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The Integrations of Biomaterials and Rapid Prototyping Techniques for Intelligent Manufacturing of Complex Organs

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Additional information is available at the end of the chapter

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1. Introduction

In the human body, an organ is a composite of different tissues in an ordered structural unit to serve a common function [1]. Ordinarily, cells self-assemble into tissues before forming an organ. There are at least three different tissues in a complex organ, such as the liver, heart, and kidney. Currently, complex organ failures are the first cause of mortality in developed countries despite advances in pharmacological, interventional, and surgical therapies [2]. Orthotopic organ transplantation is severely limited by the problems of donor shortage and immune rejections [3]. Extracorporeal support systems perform some specific functions within a limited time period [4]. Cell encapsulation techniques face the problems of capsule loss, low stability, and poor efficiency [5]. Cell sheet technique cannot rescue tissues with increased thicknesses above 80 μm [6]. Decellularized matrices are hard to be repopulated by multiple cell types [7]. On the other hand, stem cell research has emerged as one of the most high-profile and promising areas of 21st century science [8-10]. Typically, autologous adipose-derived stem cells (ADSCs) represent one of the most abundant, easily cultured, rapidly expanded, and multipotent cell source [11]. It has been a long-term goal in this field to manufacture complex organs from biocompatible materials (including non-immune patient derived cells) and computer-aided design (CAD) models in a fast, easy, cheap and automatic manner.

To manufacture a complex organ, cells act like building blocks and have special functions. A comprehensive multidisciplinary effort from biology, implantable biomaterials, and rapid

prototyping (RP) technology is extraordinarily needed. A biomaterial is defined as any matter, surface, or construct that interacts with biological systems [12]. It may be an autograft, allograft or xenograft transplant material, or a nature derived or laboratory synthesized chemical component. Biomaterials are often used and/or adapted for a medical application, and thus comprise whole or part of a living structure or biomedical device which performs, augments, or replaces a natural function [13]. RP, also referred to as additive manufacturing (AM) or solid freeform fabrication (SFF), is a set of manufacturing processes which can deposit materials layer-by-layer until a CAD model with freeform geometry has been built. RP technology, which has been widely used in the automatic fabrication of complex geometric structure areas, carries the promise to become the most convenient and reliable technique for manufacturing of complex organs in the coming years [14-18].

Over the last two decades, tissue-engineering researchers have devoted themselves to seeding cells onto a porous biodegradable scaffold material to direct cell differentiation and functional assembly into three-dimensional (3D) tissues [19]. This strategy has achieved a great success in simple tissue/organ regeneration [20]. However, it is extremely difficult for this strategy to be used in creating a branched vascular system or a complex organ regenerative template mimicking the native ones with similar mechanical and biological properties. Similar to building a nuclear power plant for complex organ manufacturing, there is a significant gap between simple tissue/organ engineering and complex organ manufacturing approaches both in fabrication technique employed and ultimate goal achieved (Table 1) [14-18].

Complex organ manufacturing	Nuclear power plant building
Cells	Bricks, nuclear reactors
Synthetic, natural polymers	Steel
Crosslinking agents	Cements
Vascular systems	Water and light pipes
Nerve system	Electric control system
Multi-nozzle rapid prototyping machines	Cranes
CAD models	Blueprints
Construction	Architecture

Table 1. Analogues between complex organ manufacturing and nuclear power plant building.

The ultimate goal of complex organ manufacturing is to fabricate hybrid biomaterial (including living cells, even gene/protein) structures over a range of size scales (i.e. from a few micrometers to a few millimeters). We herein provide insights into some special integrations of biomaterials and RP techniques towards the purpose of intelligent freeform manufacturing of complex organs. The most successful and promising integrations have been highlighted; meanwhile the future development directions have been highlighted.

2. Biomaterials and RP techniques in thousands of postures

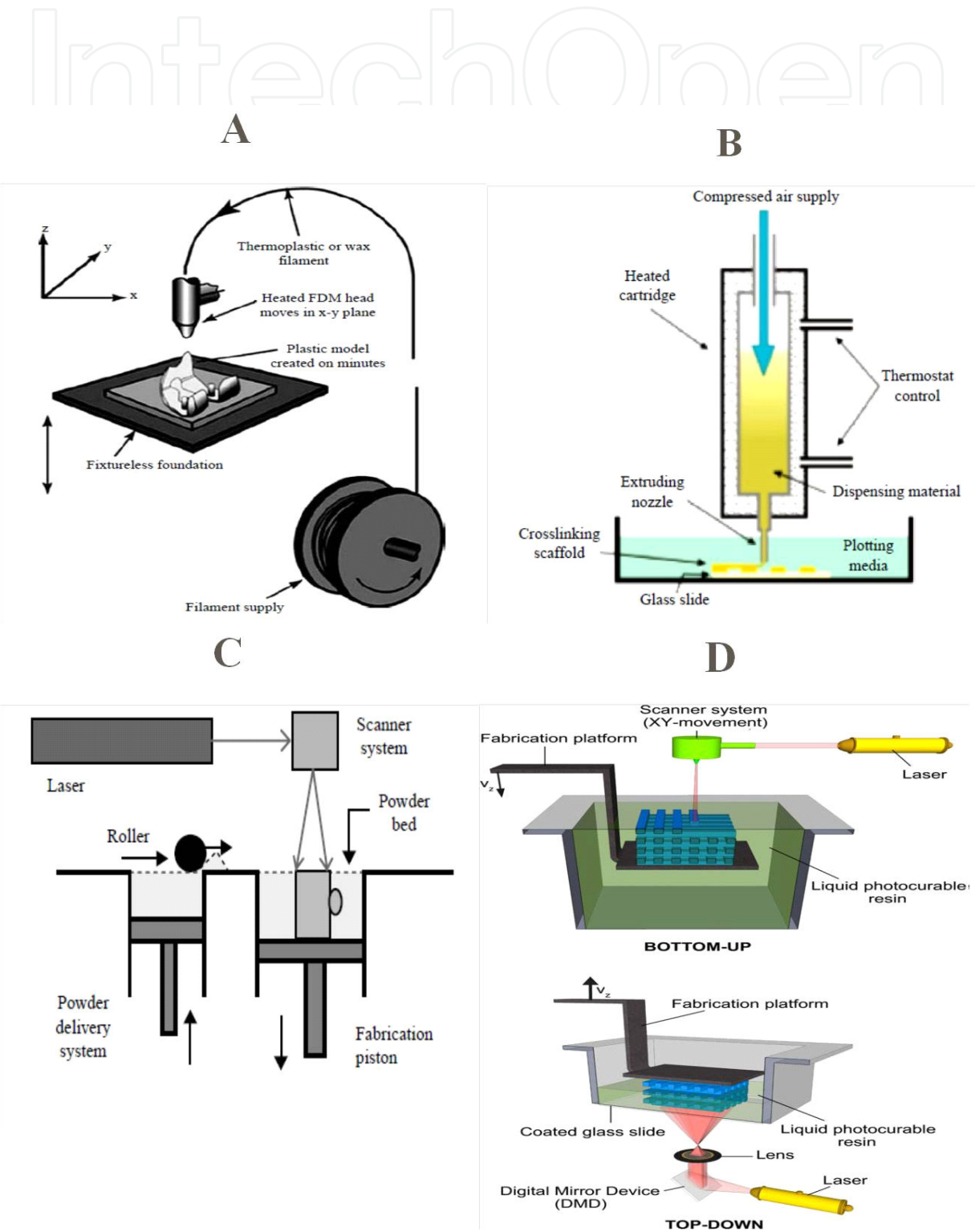
As stated above, biomaterials, usually acting as synthetic frameworks (referred as scaffolds, matrices, or constructs), can be categorized into different groups according to their supply sources, existence states, chemical properties as well as biomedical applications. Typically, patient specific blood, cells (especially stem cells), acellular matrices, tissues and organs are a kind of biomaterials with no immune reactions. More than 100 implantable biomaterials have been reported in different forms, such as bulks, blocks, membranes, sheets, beads, hydrogels, fibers, sutures, plates, nets, meshes, tubes, non-woven fabrics, porous scaffolds (or sponges), heart valves, intraocular lenses, dental implants, pacemakers, biosensors, etc [21-23]. However, very few of them are suitable for complex organ manufacturing purposes. For biomedical applications, biocompatibility, biodegradability and processing ability are among the most crucial issues one should consider. In most cases the implantable biomaterial has to be nontoxic, biocompatible, and biodegradable. Therefore, stringent criteria must be met before proceeding to clinical applications.

Especially, hydrogels are a family of natural or synthetic polymers with high water contents. During the last twenty years, hydrogels have been an important class of soft tissue repair materials or cell delivering vehicles that can be fabricated in the form of 3D micro-periodic structures by colloidal templating [24], interference lithography [25], direct-writing [26], ink-jet printing [27], and two-photon polymerization (2PP) [28].

In the last four decades, significant advances have been made in the progress of scaffold fabrication techniques for biomedical applications. For example, synthetic and natural biodegradable polymers, such as polylactic acid (PLA) [29], poly(lactic/glycolic) acid (PLGA) [30], collagen [31], hyaluronic acid [32] and chitosan [33], are often used as pure implantable biomaterials or tissue engineering scaffolds.

In parallel with the development of biomaterials, the number of commercial RP techniques has expanded rapidly during the last decade. More than 30 different RP techniques have been applied in the most diverse industries. Several companies are now using RP technologies for plastic, wood and metal product manufacturing. For example, Siemens, Phonak Windex, and other hearing aid manufactures use selective laser sintering (SLS) techniques to produce hearing aid shells. Align technology uses SLS techniques to fabricate molds for producing clear braces ("aligners"). And Boeing and its suppliers use SLS techniques to produce ducts and similar parts for F-18 fighter jets [34]. Around 20 of the RP techniques have been adapted in the field of regenerative medicine [35]. Basically, these adaptations can be classified into three major groups hinged on the RP working principles (Figure 1): (i) nozzle-based extruding/assembling/deposition systems, e.g. fused deposition modeling (FDM) (Figure 1A) [36], pressure assisted manufacturing (PAM), low-temperature deposition manufacturing (LDM), and bio-plotters (3DB) (Figure 1B) [37,38], which deposit materials either thermally or chemically through pens/syringes/nozzles; (ii) laser/photolithography-based writing systems, e.g. laser-guided direct writing, which arrange materials/cells by laser beams [39,40] or photopolymerize a liquid (resin, powder, or wax) in stereolithography (SLA or STL) (Figure 1C) [36,41], or sinter powdered material in SLS systems (Figure 1D)

[42]; (iii) printing-based inkjetting systems, e.g. 3D printing (3DP) systems and wax-based systems, which print a chemical binder onto a powder bed and print two types of wax materials in sequence (Figure 1 E) [36].



E

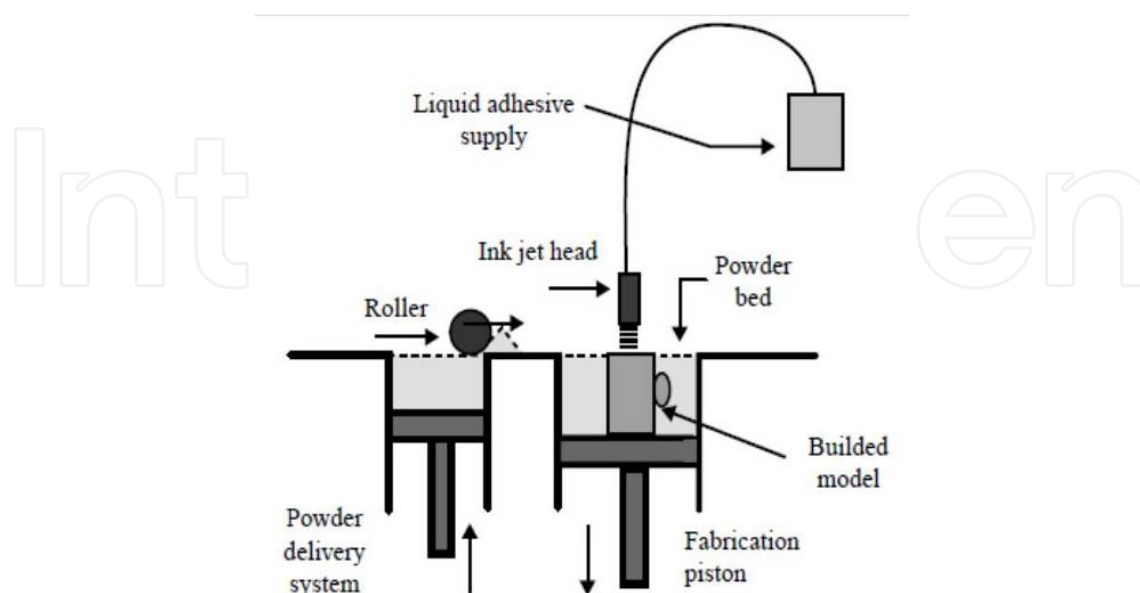


Figure 1. Working principles of various rapid prototyping systems: A) Schematic illustration of the nozzle-based FDM process [36]. B) Scheme of a nozzle-based 3D-Bioplotter heated cartridge setup [37,38]. C) Schematic of the laser-based SLS techniques [36]. D) Schemes of two laser-based of stereolithography (SLA or STL) setups [39]. Upper: a bottom-up system whereby the laser scans the surface for the curing of the photosensitive materials. Bottom: a top-down setup with dynamic digital light projection to cure a complete 2D layer at once. E) Schematic of the 3DP systems [36].

Although most of the adapted techniques can be used in building complex geometrical shapes with CAD modelling, every technique group is subjected to a limited biomaterial incorporation ability and has its own drawbacks in creating 3D living organs. For example, Chu and coworkers have developed design-for-manufacturing rules for their lattice mesostructure fabrication technique with a STL system. Lattice structures tend to have geometry variations in three dimensions [43]. However, this system is not fully capable of creating a branched vascular system, which is vitally important in the context of organ manufacturing to direct spatially heterogeneous tissue development. On the other hand, Arcaute and coworkers have encapsulated human dermal fibroblasts in a synthetic poly(ethylene glycol)-dimethacrylate hydrogel by a SLA technique. Without porous structures and biodegradable properties of the synthetic polymers, it is hard for the cells to form tissues inside the hydrogel [44]. The integrations of biomaterials and RP techniques can form a huge “family tree” with many different combinations. Figure 2 summarizes the integrations of biomaterials with RP techniques and their potential usages in complex organ manufacturing.

Currently, as the concepts of “factory in a box” and “desktop manufacturing” are expanding, new applications of RP techniques in architectural design and 3D construct building increase speedily. Among the most popular RP techniques, the Fab@Home equipments with an average price of about 3000 US dollars are among the most convenient and cost effective RP instruments used in biomaterial fabrication field [45].

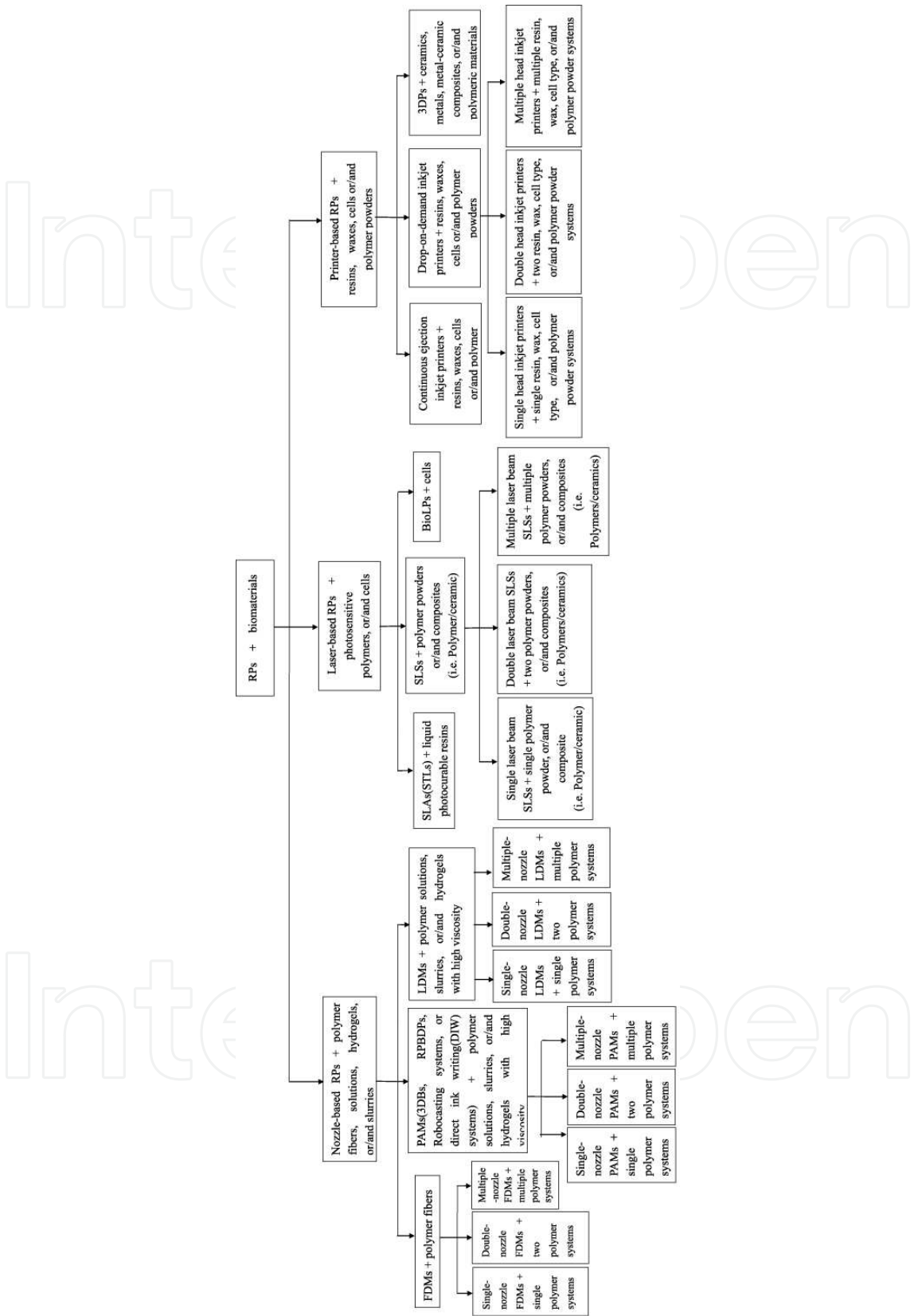


Figure 2. A “family tree” indicates various integrations of biomaterials and rapid prototyping techniques.

At present, the concepts of “scaffolds”, “tissues”, and “organs” are rather confused both in scientific and industrial areas. Most researchers and manufacturers in the area of tissue engineering like to label their RP products as “scaffolds”, “tissues”, or “organs”. It is reasonable to describe an acellular porous 3D structure with a micro-scale internal architecture but without cells as a “scaffold”. However, those with living cells incorporated should be defined as “constructs”. Especially, those with cells have already connected to each other to perform special functions should be called “tissues”. As described in the beginning of the introduction section, those with more than three different tissue types inside a construct should be called “organs”. Simple organs, such as the bladder and blood vessels, should have less than or equal to three tissue types, while complex organs, such as the liver, heart, and kidney, should possess more than three tissue types. With these definitions, it is easier to distinguish which RP technique will be useful in complex organ manufacturing.

3. The integrations of biomaterials with RP techniques for complex organ manufacturing

The ability to put material only into a specific location where it is desired could have a profound impact on how parts are designed and manufactured [46]. Similarly, the ability to put different biomaterials (including different cell types) to exact sites where they are desired could have a profound impact on how complex organs are designed and manufactured. For example, in a complex organ, such as the liver, at least three different cell types (hepatocytes, stellate cells, and Kupffer cells) are required that function in a construct along with the three common cell types of a vascular system. The fundamental unit of the liver, the acinus, has a typical radius of 500 μm . Within this structure at least six cell types interact with one another to coordinate the diversity of liver functions [46]. The spatially heterogeneous arrangements of multi-tissues make all the traditional, or existing techniques incapable of completing this ambitious task.

Over the last ten years, the integration of biomaterials with RP techniques in creating special 3D constructs for various biomedical applications has emerged. The ability to use data from clinical imaging techniques like magnetic resonance imaging (MRI), computerized tomography (CT) or patient-specific data makes RP techniques particularly useful for biomedical applications. Several research groups have adapted different RP techniques to assemble (or print) cell-laden constructs directly from computer-programmed design models with high resolution (Table 2) [47-64]. Six unique intelligent RP devices as well as their primary products are shown in Figure 3[47-52, 65-67]. These processes have demonstrated some possibilities in the area of complex organ manufacturing. The pros and cons of these techniques in complex organ manufacturing are outlined in Table 2. Those with only porous 3D scaffolds are not included here because these integrations have been reported extensively in the former reviews [36,37,44,68-79].

Technique	Pros	Cons	Refs
3D inkjet bioprinting (3DP) in and Pittsburgh Clemson University, USA	Several thermosensitive hydrogels can be used as biopaper; low viscosity cell suspensions or aggregates can be used as bioink; Cell viability greater than 85%.	Complex 3D constructs are difficult to realize; limited feature height (< 5 μm); lack of structural support for cell layer or cell aggregates; tissue formation into lines depends on cell or cell aggregate fusion or assembling; poor mechanical properties.	[47, 48]
3D direct-write bioprinting in University of Cornell and Arizona, USA.	Low and high viscosity hydrogels, including type 1 collagen and alginate can be used; high cell viability (up to 98%); flexible geometric shapes.	Cell viability depends largely on the inner diameter of the gauge tip, collagen concentration and extraction environments; difficult to control the collage gel state.	[49, 50]
3D fiber deposition (3DF) in University Medical Center Utrecht, The Netherlands.	High viscosity hydrogels, such as Pluronic F127, Matrigel, alginate and agarose, can be used; multiple cell types can be incorporated; homogeneous and heterogeneous structures can be created.	Limited materials can be used; limited height of 3D construct (< 10 μm); difficult cell-cell interactions; poor mechanical properties.	[51, 52]
3D single/double syringe cell assembling (or pressure assisted manufacturing (PAM)) in Tsinghua University, China.	Gelatin-based hydrogels can be used; a wide range of biological components can be incorporated; variable and hybrid geometric shapes; high cell viability (more than 98%); easy for long-term storage and transportation.	Limited materials can be used; weaker mechanical properties.	[53 - 60]
Double-nozzle low-temperature deposition manufacturing (DLDM) in Tsinghua University, China.	A wide range of biomaterials including both synthetic and natural polymers can be used; a wide range of biological components can be incorporated; arbitrarily hybrid geometric shapes; high mechanical properties; easy for long-term storage and transportation.	Material viscosity and temperature dependent.	[61 - 64]

Table 2. Comparison of different cell-laden rapid prototyping techniques in complex organ manufacturing

Due to the heterogeneous properties of complex organs both in geometrical structures and material components, emphases should be given to those RP techniques with further development possibilities in the further integrations of biomaterials and equipments. In the following part of this section, some special two or multiple syringe/nozzle techniques are highlighted. In Harvard Medical School, Lee and coworkers have printed a collagen hydrogel precursor, fibroblasts and keratinocytes into a quasi 3D structure for skin repair using a

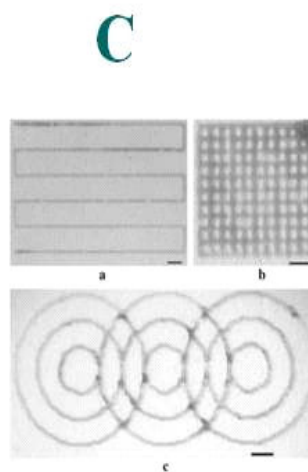
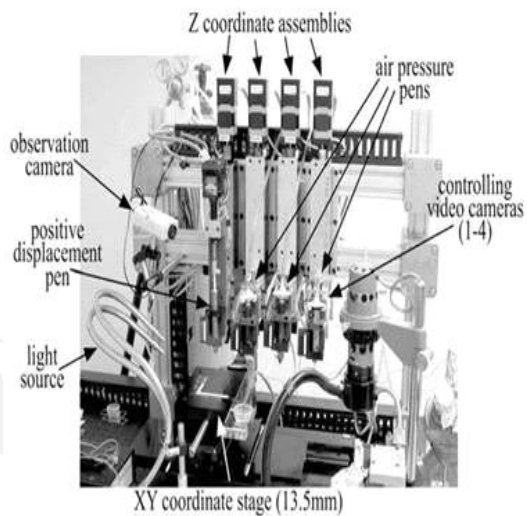
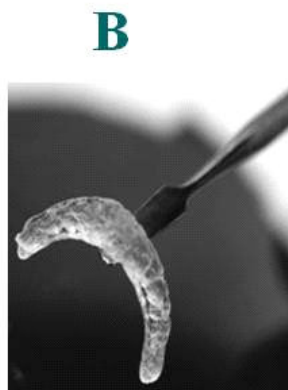
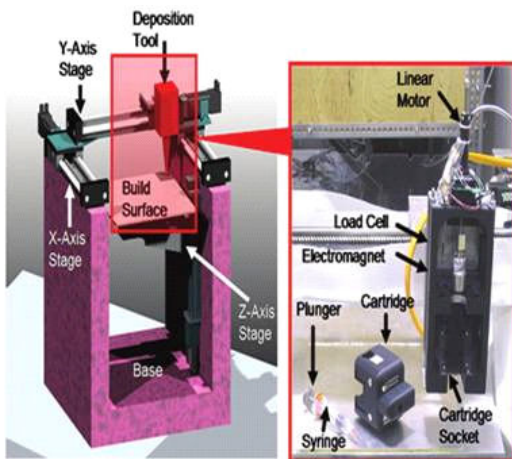
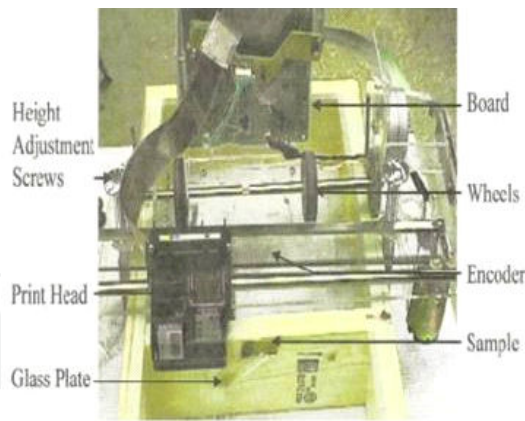
robotic platform (Figure 3D) [65]. The procedure involves printing a layer of liquid collagen to act as a hydrogel precursor. The liquid collagen is crosslinked with a nebulized aqueous crosslinking agent (sodium bicarbonate) to form a hydrogel that provides structural integrity for the subsequent cell suspensions. In fact, this technique is an extension of the above mentioned 3DP or 3DB robotic system with additional syringes as “cartridges” to load two cell suspensions and hydrogel precursors. Highly viable proliferation of each cell layer (85% for keratinocytes and 95% for fibroblasts) was observed on both planar and non-planar surfaces. For thin tissue/organ (such as skin/bladder) manufacturing, this technique is a right choice. However, for complex organ manufacturing, some intrinsic shortcomings, such as limited printing height, and difficult to control the collagen gelation process, made this technique almost incapable.

In University Medical Center Utrecht, The Netherlands, Prof. J Alblas’s group, a special bio-scaffolder pneumatic dispensing system (SYS + ENG) was adapted for printing cell-laden bone tissue repair hydrogels. High viscosity alginate (10% w/v) and BD Matrigel™ (10% w/v) hydrogels were employed. A limited ten-layer rectangular 3D construct of 10 ×10 mm with spacing between fibers of 0.8-2.5 mm and a thickness of 100 μm was fabricated and subsequently crosslinked in a CaCl₂ solution [51]. In spite of the limited height, the interconnected channels are still necessary for oxygen and nutrient delivery, as well as for tissue formation and vascular ingrowth. There are two critical drawbacks of this technique in complex organ manufacturing. The first is the poor mechanical properties of the cell-laden alginate or matrigel hydrogel for use as vascular systems. The second is that low viscosity hydrogels (including alginate and matrigel) are hard to be assembled into 3D constructs.

In university of Missouri, Norotte and coworkers used agarose rods as a molding template to print multicellular spheroids with their special bioprinter to form a tubular cell-laden structure (Figure 3E) [66]. After the fabrication stage, they manually pulled the agarose rods out of the tube, and concluded that it is a time consuming and labor-/spheroid- intensive procedure.

4. Some outstanding achievements made in Tsinghua University

In parallel with the above mentioned RP approaches, a series of RP technologies have been explored extensively by Professor XH Wang’s group at the Center of Organ Manufacturing, Department of Mechanical Engineering, Tsinghua University, China. State-of-the-art of the layer-by-layer modeling, material incorporation, and manufacturing principles of these techniques can be found in some of the pertaining references. The advantages and disadvantages of these approaches to be used in complex organ manufacturing have also been listed in Table 2. Previous studies have demonstrated their abilities to engineer complex 3D tissues using various single/double nozzle/syringe RP systems. In the following section some technical specifications are highlighted.



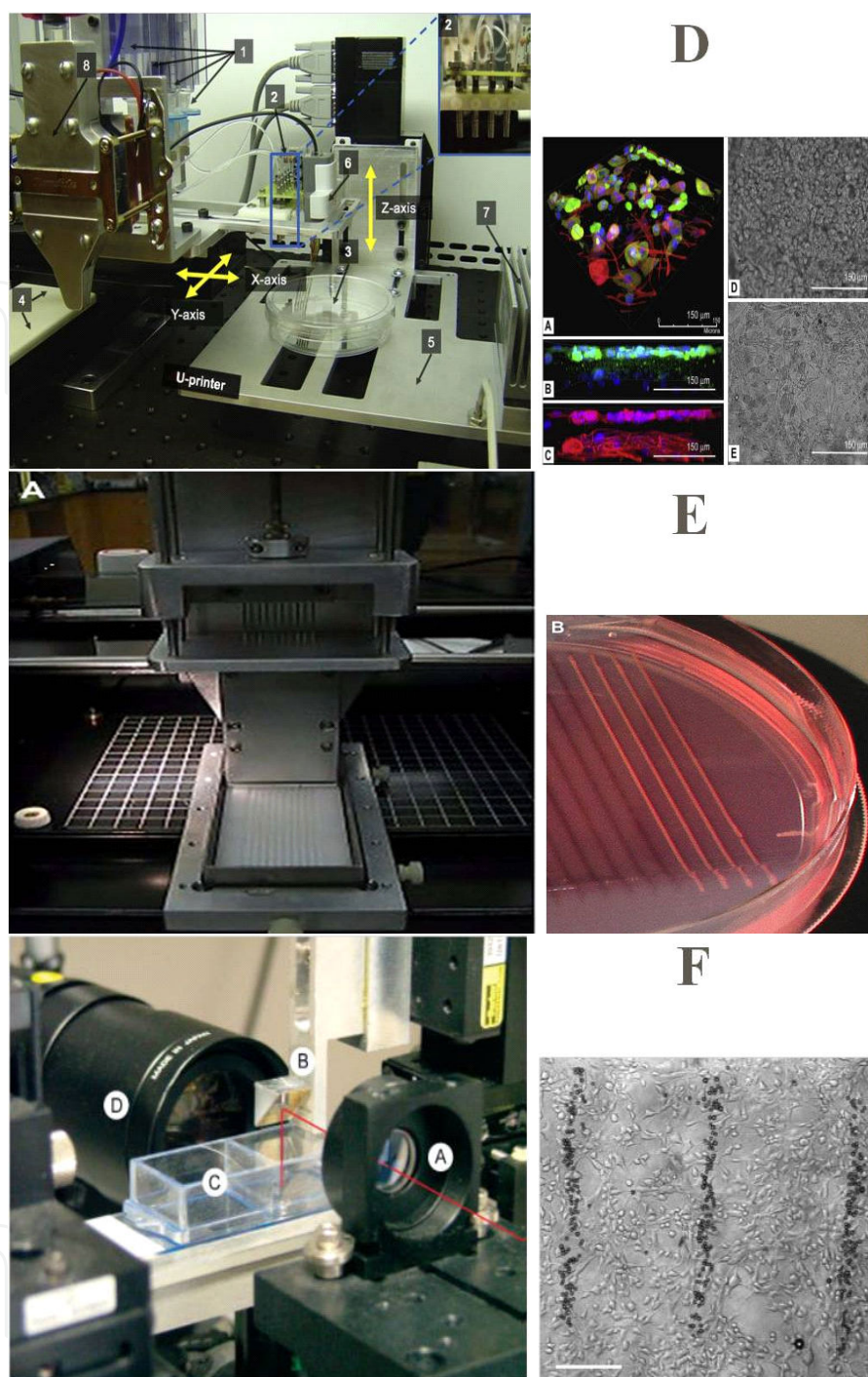


Figure 3. Several unique intelligent rapid prototyping devices and their functional cell-laden products: A) The inkjet cell printer and its bagel-like quasi-3D structure developed in Clemson University, prof. T Boland's group [47]. B) The robotic printing platform and its crescent construct made in Cornell University, prof. LJ Bonassar's group [49]. C) The direct-write system and its preliminary 3D figures developed in University of Arizona, prof. SK Williams' group [50]. D) A modular tissue printing platform with 4 'cartridges' to load cell suspensions and hydrogel precursors developed in Brigham and Women's Hospital, Harvard Medical School, Prof. S.-S. Yoo's group [65]. E) A bioprinting tubular structure with cellular cylinders developed in University of Missouri, Columbia, USA, Prof. G Forgacs' group [66]. F) A laser-guided direct writing (LGDW) system and its patterned factor-linked beads on a stem cell monolayer with micrometer accuracy (Bar = 200 μ m) developed in University of Minnesota, prof. D.J. Odde's group [67].

4.1. The single syringe cell assembling technique

Figure 4 shows some of the cell assembling results using our first generation cell assembling system. A gelatin based hydrogel system, such as gelatin, gelatin/chitosan, gelatin/hyaluronan, gelatin/alginate, gelatin/fibrinogen or gelatin/alginate/fibrinogen, was integrated with a single syringe cell-assembling machine to obtain the necessary space and stabilizing factors for cell survival and tissue formation [53-58]. A single cell type was deposited at an ambient temperature (1~10°C) layer by layer in a chamber as the sol state material was transferred into a hydrogel. Grid hepatic tissues, endodermis, and adipose tissues have been regenerated by using this single syringe cell-assembly machine at about 8°C. The gelatin based hydrogel network provided stabilization support for the 3D constructs during the fabrication and post culture stages. This mild deposition temperature is favorable for biological property preservation as increased Joule heating can result in loss of cell viabilities and bioactivities. During the culture period, the gelatin based hydrogel served as both a mass transportation template for tissue development and an extracellular matrix accommodation mimicking the microenvironment in native organs. The use of the natural gelatin based hydrogels was clearly highlighted the distinct advantage of this cell assembly technique for fabricating living tissue analogs. A shortage of the single nozzle/syringe systems was that, these systems lack the ability to easily create parts with spatial heterogeneous materials. Consequently, two double nozzle/syringe RP systems have been explored to deposit different materials at different temperatures.

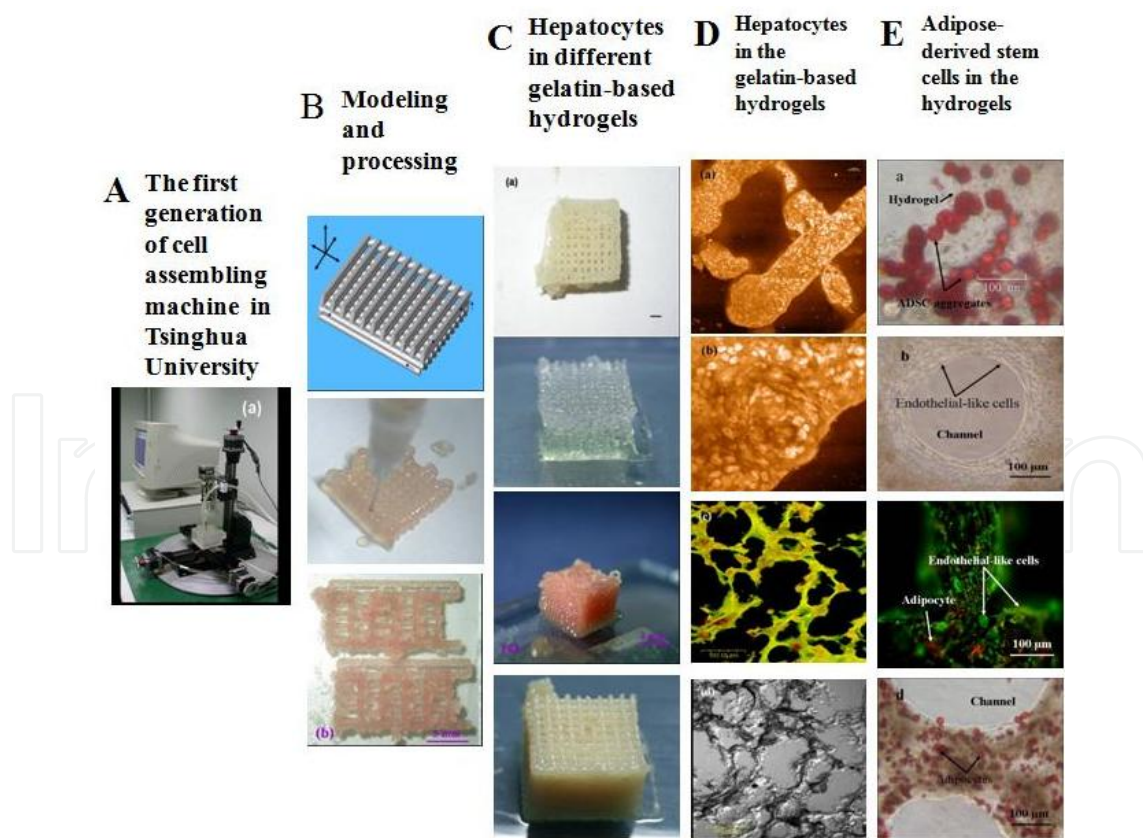


Figure 4. Hepatocyte and adipose-derived stem cell (ADSC) assembling based on the first generation of cell assembling technique developed in Tsinghua University, prof. XH Wang' group [53-58]

4.2. The double syringe cell assembling technique

Different from the above single syringe cell assembling technique, a double syringe cell assembling technique was developed in Tsinghua University with a updated software and hardware. Gradient and cylindrical architectures consist of two different cell-laden hydrogels have been fabricated at a temperature range of 8 – 10°C [59,60]. Two cell lines encapsulated in the similar gelatin-based hydrogels were put into different regions or compartment in a construct (Figure 5). The embedded branched networks enable culture medium to flow through the entire construct with unparalleled geometric complexity. However, there is a fatal shortcoming of this system to be used in complex organ manufacturing. The mechanical weak properties of the gelatin-based hydrogel made it impossible to connect the branched construct to an *in vivo* vascular system to endure anti-suture anastomosis and blood pressure even after a long-term *in vitro* culture period.

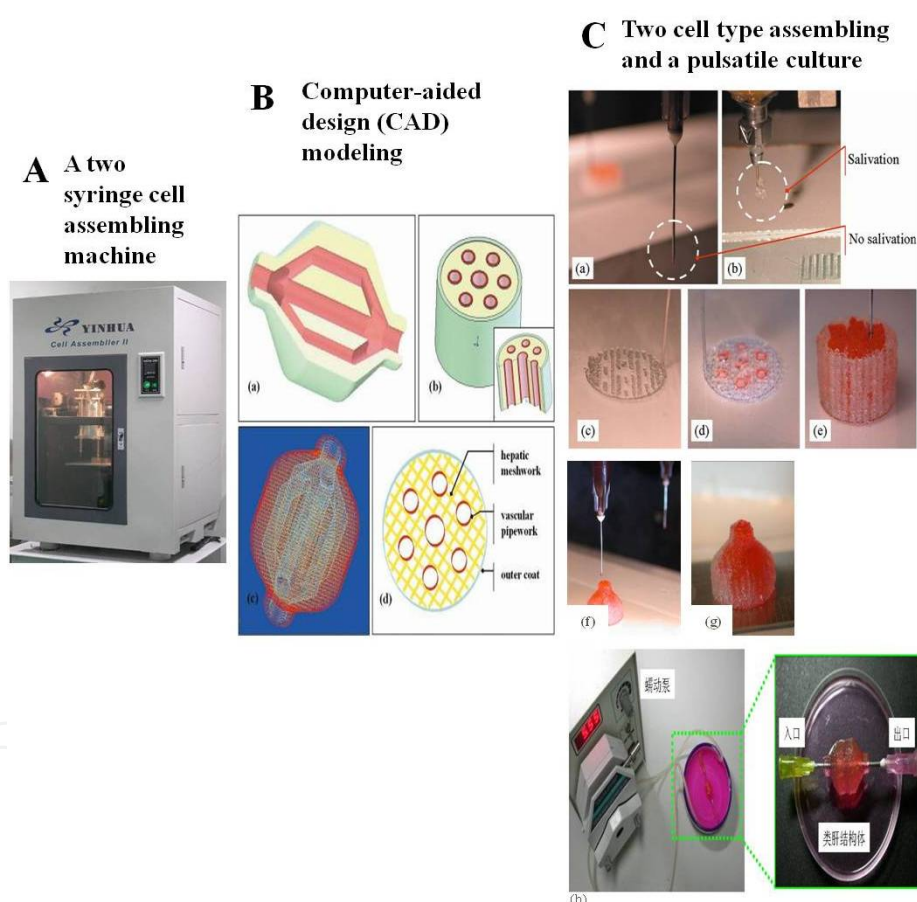


Figure 5. Cell assembling based on a two syringe RP technique developed in Tsinghua University, prof. XH Wang' group. Two different cell types in the gelatin-based hydrogels can be assembled simultaneously into a construct [59-60].

4.3. The combination of cell assembly and cryopreservation techniques

With the advantages of the gelatin-based hydrogel, cryoprotectants (e.g. dimethyl sulfoxide (DMSO), glycerol, and dextran-40) can be incorporated into the cell/hydrogel system and the

constructs can be stored at low temperature (below -80°C) directly after the fabrication stage (Figure 6). This incorporation technique represents a significant advancement towards the cell-laden product storage and transport, potentially resulting in labor and resource saving, clinical availability and medical convenience [80-82]. With the gelatin-based hydrogel various bio-factors including macromolecular cell growth factors, small chemical regulators, and even genes/drugs can be easily incorporated to the deposition or assembling systems. This approach is suitable for some special natural thermosetting polymers' (e.g. gelatin and agarose) deposition and opens a new avenue for complex organ manufacturing.

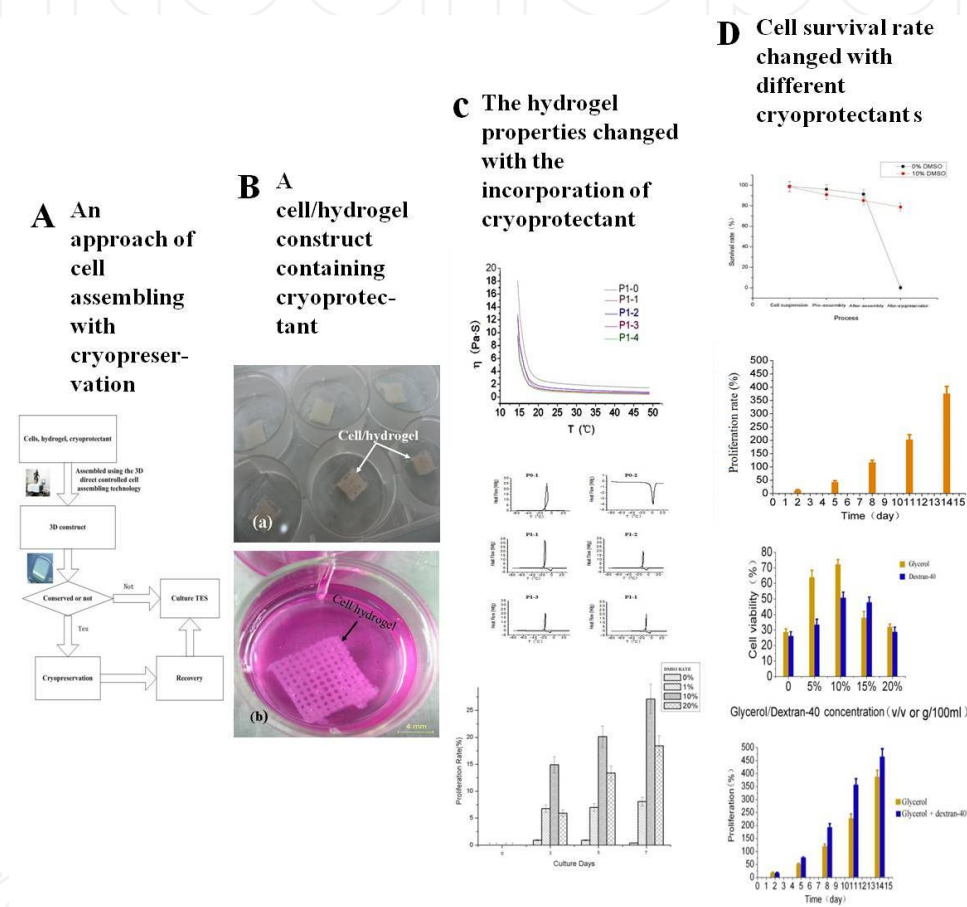


Figure 6. The combination of cell assembly and cryopreservation techniques, developed in prof. XH Wang' group [80-82].

4.4. The double-nozzle low-temperature deposition manufacturing (DLDM) system

The creation of a geometrically complex branched vascular system is a subject of broad fundamental and technological interest in complex organ manufacturing. With the DLDM system it is easy to deposit two different material systems, especially both synthetic and natural polymer systems simultaneously in a construct (Figure 7). Grid, tubular and elliptic structures with both synthetic and natural polymers, such as PU/gelatin and PU/collagen, have been produced at a low-temperature range of -20 - -30°C [61-64]. As shown in Figure 7C, PU

and collagen were successfully assembled into a tubular double layer construct. In Figure 7D, an elliptic hybrid hierarchical PU-cell/hydrogel construct with branched and grid internal channels was realized. Cells can survive the heterogeneous fabrication, polymerization/crosslinking, and even storage stages with a high recovery proliferation ability. Figure 7D demonstrates that the external out coat was made of a PU/tetraglycol solution to provide mechanical support for the whole construct. The internal branched and grid channels were made of a cell/dimethyl sulfoxide (DMSO) containing gelatin/alginate/fibrinogen hydrogel to encapsulate ADSCs. Both the out coat PU and compartment cell/hydrogel layers possess microporous, which permit water, oxygen and other small molecules to pass. During the fabrication stage, a low temperature in the range of -20 - -30 °C around the nozzles is an important factor to control the sol-gel transformation of the material systems. If the temperature is set too high, the deposited fiber (strand) cannot solidify to form a stable 3D structure. On the other hand, if the temperature is set too low, the fiber is frozen too quickly to fuse with the previous deposited layer. An optimum deposition temperature has played a central role in putting the heterogeneous material systems at the desired locations in the construct.

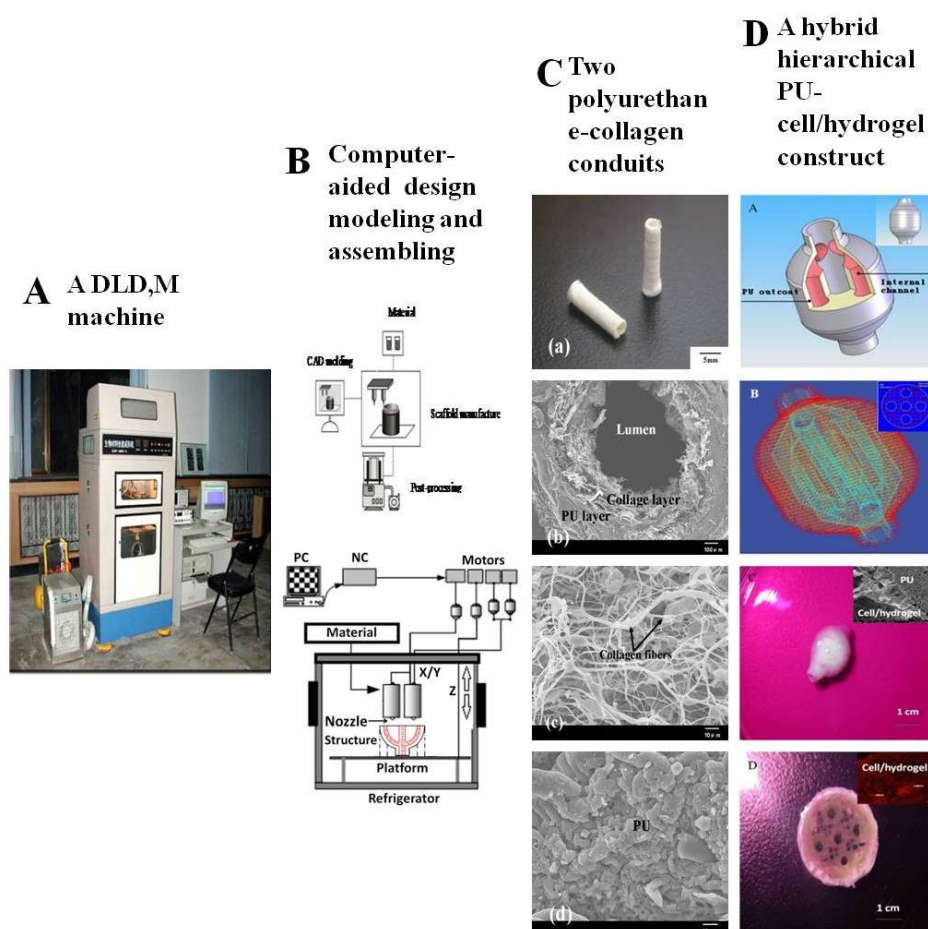


Figure 7. A DLDM technique developed in Tsinghua University, prof. XH Wang' group. An elliptical hybrid hierarchical polyurethane and cell/hydrogel construct was fabricated using the DLDM system [61-64].

This DLDM technique has demonstrated some outstanding merits in complex organ manufacturing with two different material systems that are technologically and biologically incapable to produce using the other existing or traditional RP techniques. The potential applications of the assembled elliptic hybrid hierarchical constructs are diverse, such as cell-cell interaction analyses, stem cell differentiation tracing (pursuing), chemical drug screening, and pathogenic mechanism studies. The synthetic PU system can provide elaborate compartments for cell/hydrogel accommodation. In these compartments, the composition of the cell/hydrogel mixture becomes the key factor in ensuring spatially uniform cell distribution, survival, proliferation and differentiation. By encapsulating the cell/hydrogel mixture in the PU compartments, the composition and proportion of hydrogel components can be easily adjusted to meet the necessary requirements for mimicking the natural cellular arrangements. A maximal cell density (hydrogel-poor and cell-rich) can be easily achieved in the compartment. The use of gelatin-based hydrogel can even be avoided completely in this system, irrespective of stabilization of the construct. Compared with the pure cell/gelatin/alginate/fibrin construct made by the single/double RP systems, the hybrid hierarchical network can provide much higher mechanical stability and pressure resistance abilities when it is applied to *in vitro* pulsatile cultures and *in vivo* blood vessel anastomoses. Some experiments have proved that the 3D constructs with intrinsic interconnected branched and grid channels were easily adapted to an *in vitro* pulsatile culture and *in vivo* implantation system [83-86].

5. A four-nozzle low-temperature deposition manufacturing (FLDM) system

At present, a FLDM system is under development in professor XH Wang's group [18]. Figure 8 demonstrates the outlook of the machine and a primary try on a liver lobe like structure construction. Compared with the DLDM RP system, two more nozzles have been equipped. Thus, two more cell types can be incorporated simultaneously into a construct. This amplified integration possesses some outstanding advantages towards complex organ manufacturing: (i) hierarchically organization of multiple population of cells and growth factors in a more intricate physiologically mimicking geometry; (ii) simultaneously deposition of one scaffold material, a vascular system with two main cell types, and one parenchymal cell type in a more elegant native tissue-specific phenotype; (iii) computer definition of the fluid paths and macro/microstructures in a more patient specific manner; and (iv) spatial distribution of multi-tissue boundaries and fluorescent biomarkers in a more controllable pattern. This FLDM RP system makes it possible to partially control over the design, modeling and fabrication of a highly hierarchical liver lobe like construct in a rapid, convenient, and cost effective manner.

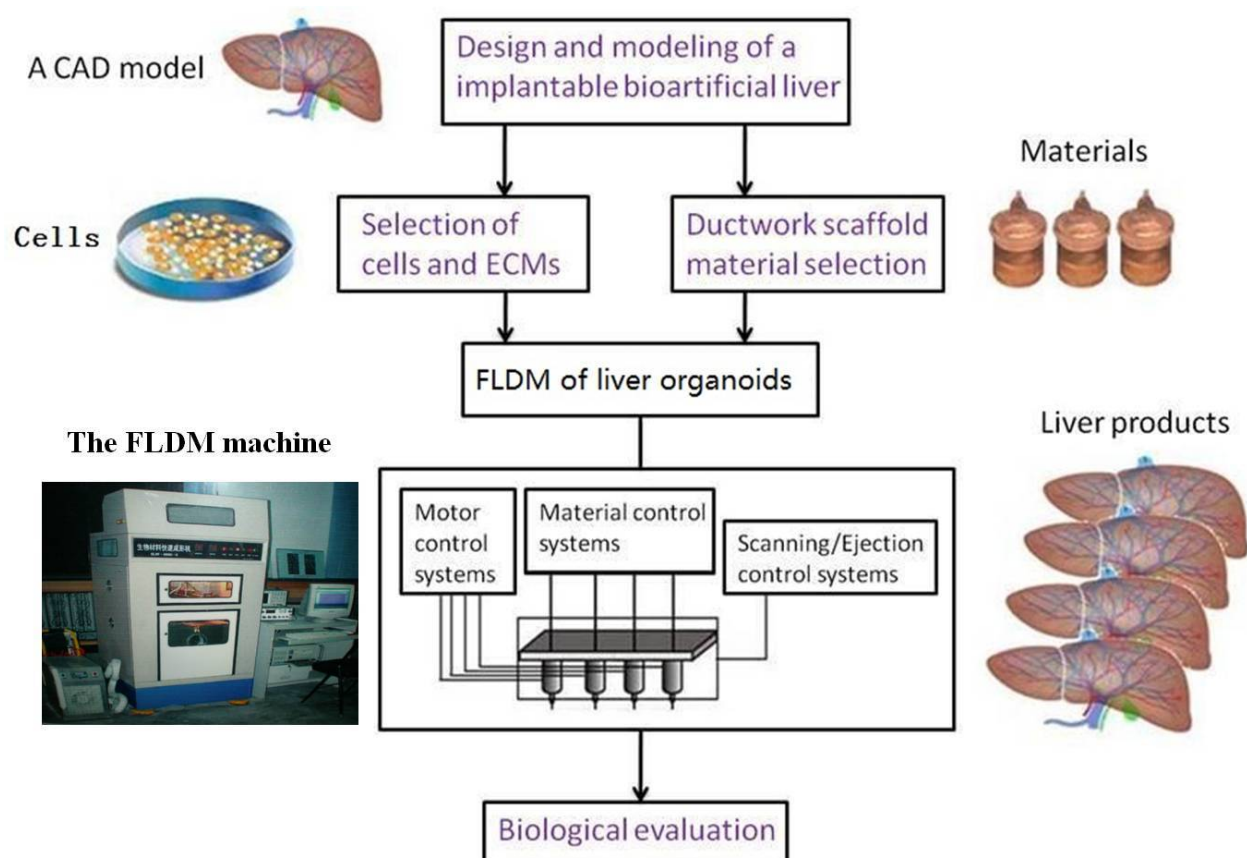


Figure 8. A schematic description of the modeling and manufacturing processes of four liver lobe-like constructs with a FLDM RP system developed in Tsinghua University, prof. XH Wang' group [18].

6. Emphases to some technical specifications

Theoretically, RP technology is able to produce any required complex shape. The standard modeling and deposition technologies enable the hybrid hierarchically ordered patterns to be generated in an automatic, convenient, and inexpensive manner. Again, we use the liver as an example. In a liver lobe at least 6 different cell types are structured as repeated units. These units can achieve high oxygen exchange and nutrient supply for a mass of cells where the cell sizes are in the range of $\sim 20 \mu\text{m}$. This geometry enables a high degree of processing optimization, which provides the opportunity for RP designers and manufacturers to control readily the distribution of different cells in a construct. Stimulated by this motivation, many groups have tried different RP systems with only thin or quasi-3D cell containing structures so far. Someone even claims to use scaffold free cell aggregates to print organs. This has been proven to be a time-consuming process and cells can not find their respective places in a complex organ without the support of scaffold materials.

Different from all the existing techniques, the gelatine-based hydrogel has been explored extensively as an internal scaffold material with the single/double syringe/nozzle RP techniques in the author's own group Tsinghua University. Aqueous gelatin solution is an amorphous natural hydrogel in which cells can be encapsulated, extruded and deposited at desired positions [87-90]. This solution is flexible with a gelation temperature of 20°C and allow the diffusion of hydrophilic substrates. The sol-gel transform property makes it possible to deposit the gelatin-based cell-laden hydrogel at a large range of temperature (from 20°C to -30°C). However, this hydrogel is not stable at 37°C. The mechanical properties of the gelatin-based hydrogels are notoriously inadequate and prohibit the use in stress-loaded implants. To improve the stability of the gelatin-based hydrogels, alginate and fibrinogen were incorporated. Sodium alginate (composed of mannuronic and guluronic (G) dimmers) is a biocompatible and biodegradable polymer, and has been widely used in cell encapsulation technology, although the biocompatibility of the alginates in relation to their composition is a matter of debate. Sodium alginate can be reversibly cross-linked by divalent cations, such as Ca^{+2} and Mg^{+2} , to form a relatively stable hydrogel. Fibrin, derived from blood fibrinogen, is another natural biocompatible and biodegradable polymer, which has been widely used as sealant and adhesive during surgery. With the catalyzing of thrombin smaller fibrinogen molecules can polymerize to form a stable fibrin hydrogel.

In addition to being able to build complex structures with precision and accuracy, it is equally important that the mechanical properties of the supporting materials are suitable for the intended applications. A novel linear elastomeric polyurethane from soft polycaprolactone (PCL) and polyethylene glycol (PEG) segments, and hexamethylene diisocyanate (HDI) chain extender has been synthesized in the authors' own group and used as an external scaffold material. This PU possesses tunable biodegradability, excellent biocompatibility and compatible mechanical properties with animal veins [91,92]. Long-term *in vivo* biocompatibility and biodegradability of the PU have been proven with a rabbit model. It has successfully repaired nerve and vein defects without any detected side effects, such as thrombosis, inflammation, intimal hyperplasia, and calcification. The excellent mechanical properties, biocompatibilities, adjust abilities and processing abilities have made this kind of polymer to be outstanding from the other existing synthetic scaffold biomaterials, such as polyhydroxybutyrate (PHB) [93], poly(D,L-lactic-co-glycolic acid) (PLGA) [94], and poly(tetrafluoroethylene) (PTFE) [95].

To date, the most widely recognized advantage of the RP technology (i.e. layered manufacturing methodology) is the relative ease of automatically manufacture of complex geometric shapes with heterogeneous structures composed of multi-material regions. Complex organ manufacturing aims to automatically produce complex organs directly from CAD models with high sophisticated RP techniques. Since the earlier concept of complex organ manufacturing using both synthetic and natural scaffold biomaterials and multi-nozzle RP techniques was first introduced in 2007, the present technique was developed gradually [13-18]. As displayed in Figure 7D, the virtual elliptic construct with branched fluidic network has been designed and fabricated according to a pre-defined CAD software. The integration of the DLDM RP technique with the cell cryopreservation technique together with the mechan-

ically strong enough synthetic PU scaffold enables us to efficiently produce spatially heterogeneous cell-laden tissue/organ substitutes that would otherwise be challenging to achieve [61-64]. This integrated technique therefore has the potential to lead a big revolution in the fields of tissue engineering and regenerative medicine.

It is expected that in the following several years these integrated RP technologies will see their major break-through development stage and play a key role in complex organ manufacturing area. With the proper integrations of biomaterials and enabling RP techniques, it is possible for us to address all the challenges involved in complex organ manufacturing and to make the realization of complex organ manufacturing both feasible and practical. These proper integrations also benefit some of other related areas, such as high throughput drug screening, stem cell differentiation induction, fluorescent dye discovering, energy metabolite model establishing and cancer/stem cell behavior controlling.

7. Concluding remarks

The goal of complex organ manufacturing is to directly fabricate multiple cell types into an organ substitute using a multiple nozzle RP system. Analogous to the process of building a nuclear power plant, complex organ manufacturing requires the ability to control the positions of many cell types, internal/external scaffold materials, and even cell growth factors on the nano-, micro-, and macro-scales with respect to each others. The integrations of biomaterials and RP techniques have significantly improved the ability to manufacture cell-laden constructions with predefined geometries under the instructions of CAD models or medical data (for example, patient-specific images). Especially, recent advances in DLDM and FLDM RP techniques in Tsinghua University have leveraged these progresses. Although still at its infant stages and associated with numerous problems, ever-increasing evidence supports the intriguing hypothesis that the integrations of multiple biomaterials (including multiple cell types) and multiple nozzle RP techniques will eventually change the traditional practices and make the dreams of complex organ manufacturing come true. It is expected that in the future, most of the reconstructive disciplines of complex organ manufacturing will be fully revised by the development of new multiple nozzle RP systems with optimal safety, easy manipulation ability and maximum reliability. Multiple nozzle RP techniques will undoubtedly play an important role in the future complex organ manufacturing area. Cells in the engineered construct will potentially behave as comfortably as in their natural *in vivo* environment. Further studies are therefore needed to elucidate and determine the fundamental structure-function relationships of diverse tissues in a complex organ, the nutrition supply systems and the heterogeneous structural cues to promote full functional realization in a complex organ. Ever increasing evidences have indicated that with the right integrations of biomaterials and RP techniques, a brand-new era of complex organ manufacturing like the rising sun, is on the horizon.

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