

INKJET PRINTING: AN EMERGING TECHNOLOGY FOR 3D TISSUE OR ORGAN PRINTING

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Abstract

The typical scaffold-based tissue engineering approach, though promising and still considered as a paradigm in tissue engineering, faces some challenges: immunogenicity, degradation rate of the biomaterials, toxicity of degradation products, inflammatory responses of host tissues, mechanical mismatch with surrounding tissues, cell cultures with multiple cell types & specific localization, suitable fabrication method of scaffold, and proper vascularization are some key issues which may affect the long term behaviour of the tissue engineering construct and its primary biological functions. To overcome these drawbacks & for successful reconstruction of defective tissues, new manufacturing methodologies under the principle of rapid prototyping have emerged that enabled the fabrication of structures more close in architecture to biological tissues. A more exciting approach is bioprinting also referred to as organ printing which could be defined as the use of rapid prototyping strategy for patterning and assembling biologically relevant materials like biomolecules, cells, tissues, biodegradable biomaterials in order to produce functional living tissues or organs. In this strategy functional 3D tissue is printed layer-by-layer that could be later implanted in vivo where in situ printing has also been proposed and demonstrated in some areas like skin and cartilage. This article presents a general approach of inkjet printing technology with a specific focus on its successes, limitations, & prospects for the future as a bioprinting technology in 3D tissue or organ printing.

Keywords: Bioprinting, Inkjet Technology, Tissue engineering

Introduction

The term ‘Tissue Engineering’ was coined to mean “the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues & the development of biological substitutes to restore, maintain, or improve tissue function” (Skalak, 1989). The classical tissue engineering approach first proposed by Langer & Vacanti is based on using pre-formed solid scaffolds and isolated cells in which porous 3D scaffolds are generally seeded with cells and occasionally with signaling molecules or subjected to biophysical stimuli in the form of a bioreactor (I. Martin, 2004). These cell-seeded scaffolds are either undergo a pre-implantation differentiation culture *in vitro*, to synthesize tissues and then transplanted or are directly implanted into the injured site, using the body’s own systems, where tissue regeneration is induced *in vivo* (Fergal, 2011).

Though this approach has brought some unprecedented success in tissue engineering but there are still some challenges in scaffold-based tissue engineering. First, complications posed by host acceptance such as immunogenicity, inflammatory response, mechanical mismatch. Second, problems associated with cell cultures such as cell density, multiple cell types, specific localization (T. Billiet, 2012). Third, often uncontrolled and imprecise scaffold geometries (S. Wust, 2011). Fourth, the preformed rigid scaffolds are not optimal for engineering contractile tissues like heart vascular tubes or capillaries (T. Boland, 2006).

The fabrication route for preparing scaffold is important issue to incorporate all the requirements of scaffold and for successful tissue engineering. Traditional porous scaffolds have been made by using some techniques: salt leaching, gas foaming, porogen melting, electrospinning, fiber deposition, molding and freeze-drying. All these techniques can not sufficiently provide scaffold with homogeneous porous structure, porosity, pore interconnectivity, & precisely controlled geometries of scaffold (S. Wust, 2011). However the development of rapid prototyping method since the 1980s has allowed a true engineering of the scaffold by enabling fabrication of fine-scale internal porous structures with the desired complexity (B. Derby, 2012). In this rapid prototyping techniques which is sometimes referred to as 3D manufacturing or solid free form (SFF) fabrication, complex hierarchical scaffold designs can be create by adding material layer-by-layer (T. Boland, 2006), hence, it is now generally referred to as additive manufacturing (AM). This is considered as a promising technique to control over mechanical properties, biological effects, & degradation kinetics of the scaffolds due to micro to millimeter features of the hierarchical designed scaffolds (T. Boland, 2006). And the vascularization issue, one of the major and critical problems with typical

tissue engineering construct can be resolved by engineering small diameter vessels & capillaries within the scaffold through a combined SFF & cell placement approach (T. Boland, 2006).

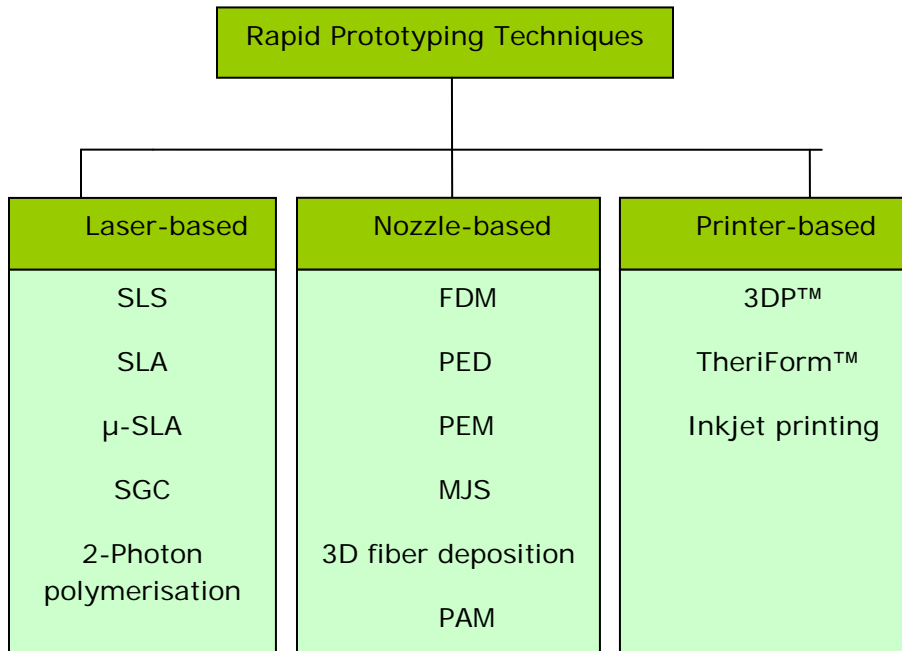


Figure 1: Classification of RP techniques with biomedical applications (T Billiet, 2012)

SLS-Selective Laser Sintering; SLA-Stereolithography; SGC-Solid Ground Curing; FDM-Fused Deposition Modelling; PED-Precision Extrusion Deposition; PEM-Precise Extrusion Manufacturing; MJS-Multiphase Jet Solidification; PAM-Pressure-assisted microsyringe; LDM-Low Temperature Deposition Modelling.

Another prospect to tissue engineering is the “Bottom-up” or “scaffold-free” tissue engineering. The most fascinating thing is that, during embryonic maturation, tissues and organs are formed without the need for any solid scaffolds (T. Billiet, 2012). The formation of a final structure with autonomous organization of components, is called self-assembly & in the field of scaffold free tissue engineering the premise concerning the self-assembly & self-organizing capabilities of cells and tissues is worked out (T. Billiet, 2012). This methodology possesses some potential advantages over scaffold-based tissue engineering: (B. Derby, 2012)

-As there is no scaffold, so no problems associated with materials or degradation product compatibility.

-Better intercellular communication is possible because cells are cultured in more similar conditions to the 3D environment of the body.

-Cell clusters that serve complex functions in organs & are very sensitive to the environment e.g. hepatocytes are less likely to redifferentiate and lose functions.

The self-assembly concept is used in cell sheet technology which is a typical solid scaffold-free tissue engineering. Stacked or rolled layers of engineered tissue can also be fused as a result of the tissue fusion process and form thicker constructs (V. Mironov, 2009, p-4). The first completely biological tissue-engineered vascular graft was built by L’Heureux N et al. by using cell sheet technology (L’Heureux N, 1998).



Figure 2: Biofabrication methods in tissue engineering; (a) Cell sheet technology-cell sheets are rolled into a tubular construct; (b) Embedding cells into a 3D hydrogel; & (c) Cell seeding in a porous solid biodegradable scaffold (V Mironov, 2009).

However, rapid prototyping technologies offers another more fascinating approach on scaffold-free tissue engineering, and is commonly termed as “Organ Printing”(T. Billiet 2012). Organ printing refers to the computer-aided 3D tissue engineering of living structures based on the simultaneous deposition of cells and hydrogels supported by the principles of self-assembly (V. Mironov, 2006). The organ printing approach includes three technological steps: (1) developing design files for organs, (2) simultaneous deposition of cells & hydrogels, & (3) organ conditioning and accelerated tissue & organ maturation (V. Mironov, Bioprinting: A Beginning, 2006).

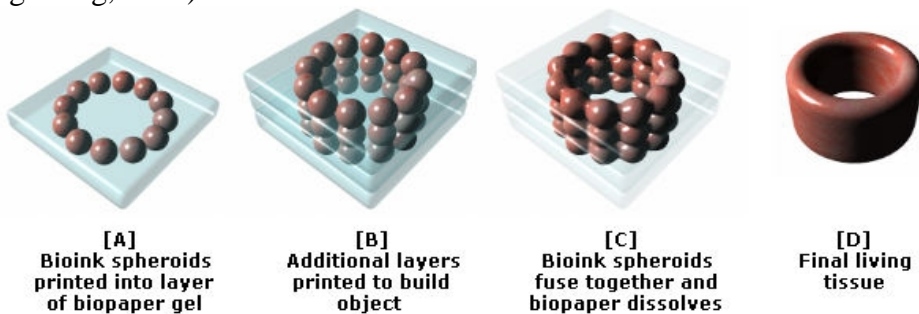


Figure 3: Stages of organ printing (explainingthefuture.com/bioprinting.html).

Three main technologies used for organ printing are inkjet printing, laser forward transfer, & microextrusion/filament plotting (B. Derby, 2012). For efficient drop ejection, inkjet printing requires a low material viscosity where in laser forward transfer cells are required to be immobilized in a gel and microextrusion methods have a wide range of fluid viscosities but offer a lower spatial resolution (B. Derby, 2012).

Inkjet Printing Technolog

Inkjet printing is defined as a non-contact printing technique that receives digital data from a computer representing an image or character, and reproduces this digital pattern onto a substrate by using tiny ink droplets (T. Boland, 2006). Depending on the mechanism of droplet formation, the inkjet technology is broadly divided into two groups, (1) continuous, & (2) drop on demand, which are then further classified in the following ways:

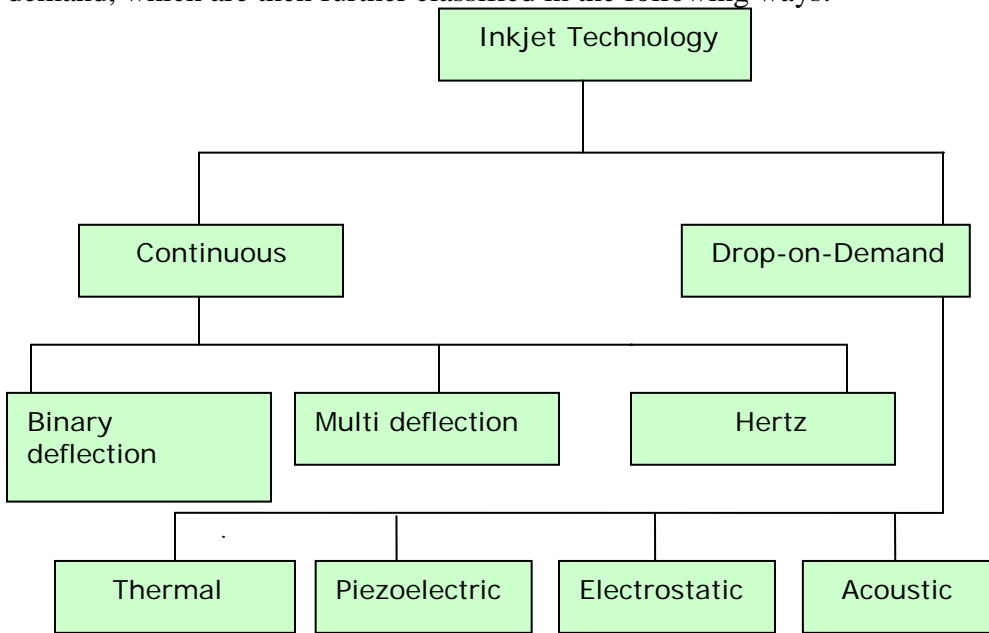


Figure 4: Classification of Inkjet Printers (Hue P Le, 1998)

In drop on demand inkjet printer drops are ejected on demand, for example, when an image pixel is ON (Sridhar A, 2012). In this system multiple actuation mechanisms are used like thermal, piezoelectric, electrostatic, & acoustic. Their drop formation mechanisms are discussed below.

In thermal inkjet printers, the print head is heated by heating elements, small air bubbles are formed that produce pressure pulses to eject ink droplets out of the nozzle with various volumes from 10 to 150 pl (Cui

X., 2012). The droplet size varies according to the applied temperature gradient, frequency of current pulse & ink viscosity (Cui X., 2012)

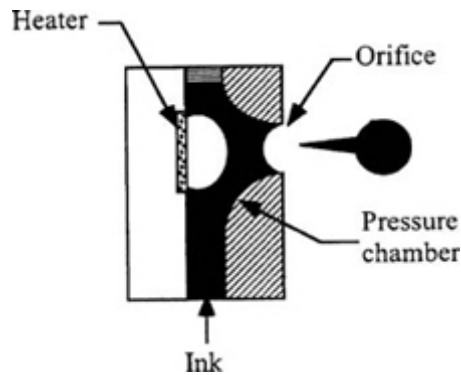


Figure 5: Printhead of thermal inkjet (Hue P Le, 1998).

In the piezoelectric inkjet method, applying a voltage pulse to a piezoelectric material induces a deformation of the shape which in turn causes the ink volume change in the pressure chamber to generate an acoustic pressure wave that propagates toward the nozzle and break the liquid into droplets into regular intervals (Murphy S. V., 2014; Hue P Le, 1998).

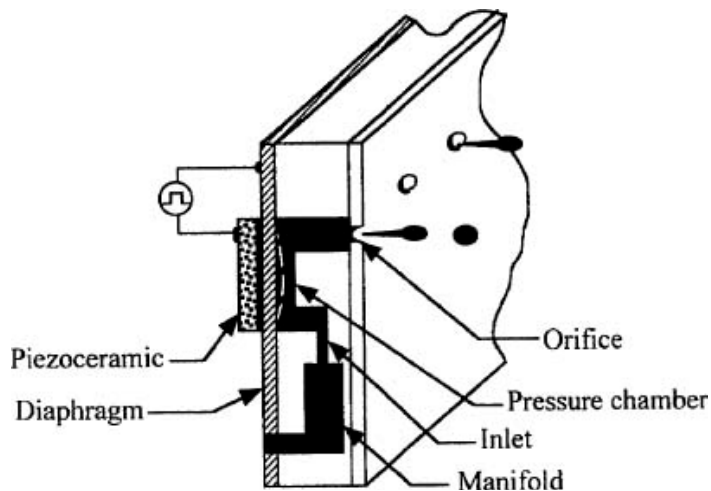


Figure 6: The basic configuration of a piezoelectric printhead (Hue P Le, 1998)

Electrically actuated inkjet system uses an electric field that exists between inkjet set up & substrate. Mechanism of droplet formation involves the complex interaction of surface tension ratio between ink & nozzle, and electric field. The signal fed to printhead balances force to create ink droplet (Sridhar A, 2012).

In an acoustically actuated inkjet printer, discrete droplets of uniform size are ejected from an air-liquid interface due to acoustic radiation pressure associated with an ultrasound field. The size of droplets and the rate of ejection can be manipulated by adjusting the ultrasound parameters like pulse, duration & amplitude (Fang Y, 2012, Murphy S V, 2014).

Bioprinting with the aid of drop-on-demand inkjet technology

For bioprinting applications, the first inkjet printers were the modified versions of commercially available 2D-ink based printers where the ink in the cartridge was replaced with biological material and the paper was replaced with an electronically controlled elevator stage for providing the control of z-axis. Now the inkjet-based bioprinters are custom designed to handle and print biological materials with increasing precision, resolution, & speed (Murphy S V, 2014). Inkjet printing offers a technology to subtly combining and orchestrating cells, growth factors & scaffolds into an architecture which would allow their unfettered interaction, especially where distinct cell types are necessary in anatomically exact locations to gain biological function (T Xu, 2004). T Boland et al. (T Boland, 2006) have demonstrated the simultaneous printing of cells and biomaterials that allows precise placement of cells and proteins within 3D hydrogel structures.

By modifying commercial thermal inkjet printer researchers have been made it possible to print biomolecules onto target substrates, resulting in the creation of DNA chips, protein arrays, & cell patterns with appropriately maintaining their bioactivities (T Xu, 2004). Viable cells can be delivered to a precise target location on the scaffold material with the help of computer assisted deposition. Also by using multiple nozzles, different cell types as different bioinks can be delivered to exact positions to mimic structures of the original tissue. Thus the printing of dissociated cells onto specific patterns, & then their subsequent fusion, may allow the development of replacement tissue or even the whole organ (T Xu, 2004).

Several studies regarding thermal inkjet printers have demonstrated that the heat and mechanical stress generated in thermal inkjet printheads minimally affects the viability of several cell types including cell lines, hamster ovary cells, muscle cells, & stem cells (B Lorber, 2014). In the thermal inkjet printers, although the temperature rises to 200-300°C in each nozzle due to localized heating, it lasts only for few microseconds during printing & ejected mammalian cells are heated for only 2 microseconds with a temperature rise of 4-10°C above ambient which does not have a substantial impact on the cell viability or post printing function of mammalian cells demonstrated by T Xu et al. (T Xu, 2004). They directly printed Chinese Hamster Ovary (CHO) & embryonic rat motoneurons by using a modified Hewlett Packard (HP) thermal inkjet printer where soy agar

& collagen hydrogels were used as the culture substrate or “Bio-paper” to prevent cells from drying and to provide nutrients for cell growth. Their study indicated that, CHO cells and primary motoneurons can be delivered successfully through the modified HP inkjet printer where most of these cells were survived (>90%) during printing and once deposited on the gel, the CHO cells returned to their normal shape and morphology to a great extent. And the outgrowth of the motoneurons processes & the establishment of polarized morphologies indicated their survival.

In another study T Xu et al.(T Xu, 2006) have demonstrated that, embryonic hippocampal and cortical neurons maintained their basic cellular properties and functions, including normal, healthy neuronal phenotypes & electrophysiological characteristics, after being printed with thermal inkjet printing. Their findings have made the inkjet printing a rapidly evolving technology as a digital biofabrication method to build functional neural tissues that may eventually be applied in neural tissue engineering.

Also in one experiment T Xu et al.(T Xu, 2013) developed a versatile method to fabricate complex and heterogeneous 3D tissue constructs by using simultaneous inkjetting of multiple cell types. For this study they used modified thermal inkjet printer. Human amniotic fluid-derived stem cells (hAFSCs), canine smooth muscle cells (dSMCs), & bovine aortic endothelial cells (bECs) were separately mixed with ionic cross-linker CaCl_2 and loaded into separate ink cartridges and then delivered layer-by-layer to pre-determined locations in a sodium alginate-collagen composite loaded in a chamber under the printer. A solid composite gel was formed rapidly due to the reaction between CaCl_2 and sodium alginate and the printed cells were embedded in designed areas within the gel. The printing process was repeated for several times, resulted in a complex 3D multi-cell hybrid construct. The biological functions of the constructs were evaluated both in vitro and in vivo. Each type of printed cell maintained their viability and normal proliferation rates, phenotype expression, & physiological functions within the heterogeneous constructs and the constructs were able to survive and mature into functional tissues with adequate vascularization in vivo.

Printing mammalian cells through piezoelectric technology is somewhat challenging because to minimize ink leakage & to prevent mist formation, commercial piezo-printers use more viscous ink which call for the more power and higher vibration frequencies to eject ink droplets. This high power sources and higher vibration frequencies can break & damage the cell membranes (T Xu, 2004)). The potential damage to the cell membrane and cell lysis were well documented after sonification at 15-25 KHz which is within the range of frequencies employed by piezoelectric inkjet bioprinters (Cui X, 2012).

However, recently piezoelectric printhead was successfully used to print viable cells derived from the eye, or any other part of the mature adult central nervous system (CNS), which is an important step in the development of tissue grafts and may aid to cure the blindness. The pioneer of this investigation was B Lorber & his research group (B Lorber, 2014) .They extended the piezoelectric printing technology to print cells (RGC) & glia. The effects of printing process on cell survival and the growth of these cells in culture were also investigated. They found no evidence of cell destruction during ejection and drop formation by imaging the printhead nozzle, the area where the cells experience the greatest shear stress and rate. The viability of the cells was also unaffected by the printing process. When cultured the same number of printed and non-printed RGC/glial cells, there was no significant difference in cell survival & RGC neurite outgrowth. The use of a glial substrate significantly increased RGC neurite outgrowth which was retained when the cells had been printed.

Also R E Saunders (R E Saunders, 2008) used a piezoelectric actuated inkjet printing to print suspensions of human fibroblasts cells from a well-characterized cell line (HT 1080) in order to investigate the cell behaviour that exposed to the mechanical and fluid stresses associated with the printing process. By varying the amplitude and rise time of the electric pulse it was possible to alter the stresses experienced by the cells. They found that the amplitude of the pulse has a small influence on cell survivability with regression analysis showing cell survival rates falling from 98% with a 40V pulse to approximately 94% with an 80V pulse. And the rise time of the pulse had no influence on the cell survival. Also the post-printing cell viability was assessed using the Alamar Blue metabolic assay and the survived cells were unaffected by the printing process. However, it was found that after about 20 minute printing, some cell agglomeration or sedimentation affected the printing performance, so inkjet printing requires the cell suspensions to be stable over several minutes during printing process.

The thermal inkjet printers are usually more convenient in terms of modification, access, & maintenance than piezoelectric inkjet printers (Cui X, 2012). The thermal inkjet printers also possess low cost, wide availability & high printing speed (Murphy V S, 2014). E A Roth et al. investigated a method to apply high throughput inkjet printing to control cellular attachment and proliferation by precise, automated deposition of collagen protein. The results showed that commercial thermal inkjet printing technology can be used to create viable cellular patterns with 350 micrometer resolution through the deposition of biologically active proteins and has a potential to be adapted to tissue engineering and colony patterning applications.

Acoustic inkjet printers have the capability to generate and control a uniform droplet sizes and ejection directionality and avoid the exposure of cells to heat or pressure stressors. Furthermore, an open-pool nozzleless ejection system can eliminate the shear stress imposed on cells at the nozzle tip which reduces the potential loss of cell viability & function with avoiding the problem of nozzle clogging (Murphy S V, 2014).

Electrostatic inkjet technology can eject bio-inks without generating significant heat which ensures the cell viability. M Nakamura et al.(M Nakamura, 2006) investigated the feasibility of microseeding with living cells through electrostatically actuated inkjet system. Suspension of bovine vascular endothelial cells was ejected onto the culture disks safely. The number of cells in each dot was dependent on the concentration of the cell suspension and ejection frequency chosen. After the completion of ejection the cells were incubated for a few hours & they were adhered to the culture disks.

One problem with inkjet printer was that as the inkjet droplets are very small, they dried immediately after ejection. While the printing substrate is wet, drawing pattern is very difficult because of blotting, mixing, & diffusion of ink. C Henmi et al.(C Henmi, 2007) have developed a gel formation technique, applying electrostatically actuated inkjet head. In this experiment, gel precursor (sodium alginate) was ejected onto the substrate of gel reactant (CaCl_2) by inkjet. The inkjet droplets formed hydrogel beads at the landing position. By using cell suspension with sodium alginate solution, they succeeded to embed individual living cells in alginate hydrogel beads. Their study demonstrated a gelation technique enabled to make 2D & 3D patterning of hydrogel structure containing living cells with no drying, no blotting even in aqueous medium.

Researchers have already demonstrated the feasibility of hydrogel-based printed constructs for extended in vitro cultures but for immediate therapeutic application printing these constructs remains challenging, partly due to storage issues & poor surgical handling. These limitations could be technically overcome by in situ printing i.e. printing the cells or biomaterials directly into the body (P G Campbell, 2007). Notable examples of in situ printing are the regeneration of skin and cartilage. The higher printing speed assisted the direct deposition of cells and materials directly into the lesions of skin or cartilage (Murphy S V, 2014).

Printed 3D constructs from naturally derived biomaterials lack structural integrity and adequate mechanical properties for use in vivo, which limited their applications in load bearing tissue engineering constructs such as cartilage and bone. T Xu, K W Binder & their research group (T Xu, 2013) developed a novel hybrid inkjet printing/ electrospinning system to fabricate layered cartilage tissues where electrospinning of PCL fibers was

alternated with inkjet printing of rabbit elastic chondrocytes suspended in a fibrin-collagen hydrogel. They built five layered 1mm thick cartilage tissue construct and evaluated four key parameters: cell viability, maintenance of the layered structure, mechanical properties, & formation of cartilage-specific extracellular matrix. They observed that, chondrocytes survived (>80%) within the printed hybrid construct, one week after printing and cells were proliferated & maintained their basic biological properties. The printed scaffolds demonstrated enhanced mechanical properties compared to printed alginate or fibrin-collagen gels or electrospun PCL fibers alone. Moreover, the deposition of collagen and glycosaminoglycans demonstrated the formation of cartilage-like tissue both in vitro and in vivo.

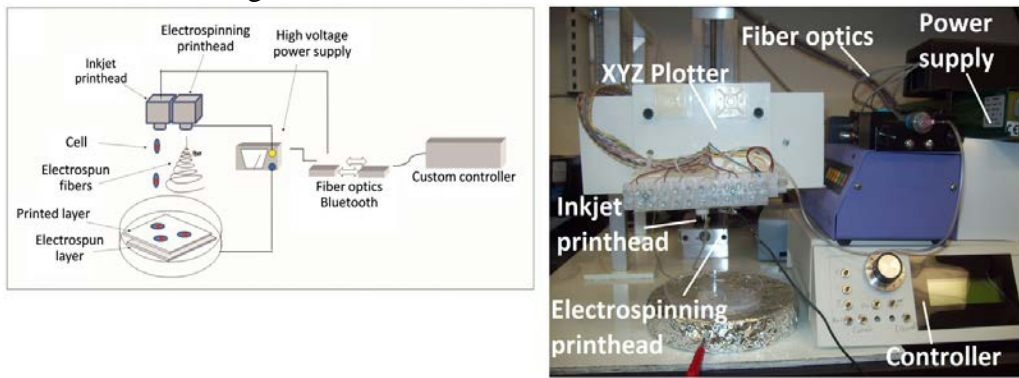


Figure 7: Hybrid printing system; (a) Schematic representation; & (b) The actual prototype (T Xu, 2013).

Where successfully building a thick tissue construct like kidney, liver, and heart with appropriate vasculature is a real challenge in tissue engineering field, recent progress in cell printing offers some promise to build complex 3D structures and it has been demonstrated by T Xu (T Xu, 2009) that precise human microvasculature can be build with suitable bio-ink. In this study, for microvasculature construction human microvasculature endothelial cells (HMVEC) and fibrin were taken as bio-ink.

Though the inkjet bioprinting technology offers high-throughput capabilities, flexibility, ease of use and low cost, one common limitation of bioprinting through inkjet technology is that the biological material should be in a liquid form to make droplet and then the printed liquid must form a solid 3D structure with structural organization and functionality. Researchers have addressed this problem by using cross-linking in bio-ink that after deposition can be cross-linked using pH, chemical or ultraviolet mechanisms (Murphy S V, 2014). But the requirements for cross-linking often slowed the printing process and chemically modify the naturally occurring ECM materials. Furthermore, some cross-linking mechanisms involve products or conditions that are toxic to cells which reduces the cell viability and

functionality (Murphy S V, 2014). Difficulty in achieving higher cell density is another problem as low cell concentration often facilitate the droplet formation, avoid nozzle clogging, reduce shear stress, and also facilitate some hydrogel cross-linking mechanisms (Murphy S V, 2014)

Conclusion and future outlook

It is necessary to create suitable microenvironment for cells which includes not only arrange single type of cells but also multiple types of cells and biomaterials, such as extracellular matrices and growth factors, around individual cells. Inkjet bioprinting technology has a potentiality to control cellular functions and tissue growth by introducing the concentration gradient of cells, growth factors, or biomaterials throughout the 3D structure which can be done by changing the drop densities or sizes (Murphy S V, 2014). It has been reported that by using inkjet printing gradient pattern of growth factors were created on 2D fibrin film and cell proliferation was increased within the growth factor patterned region (C Henmi, 2007).

For cell patterning through inkjet method it is vital to use stable, aqueous non-cytotoxic bio-inks that act as cross-linking agents (T Boland, 2006). The cell density of the printed structures and the printing speed can be increased by optimizing the rheological and surface properties of the bio-inks with designing printers optimized for these properties (T Boland, 2006).

Controlling the placement of cells or molecules within a construct will not ensure their subsequent self-assembly into a functional tissue. It is necessary to provide additional environmental cues such as appropriate mechanical stresses, oxygen tensions, nutrients and other factors. That's why continuous development of more sophisticated bioreactors will be a critical issue (P G Campbell, 2007).

Additionally, inkjet bioprinted constructs should be cost-effective with improved therapeutic outcomes over existing "off- the-shelf" solutions, like allografts or synthetics or simple scaffold constructs (P G Campbell, 2007). Furthermore, to be regarded as a viable biofabrication method, generic problems and long lasting effect of the construct in vivo must also be considered.

The final and most important thing is that for designing a sophisticated bioprinted construct, we have to enhance our understanding about the fundamentals of structure-function relationships in tissues and the underlying biology of regeneration. However, rapid progress with inkjet bioprinting technology throughout the past few years have made the promises that in the future inkjet bioprinting technology will enable to make more sophisticated, thick, vascularized 3D tissue constructs which is a long term interest and worthwhile pursuit in the field of tissue engineering.

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