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Thermal Inkjet Printing in Tissue Engineering and Regenerative Medicine

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Abstract

With the advantages of high throughput, digital control, and highly accurate placement of cells and biomaterial scaffold to the desired 2D and 3D locations, bioprinting has great potential to develop promising approaches in translational medicine and organ replacement. The most recent advances in organ and tissue bioprinting based on the thermal inkjet printing technology are described in this review. Bioprinting has no or little side effect to the printed mammalian cells and it can conveniently combine with gene transfection or drug delivery to the ejected living systems during the precise placement for tissue construction. With layer-by-layer assembly, 3D tissues with complex structures can be printed using scanned CT or MRI images. Vascular or nerve systems can be enabled simultaneously during the organ construction with digital control. Therefore, bioprinting is the only solution to solve this critical issue in thick and complex tissues fabrication with vascular system. Collectively, bioprinting based on thermal inkjet has great potential and broad applications in tissue engineering and regenerative medicine. This review article introduces some important patents related to bioprinting living systems and the bioprinting in tissue engineering field.

Keywords

Tissue engineering; hydrogel; gene transfection; growth factor; inkjet printing; microvasculature; photopolymerization

1. INTRODUCTION

Tissue or organ failure due to aging, diseases, accidents, and birth defects is crucial in today's medicine. The clinical treatments for the lost or failed organs mostly depend on the organ transplantation from deceased or living donors, including kidney, pancreas, liver, intestine, heart, lungs, etc. However, human organ donor shortage remains a critical issue in the world. There were 154,324 patients waiting for a transplantable organ in the United States alone in 2009. Among them, only 27,996 patients received organ transplantation and 8,863 patients died while they were still on the waiting list. That is about 25 people died everyday when they were waiting for a suitable organ donor (data from: OPTN & SRTR

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CONFLICT OF INTEREST

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Annual Data Report 2010, Health Resources and Services Administration, U.S. Department of Health and Human Services). This number increases each year due to more and more patients added into the waiting list. The cost of the organ replacement surgery as well as the follow-up expenses is more than 300 billion US dollars a year.

Therapies based on tissue engineering and regenerative medicine are particularly attractive to solve this organ donor shortage by repairing or replacing injured or diseased organs using cell transplantation and biomaterial scaffold with growth factor stimulation. Furthermore, the engineered tissues can serve as better physiological replicas for understanding basic biology. Tissue engineering and regenerative medicine are normally interchangeable terms. The term of tissue engineering is used to describe approaches that are aimed at fabricating tissue replacement *in vitro* that can be implanted into the human body. The term of regenerative medicine is sometimes used for stem cell technology [1–3]. Thus, tissue engineering has a much broader meaning and has been used for a wide variety of approaches, including replacement, repair, and the tissue or organ regeneration.

The industry associated with tissue engineering field has had “ups and downs” during the past two decades. The most tissue engineering products developed during 1990s were skin replacements. The leading tissue engineering companies were Advanced Tissue Sciences (ATS) and Organogenesis (OI) but both of them entered bankruptcy in the early 2000s. ATS no longer exists today and OI has reinvented to become a profitable company. Actually there has been a renaissance of this industry in the past five years. The total industrial activity was US$2.4 billion in 2007 according to the available data [4].

The traditional tissue engineering approach of seeding the isolated cells to the pre-formed solid and rigid scaffolds was introduced in 1993 by Langer and Vacanti [5]. The isolated autologous cells are expanded in monolayer and then seeded onto porous biodegradable scaffolds. A bioreactor is usually required to culture the fabricated organ construct *in vitro* before it can be implanted back to the human body. This approach has generated some significant successes in constructing avascular, aneural, alymphatic, thin, and hollow organs [6, 7]. These engineered tissues are nourished by the diffusion from host vasculature; however, the most demanding organs for transplantation (> 90%) are thick and complex organs, such like kidney, liver, and heart (OPTN & SRTR Annual Data Report 2010). When the thickness of the engineered tissue exceeds to 150–200μm, it will surpass the oxygen diffusion limitation. Therefore, functional vasculatures must be created into the fabricated tissues to supply the cells with oxygen and nutrients, and also to remove the waste products from the cells [8]. However, the conventional tissue engineering approach is not capable to create these thick and complex tissues due to these limitations:

1. The cell seeding and penetration is not effective to the pre-formed scaffold. Tissue formation or maturation is not uniform throughout the scaffold on the time scale of months. Although scaffold design has been significantly improved for effective cell seeding and migration, the approaches are still far from optimal [9–11].
2. Multiple cell types are usually required to fabricate organs with complex structure. However, the precise placement of cells and growth factors in 3D is still far from being resolved.
3. Vascular or microvascular system is crucial for thick and complex tissue engineering [12], which must be fabricated simultaneously with scaffold construction. However, the traditional approach is not able to construct the vascular system with pre-designed 3D patterns.

One promising approach to solve these critical limitations for tissue engineering is bioprinting based on thermal inkjet printing technology, which is a combination of solid

freeform fabrication and precise cell placement in 2D and 3D. There are quite a few patents regarding printing biological systems recently and some of them are applicable for tissue engineering approaches [13–18]

2. INKJET PRINTING

Inkjet printing is a non-contact printing technique that reproduces digital pattern information onto a substrate with tiny ink drops [19]. There are thermal, piezoelectric, and electromagnetic approaches to create drops on demand [20]. Most inkjet printers employ heat or mechanical compression to eject ink drops. In thermal inkjet printers, small air bubbles generated by heating in the printhead collapse to provide pressure pulses to eject ink drops with various volumes from 10 to 150 pL out of the nozzle [21–23]. The size of droplets varies due to the applied temperature gradient, frequency of current pulse, and ink viscosity [21–23]. As for the piezoelectric inkjet printers, the actuator of polycrystalline piezoelectric ceramic in each nozzle provides the transient pressure to eject the ink drops onto the substrate [24]. These inkjet printing technologies have been widely used in electronics and micro-engineering industries for printing electronic materials and complex integrated circuits [25]. Recently, inkjet printing has also been successfully applied in biomedical field. Although biological molecules and structures are usually assumed to be fragile and sensitive, DNA molecules have been directly printed onto glass slides using commercial available inkjet printers for high-density DNA microarray fabrication [26, 27]. Challenges still exist when using inkjet printers to print mammalian cells. The well-documented damage to the cell membrane and cell lysis after sonification at 15–25 kHz is within the range of frequencies employed by piezoelectric inkjet printers [28]. As for thermal inkjet printers, although the heating element in each nozzle raises the local temperature to 300°C and lasts for a few microseconds during printing [23], ejected mammalian cells are heated for only 2 μs with a temperature rise of 4–10°C above ambient and an average cell viability of 90% [29]. Therefore, thermal inkjet printing technology is more biocompatible to the living system comparing to piezoelectric printing. Furthermore, the thermal inkjet printers are usually more convenient than piezoelectric inkjet printers in terms of modification, access, and maintenance. Therefore, many research groups including us are utilizing thermal inkjet printers for tissue engineering and regenerative medicine applications.

3. FEASIBILITY OF MAMMALIAN CELL PRINTING USING THERMAL INKJET

A number of other bioprinting approaches are currently available using various mechanisms, however, these approaches either have difficulties to manipulate singe cell which is critically important for neuron regeneration and fabricating tissues requiring a higher degree of cell organization for specific anatomic structures [30, 31], or time consuming with low throughput [32, 33]. Instead of moving the cells directly, biological laser printing uses laser energy to vaporize the solution of biological samples and eject the remaining substances [34]. However, this technology may cause over-drying leading to the failure for biological systems. The high cost of these systems is also a concern for basic tissue engineering research. By contrast, bioink prepared for thermal inkjet printing is usually water based in order to minimize the clogging of the printhead. This allows the printer to freely deliver cells from single cell to multiple cells by simply adjusting the bioink concentration and the printed patterns. Cells are always maintained and protected in an aqueous environment during the thermal printing process therefore it is assumed to be safe to deliver living systems.

Although thermal inkjet printing technology has been applied for cell printing in many applications, there are similar concerns that the printing process may cause cell death or

damages. In order to maintain the printing resolution, the sizes of nozzles in the printhead are usually very small. For a Hewlett Packard (HP) Deskjet 500 thermal inkjet printer with 300 dpi printing resolution, the diameter of the nozzle channel is 48 μm [35]. Smaller nozzle size is required for higher printing resolution. Due to the thermal heat and mechanical stress applied to the cells when they pass through the narrow nozzles, it is possible that the cell membrane or cellular phenotype may be damaged or altered in the printed cells [36]. Therefore, a comprehensive evaluation of cell viability, apoptosis, heat shock proteins expression, cell membrane pore sizes and cellular repair process of the printed cells is highly important to clear the potential obstacles to apply this technology. Using the modified HP printer mentioned above, the viability of printed mammalian cells at different cell concentration was varying from 85% to 95%. There was no significant difference in apoptosis ratio and heat shock protein expression level between printed and non-printed cells [29]. By adjusting the cell concentration in bioink, we can control the cell number in individual ejected ink drop. Therefore, inkjet printing can be applied in quantitative cell seeding. Cell membrane pores were observed in printed cells with dextran dye penetration study. Previous observations showed that dextran dye with molecular weight (MW) up to 40,000 was found in the printed cells. No dextran dye was found in the non-printed cells even with the lowest MW (3,000). The cell membrane pore size of printed cells was estimated as 105 Å according to the Stokes diameter of these dye molecules [29].

The pores developed during printing were transient and could be repaired by the cells in 2 hours. The transient nature of the cell membrane pores can be utilized for targeted gene delivery during the printing process [29, 37].

These observations confirm that the inkjet printing process does minor effect to the cells and the benign side effect to the cells during the printing process has the potential for simultaneous gene transfection and drug delivery when depositing cells.

4. DIRECT HUMAN CARTILAGE REPAIR USING 3D INKJET PRINTING

Cartilage defects resulting from osteoarthritis (OA), aging, and joint injury are a major cause of joint pain and chronic disability [38]. Without blood vessels, nerves, and lymphatics, mature cartilage cannot heal spontaneously. The most common clinical treatments for cartilage repair include autologous chondrocyte implantation (ACI), microfracture, and osteochondral transfer. All of these approaches are highly invasive, complicated, and do not restore the long lasting healthy cartilage [39]. Although cell transplantation based tissue engineering approaches for human cartilage repair was introduced almost two decades ago [40], the current cartilage tissue engineering strategies still cannot fabricate new tissue that is indistinguishable from native cartilage with respect to zonal organization, extracellular matrix (ECM) composition, and mechanical properties [41]. Furthermore, most current strategies of knee cartilage repair involve a procedure of removing healthy cartilage tissue around the lesion site to create artificial defects for further treatment or implantation [42]. This procedure in fact causes additional necrosis to the existing cartilage tissue and it is believed to lead to ultimate cartilage degeneration and failure of implanted tissue [43].

Direct cartilage repair with engineered tissue closely mimicking native cartilage to the site of the lesion without any additional damage to the existing healthy tissue is therefore very attractive. The ideal implanted tissue is expected to integrate with existing native cartilage and to repair lesions of different sizes and thicknesses. The multifaceted nature of this challenge requires a technique adaptable to variable physical dimensions and properties for tissue repair; bioprinting technology, based on inkjet printing, provides the necessarycapabilities.

Synthetic hydrogels formulated from poly(ethylene glycol) (PEG) macromers have been demonstrated to maintain chondrocyte viability and induce ECM deposition in proteoglycans and type II collagen [44, 45]. The compressive modulus of PEG hydrogel is tunable to match that of human cartilage [46]. In addition, PEG is water soluble with low viscosity and can be modified to be photocrosslinkable, which makes it attractive for direct printing with simultaneous polymerization during printing. A standard thermal inkjet printer was modified to precisely deposit human articular chondrocytes and poly(ethylene) glycol dimethacrylate (PEGDMA; MW, 3400) layer-by-layer into a cartilage defect within an osteochondral (OC) plug (3D biopaper) for cartilage repair (Fig. 1). For a representative defect of 4 mm diameter and cartilage thickness of 2 mm, a nominal 0.23 μL of bioink estimated to contain 1140 human chondrocytes (5×10^6 cells/mL) was printed and photopolymerized for each layer to repair the cartilage defect in a layer-by-layer assembly. The thickness of each printed layer was about 18 μm. Total firing time of printhead was 1.1 sec and the whole printing process completed in 108 sec. Compared to manual zonal cartilage fabrication which requires at least 11 min for UV exposure [47], bioprinting reduced UV exposure to cells by 80%. The viability of human chondrocytes printed with simultaneous photopolymerization was 89.2×3.6% ($n = 3$), compared to cell viability of 63.2×9.0% ($n = 3$) when exposed to the same UV light source continuously for 10 min in PEGDMA for manual fabrication [48].

The printed PEG gel remained bound firmly to the native tissue in the defect even after sectioning. An even distribution of printed human chondrocytes was obtained in the 3D PEGDMA hydrogel with simultaneous polymerization during printing. By contrast, when photopolymerization was performed after printing, the deposited chondrocytes accumulated at the zonal interface instead of their originally deposited positions due to gravity [48]. This accumulation of cells at zonal interfaces was also observed in previous reports of manually fabricating zonal cartilage [47, 49].

Printed cartilage implant attached firmly with surrounding tissue and greater proteoglycan deposition was observed at the interface of implant and native cartilage in Safranin-O staining. Printed cartilage in 3D biopaper had elevated glycosaminoglycan (GAG) content comparing to that without biopaper when normalized to DNA. These observations were consistent with gene expression results [48]. This study indicates the importance of direct cartilage repair and bioprinting successfully controlled placement of individual cells, preserved cell viability, maintained chondrogenic phenotype, and demonstrated integration with host tissue.

5. GROWTH FACTORS STIMULATE PRINTED NEOCARTILAGE FORMATION

Although bioprinting is promising to engineer zonal cartilage, previous observations showed significantly higher ECM production with initial cell seeding density at $10–20\times10^6$ cells/mL for cell based cartilage tissue engineering with bovine chondrocytes [49] and mesenchymal stem cells (MSCs) [50]. However, the maximum cell density for bioprinting is restricted as the optimal printing resolution is only achieved with bioink at or lower than 8×10^6 cells/mL [29]. These observations raise concerns that optimal cell densities for cartilage tissue engineering cannot be achieved using bioprinting approaches. Insulin-transferrin-selenium (ITS+) based medium supplemented with 10ng/mL transforming growth factor-β1 (TGF-β1) is commonly used to maintain chondrocyte chondrogenic phenotype in 3D culture [51]. Although TGF-β1 supplemented medium seems sufficient to induce persistent expression of chondrogenic ECM proteins, it is not as efficient for chondrocyte expansion [52]. Fibroblast growth factor-2 (FGF-2) supplemented medium has been shown to be beneficial for chondrocyte monolayer expansion as well as the chondrogenic capacity in terms of ECM

synthesis and chondrocyte specific phenotype expression [53–55]. We tested the role of these growth factors in chondrocyte proliferation and ECM production for the first time in bioprinted cell-laden 3D hydrogels. A successful approach would stimulate cell proliferation in 3D hydrogel without delaying neocartilage formation. The bioprinted samples were treated with FGF-2 for the first week and all samples were then cultured with standard chondrogenic medium. The FGF-2/TGF-β1 treated samples demonstrated 40% more cell proliferation compared with TGF-β1 treated group. Therefore, the FGF-2/TGF-β1 synergistic treatment, made the initial 8×10^6 cells/mL seeding density equivalent to a seeding density of over 11×10^6 cells/mL [56]. This is within the range of cell seeding density for optimal ECM production for cartilage tissue engineering [49, 50]. Thus, proper growth factors stimulation induced cell proliferation and more chondrogenic ECM deposition in bioprinted cartilage tissue while maintained the high printing resolution.

A major challenge constraining the clinical translation of autologous cell-based cartilage repair is the limited source of chondrocytes available during biopsy. A method of expanding cells after bioprinting to the density required for optimal tissue engineering without compromising the quality of the matrix is extremely valuable. One attractive approach is to directly implant harvested autologous chondrocytes or mesenchymal stem cells (MSC) for cartilage repair without *in vitro* expansion. Furthermore, in order to maximize the advantage of bioprinting which delivers cells, growth factors, and biomaterial scaffold precisely to the desired 3D position, limiting initial cell density will greatly optimize the delivery precision. Therefore, the approach developed here will be particularly valuable to supplement our bioprinting to deliver cells with optimal resolution in combination with spatially arranged growth factor gradients to fabricate cartilage tissue that recapitulates the zonal architecture of native tissue.

6. HUMAN MICROVASCULATURE FABRICATION

The current tissue engineering paradigm is that successfully engineered thick tissues must include vasculature. As biological approaches alone, such as VEGF, have fallen short of their promises, one may look for an engineering approach to build microvasculature. Layer-by-layer approaches for customized fabrication of cell/scaffold constructs have shown some potential in building complex 3D structures [57]. With the advent of cell printing, one may be able to build precise human microvasculature with suitable bioink. Human microvascular endothelial cells (HMVEC) and fibrin were utilized as bioink for microvasculature construction [58].

Endothelial cells form the whole inner lining of cardiovascular system and have a remarkable capacity to adjust their number and arrangement to suit local requirements. Most tissues depend on a blood supply and the blood supply depends on endothelial cells. Endothelial cells are the only cells to form capillaries and create an adaptable life-support system spreading into almost every region of the body. Endothelial cells extending and remodeling the network of blood vessels makes it possible for tissue growth and repair (angiogenesis) [59].

Fibrin plays a significant role in natural wound healing. Fibrin gel has been widely used as sealant and adhesive during surgery. Fibrin Glue is used as skin grafts and tissue engineered skin replacements [60]. Fibrin can be produced from the patients' own blood and used as an autologous scaffold for tissue engineering [61]. Fibrin can be polymerized using fibrinogen and thrombin solutions at room temperature [62]. Fibrin gels might promote cell migration, proliferation, and matrix synthesis through the incorporation of the transforming growth factor β and platelet derived growth factors [63]. Fibrin has also been utilized in tissue

engineering to engineer tissues with skeletal muscle cells [64], smooth muscle cells [65], and chondrocytes [66].

A standard inkjet printer was modified to simultaneously deposit human microvascular endothelial cells and fibrin to form the microvasculature (Fig. 2). The bioink and biopaper components for fibrin bioprinting were carefully evaluated for optimal condition of simultaneous deposition of cells and scaffold [58]. HMVEC with 50 unit/ml thrombin and 80 mM Ca^{2+} in PBS was used as bioink to print onto 60 mg/ml fibrinogen hydrogel (biopaper). The samples were incubated for 10 to 15 minutes after the printing to finalize the enzymatic crosslinking and cell attachment.

After 3 weeks in culture, the printed HMVEC aligned themselves in the fibrin channel and proliferated to form a confluent lining. Confocal series images at the z-axis demonstrated tubular structure of the printed human microvasculature. The endothelial cells were forming a channel or tubular structure in the printed fibrin channel [58]. This shows the printed and proliferated endothelial cells processed the critical angiogenesis function and the simultaneous deposition of endothelial cells and fibrin using thermal inkjet printing technology can be used for human microvasculature fabrication.

7. CURRENT & FUTURE DEVELOPMENTS

Taken together, bioprinting based on thermal inkjet printing technology demonstrated feasibility of printing living systems and the benign effects to the printed cells can in fact be used for more attractive applications, such like gene transfection and drug delivery. The printing system is versatile for 2D and 3D tissue application as well as avascular and vascular tissue printing. One promising clinical application is to develop a hand-held printer or printhead with digital control for direct tissue repair. By using 3D reconstructions of scanned lesions, bioprinting is able to precisely deliver cells, growth factors, and biomaterial scaffolds to repair the lesion with various shape and thickness with digital control. The successful application in microvasculature fabrication also revealed the bioprinting may be the ultimate solution to engineer thick tissues with complex vasculature and innervation.

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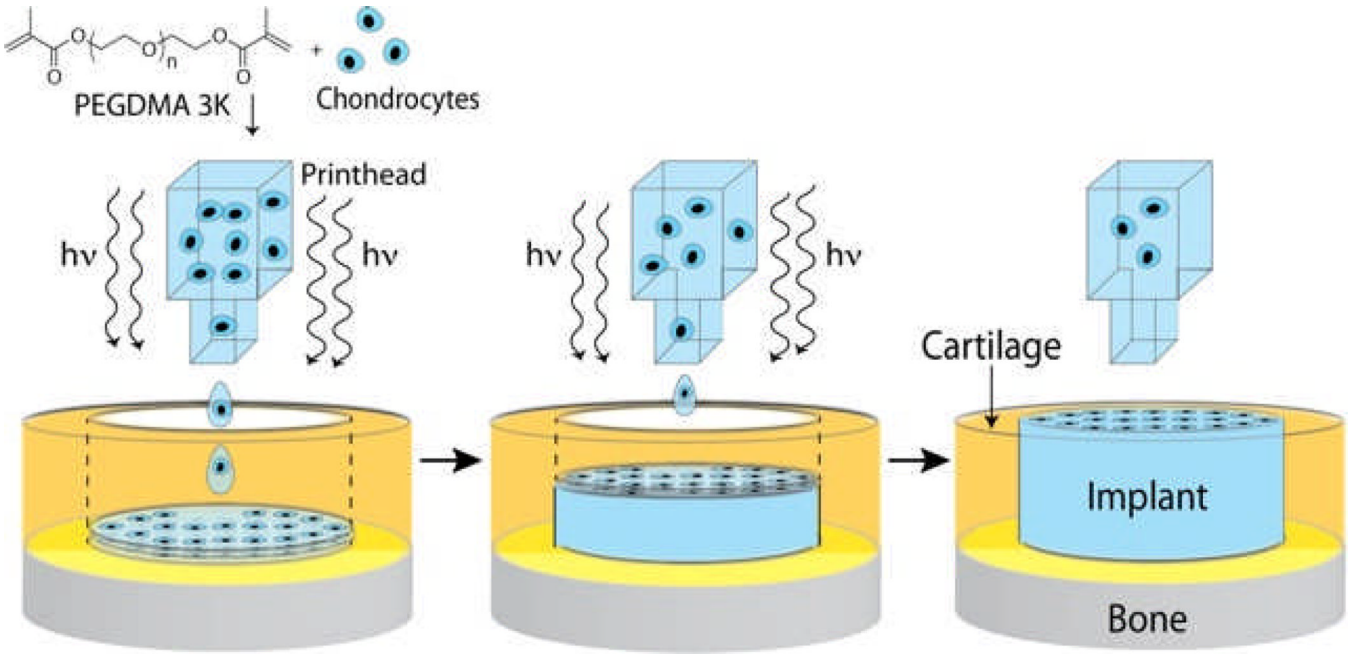

Figure 1.
Schematic of bioprinting cartilage with simultaneous photopolymerization process (adapted from [41]).

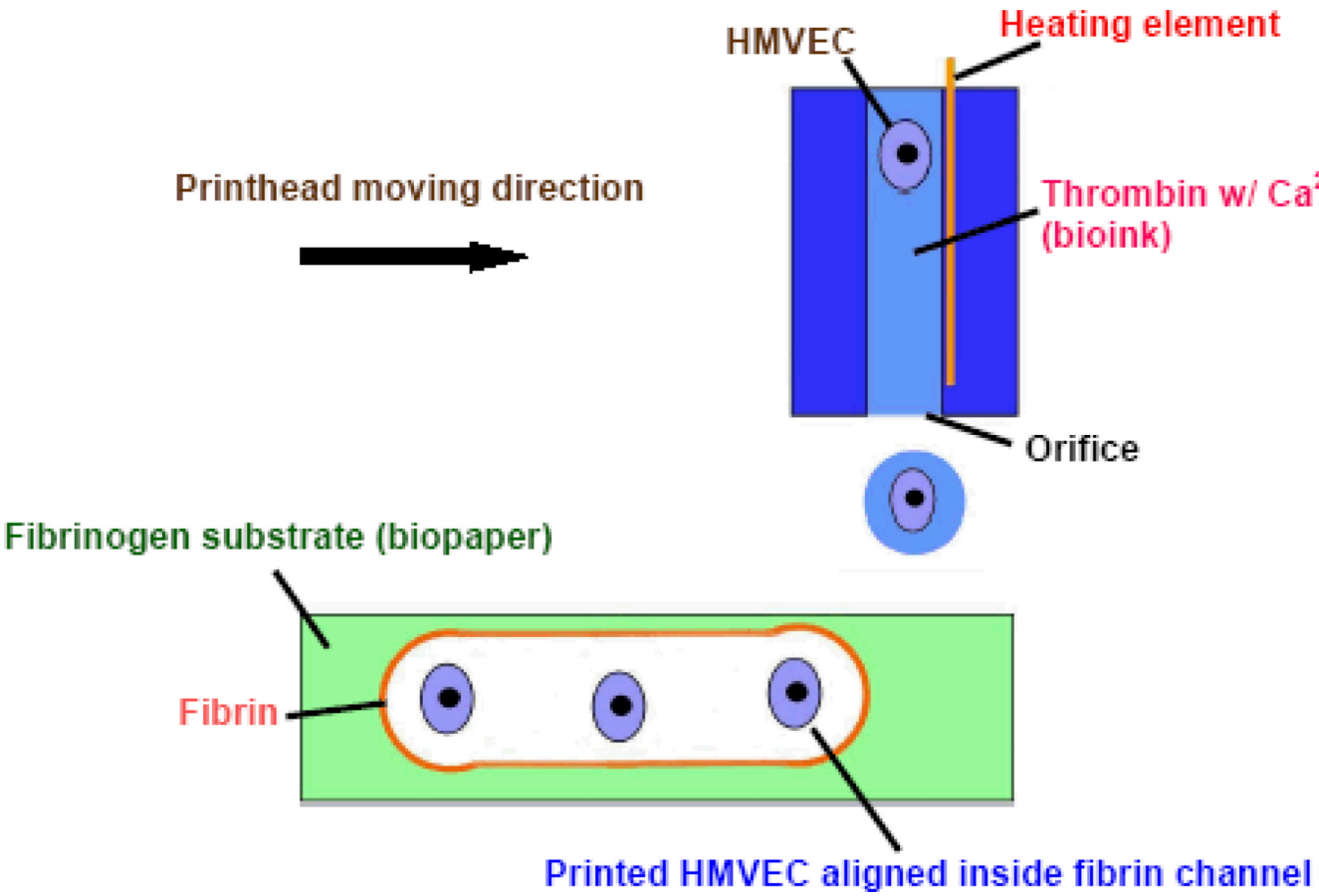

Figure 2.
Schematic drawing of postulated mechanism for simultaneous deposition of HMVEC and fibrin channel scaffold using modified thermal inkjet printer. When bioink is printed into fibrinogen substrate to form fibrin channel, the cells in the bioink are deposited into the scaffold at the same time as the fibrin channel fabrication. The printed cells are aligned inside the fibrin channels and ready for proliferation (adapted from [58]).