotency and proliferation. The cell proliferation allows the ESCs to form highly uniformed and size controllable spheroids. However, it takes longer time (5 days) to reach the same size spheroids diameter of 60–70 μm. Producing human tissues from pluripotent stem cells requires a lengthy culture period of several weeks to months. Therefore it is very important to ensure the printed construct to be free from microbial contaminations. Maintaining a high-class microbiological safety cabinet and an improved sterility system is necessary.

4. Perspectives and conclusions

Bio-printing technology still is in its infant stage. In terms of both technology and biology, a number of challenges still have to be solved. For instance, engineering challenges are the development of a fast printing process, the improvement of nozzle and cartridge design, improving resolutions, avoiding clogging problem for large size organ printing, suitable stress and temperature condition without effecting cell viabilities. A major challenge is to write a control script for computers to identify individual cells by their visual characteristics and to print them accordingly. Moreover, the scripts need to be modified for printing different types of organ for an individual patients. In terms of biology, bio-compatibility, cell viability, cytotoxicity, fusion without deformation, leak-free perfusion, high cell density, printed cell transformation to the host are major issues to be solved.

Various research groups have reported 3D printing of cell constructs. However, fabricating a full operational and long-life cell structure will be a greater challenge. A successful attempt will lead to the construction of the whole human organ. For these purposes, current medical knowledge associated with organ transplantation needs to be integrated with future 3D organ-printing platform. Engineers, biologists, chemists, computer scientists, mathematicians and physicians need to work together to solve the challenges of the bio-printing. Biologists need to address what is needed to be understood and to be developed. Based on the feedback of biologists, engineers design the printing platforms considering mathematical parameters and physical laws. Computer scientists develop corresponding software to synchronize the machines with the specific needs of users. A cell data bank needs to be established that will include cell properties and behaviours so that the biologists can use, further modify and improve them if needed. This cell database will facilitate the future commercialisation of 3D organ printing. Moreover, commercialisation of bio-printing requires large-scale bio-fabrication tools. Barnett et al. recently conducted an experiment with large-scale 3D printing where the robotic tools with six degrees of freedom allowing a large range of motion. Even though their printing material was non biological as they utilized polymeric foam to construct a large-scale statue, the knowledge and challenges from this can be taken in consideration for the future large-scale bio-printing [18].

It will be possible for surgeons to facsimile patient's specific body parts according to the needs for repair, replace or removed. Considering the state of the art of bio-printing, it might take two or three decades or perhaps more to fabricate a marketable printed human organs with high order of functionality. Nonetheless within the next decade, direct visualization and quantification of diverse medical and biological processes can be expected. For example, artificial skin printing (*in-vitro*) as a testing beds for cosmetic industries; engineered tissues and mini organ printing for toxicity/efficiency screening of pharmacological drugs; *in-vitro* tumour, cancer, trauma, and infected tissue modelling that might enables examination of identical operational conditions in human body. Customized 3D printing for dental industries, urological applications, bone vascular co-culture for orthopaedic applications, stem cell based neurological applications, personalized medicine will become increasingly a common practice.

The 3D printing of a specific tissue such as tumour for drug testing will improve the efficiency of the drug. Preclinical testing including *in-vitro* analysis to determine toxicity, absorption, distribution and metabolism on the cells and tissues will enhance the reliability of the drug. Three-dimensional bio-printing technology will hopefully one day solve the organ transplantation crisis and revolutionize health sectors including drug screening, tissue engineering, and biological testing with minimum clinical trials.

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