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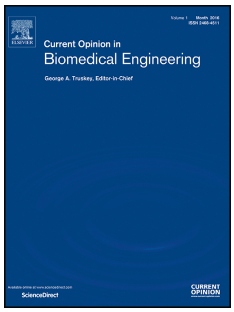

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Current developments in 3D bioprinting for tissue engineering

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Abstract

The field of 3-dimensional (3D) bioprinting have enjoyed rapid development in the past few years for the applications in tissue engineering and regenerative medicine. In this review, we summarize the most updated developments in 3D bioprinting for the applications in the tissue engineering with a focus on the printable biomaterials used as bioinks. These developments include 1) novel printing regimes have been enabled by the use of fugitive inks for the creation of intricate structures e.g. vascularized tissue constructs; 2) mechanical strength of printed constructs can be enhanced by co-printing soft and hard biomaterials; 3) bioprinted *in-vitro* models for drug testing applications are closer to reality. We conclude that the research and application of new bioinks will remain the key highlights of the future developments in 3D bioprinting for tissue engineering.

Keywords: Bioprinting, Bioinks, Vascularized tissue, Mechanical Properties, In-vitro models, Drug Testing.

Highlights

- **Novel printing regimes have been developed with fugitive inks to create vascularized tissue constructs.**
- **New developments in printable biomaterials have been focused on fine-tuning their mechanical properties.**
- **Bioprinted *in-vitro* tissue models are closer to reality.**

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Introduction

The field of 3-dimensional (3D) bioprinting have enjoyed rapid development in the past few years for the applications in tissue engineering and regenerative medicine. The rapid developments have been fuelled by the new bioprinting technologies, bioprinters, novel printable biomaterials or bioinks and exciting applications for in-vitro models or transplantable tissues. We believe the new developments in the bioinks have been the key to many recent achievements. Therefore, this article aims to review the most recent developments in the field of 3D bioprinting for the applications in tissue engineering with a focus on printable biomaterials.

Novel Printing Regimes

Fugitive inks

Some research groups are using a different type of hydrogel material "fugitive inks" along with standard bio-inks to support their bioprinted structures. After the printing is completed, the fugitive inks are transformed from a gel to a liquid by simply altering the temperature and then the resulting liquid can be drained away leaving the bioprinted structure behind. Fugitive inks are used in two ways, either around the structure (in the form of a supporting bath) to support its creation in free space [1–3] or inside the structure to enable the creation of internal channels [4].

Fugitive support baths

There have been some interesting improvements made to the technique of printing complex structures within a hydrogel reservoir to provide support. Previously when printing within a hydrogel, filler material is required to restore the resulting voids and crevasses left by the nozzle as it travels through the hydrogel material [1]. However, a new technique called Freeform Reversible Embedding of Suspended Hydrogels (FRESH) [2], or more simply Freeform Reversible Embedding (FRE) [3], has been developed which uses a supporting bath composed of a material that exhibits a Bingham plastic rheology, flowing as a viscous fluid at high shear stresses but behaving as a rigid body at lower shear stresses. Due to this property of the bath, the syringe nozzle can travel through the supporting material with negligible resistance while the extruded material is supported and the geometry of the printed structure is maintained. In FRESH the supporting material is composed of a slurry of gelatin microparticles which is melted and washed away by simply raising the temperature to 37 °C when the structure is completed [2]. While FRE utilised a hydrophilic Carbopol gel to support the 3D printing of hydrophobic PDMS prepolymer resins; after the PDMS print is cured, it can be released by liquefying the Carbopol in the presence of ionic solutions such as phosphate buffered saline solution [3].

Conventionally, supporting materials have special properties to allow them to support structures during printing i.e. Bingham plastic [2], high-density hydrophobic fluorocarbons [5] or more rigid biodegradable materials are used. A novel bioprinting scheme has been developed by Ghanizadeh Tabriz et al. [6] wherein 3D cell-laden alginate structures are built up using a three-stage cross-linking process. Partially cross-linked alginate is extruded onto a porous PMMA platform which is lowered into a bath of 100mM calcium chloride solution as each layer is completed thus further cross-linking the structure before finally treating the completed structure with barium chloride in order to extend the degradation time of the hydrogel. This technique allows for the creation of cell-laden structures with overhangs that would normally not be possible using conventional extrusion and the encapsulated cells exhibit a high survival rate.

Internal channel creation via fugitive inks

Another use for fugitive inks is the creation of perfusable channels within other structures, these materials have the mechanical stability to maintain shape while the entire tissue is printed, but can be washed away at a later time, leaving perfusable channels in whatever configuration is needed [4,7,8]. The standard process creates a regular geometric network structure first via bioprinting before cast moulding the desired bulk material around it – epoxy resin for microfluidic devices or cells suspended within a hydrogel – finally the sacrificial structure is removed and the resulting perfusable channels are lined with endothelial cells [4,9].

While the use of fugitive inks to create internal channels is not entirely novel – the Lewis group has been using this technology to create microvascular-like networks in microfluidic devices since 2003 [10] – the technology is gradually developing and the complexity of these tissue-like structures is increasing from simple blocks to more complex multi-cellular structures.

Kolesky et al. [4] showed that by using this technique, they can create a thick, perfusable tissue construct. This helps to keep cells alive all throughout the construct, and more importantly, to perfuse it with growth factors that differentiate the printed Mesenchymal Stem Cells (MSCs) toward the osteogenic lineage. Lee et al. [8] showed that by not only lining the channels with endothelial cells, but also incorporating endothelial cells within the printed construct, micro-vascularisation is created between the larger channels.

New Developments in Biomaterials for 3D Bioprinting

Biomaterials traditionally have been defined as materials used in biomedical devices, made specifically not to harm organs or tissues. However, over time, biomaterials have evolved to include a variety of materials. From rigid materials, like metals and ceramics for implants, to hydrogels for drug delivery and cell encapsulation, to nanodots and quantum particles for imaging and drug

delivery. The classifications of different biomaterials is ever expanding with the vast amount of research being done in the field of tissue engineering [11,12]. In the field of 3D bioprinting, two major groups of biomaterials for 3D printing can be determined. The first being a group of rigid curing materials, used mainly as a scaffold for cells, providing mechanical support; these materials include hydroxyapatite (HA) [13], calcium phosphate [14], Poly-(ɛ-caprolactone) (PCL) [15], and others. Some of these materials are osteogenic and promote cell proliferation on their surface, making them perfect for 3D printing bone tissue [16].

The second group consists of soft materials, usually hydrogels, into which cells can be incorporated, and printed at the same time. When cells or biochemical molecules are incorporated in these materials, it is considered a bioink. This form of bioprinting is increasing in popularity, as it allows you to not only provide a 3D environment that mimics native extracellular matrix [17], it also allows for patterning of the cells [18,19].

Constructs made in this way lack the mechanical strength to be used for the tissue engineering of hard tissues, like bone or cartilage. Visser et al. [20] proposed a solution in the form of reinforced hydrogels. A PCL microfiber scaffold with a porosity of 93-98% was made using a method called melt electrospinning writing, and subsequently infused with either alginate or gelatin methacrylate (GelMA). The constructs with the cross-linked gels show a synergistically increased stiffness, compared to the gels or the microfiber scaffold alone. Using traditional Melt printing, Daly et al. [21] were also able to reinforce their hydrogels with PCL to elevate the compressive equilibrium modulus from their hydrogels into the range of articular cartilage. Another principle used to enhance the mechanical properties of hydrogels is the use of nanofibers. These can be used to enforce a hydrogel by forming an internal random network [22] or, by aligning them, increasing the tensile strength [23] or tune the matrix compressive modulus [24].

Silk is a material that seems to be getting more and more attention as a biomaterial. Silk is a natural occurring material, referring to protein fibres produced by several insects and spiders [25]. It is mainly Silk Fibroin that is being used to create hydrogels. Gelation can happen without any important secondary structural changes; intramolecular cross-linking between protein chains happens with the aid of electrostatic interaction, hydrogen bonds and hydrophobic interactions, forming strong β-sheets [26]. The gelation time can be shortened with the aid of physical changes as lowering the pH [27], increasing the Temperature [28], sonication [29] or by adding chemical crosslinking agents [30]. Silk is a good material for bioprinting, as it has good mechanical stability [25,31], and has shown to allow cells to attach and proliferate [32]. Jose et al. show that mechanical properties, such as viscosity, yield stress, and solubility can be modified with the addition of non-toxic polyols, creating more possibilities for use in printing [33]. Schacht et al. have shown that recombinant silk can be used for 3D bioprinting by letting a silk-cell mixture gel overnight before using them with extrusion printing. By incorporating the cell adhesion peptide motif RGD they were able to increase cell adhesion and proliferation [28]. The same group showed that this silk biomaterial has enough mechanical strength to print larger, more intricate structures [34].

Another subset of materials that are looking very promising for bioprinting, are synthetic self-assembling peptides. Although short peptide groups have been incorporated in other bio-inks on numerous occasions [35–37], for instance to allow cell adhesion, there aren't many groups using just peptides for bioprinting [38]. The 3D network of nanofibers created by these peptides could resemble the native ECM, providing a good environment for cells to survive and proliferate, while maintaining structural integrity [39]. Mechanical properties, as well as stimuli-responsive gelation can be completely engineered, by modulating factors like amino acid sequence, number of repeating units and final peptide concentration [38,40]. Li et al. created a 2-part bio-ink out of polypeptides and DNA [41]. The first part contains a polypeptide, conjugated with a specific DNA sequence, and the second bio-ink contains a complementary DNA-linker, both bio-inks staying liquid until mixed. By making use of a valve-based bioprinter with multiple printing heads, the bio-inks could be deposited alternately, creating a structure with mechanically tuneable strength that is completely biodegradable and doesn't impede on cell activity in any way [41].

Applications of Bioprinting

One of the strongest features of bioprinting is the fact that it is so versatile. It can be used for basic research, research in the fields of Tissue Engineering and Regenerative Medicine, and drug testing [42].

Bioprinting of the liver has some interest as it will allow testing of drugs for drug induced liver injury [43]. Nguyen et al. [44] show that by 3D bioprinting hepatocytes at a high density with endothelial and stellate cells, in an *in vivo*-like architecture, they can create tissues that were able to show the hepatotoxicity of a compound whose hepatotoxic potential could not be assessed by any other standard pre-clinical model. Bioprinting hepatic cells for toxicity can be combined with organ-on-a-chip technology [45] by printing directly onto the chip. When bioprinting is combined with conventional 3D printing methods, it is even possible to print cells on top of a 3D printed chip, creating a one-step protocol [46]. Faulkner-Jones et al. [47] provides us with promising work regarding induced pluripotent stem cells (iPSCs), as they were able to bioprint iPSCs in multilayer constructs, while maintaining their ability to differentiate into hepatocyte-like cells. This could open the doors for patient specific drug testing and stratified medicine. Ma et al. [43] also used hepatic cells derived from iPSCs in combination with supporting cells to create a patient specific hepatic model that mimics the

native architecture. In this research, they show one of the biggest strengths of 3D bioprinting: being able to determine the location of different cell types.

Another type of tissue for which bioprinting is used to create a better *in vitro* model is cancerous tissue [48]. Current two- and three-dimensional models present limitations as the complexity of tumours are not replicated and the tissues do not possess vascular networks [49]. Using Bioprinting techniques, complex interactions such as cancer cell dynamics during vascularization can be researched [50,51]. Metastasis of cancer cells is one of the biggest dangers of tumours. However, not enough biomimetic models are available for research. Zhou et al. [13] were able to research cancer metastasis into bone by 3D bioprinting a cell-laden bone matrix, providing a microenvironment that mimics native bone tissue. Using this model, they were able to research the morphology, migration and interaction with bone stromal cells of breast cancer cells. Zhao et al. [52] used 3D bioprinting to create a new 3D tumour model that showed a higher proliferation rate and a higher chemoresistance compared to the 2D model. This shows that 3D printing can help develop *in vitro* models that are closer to reality.

Apart from drug testing, a big goal for bioprinting is to create transplantable tissues [43]. However, one of the biggest challenges of whole-organ engineering is that to create clinically relevant tissue, it needs to be vascularised [53]. Therefore, it is not remarkable that using bioprinting to create blood vessels, or vascularised tissue is a hot topic of research [54]. Bioprinting vascularization can be divided in two groups: bioprinting the actual vessels [55] and bioprinting tissue with nutrient channels [54]. By building up a vessel layer by layer, it is possible to create the intricate structures of hollow, branched blood vessels [6,56] and larger self-supporting structures [57]. Hinton et al. [2] showed they can create an entire, full-size, perfusable human right-hand coronary arterial tree out of alginate using FRESH printing. However, the real challenge of bioprinting lies in the creation of larger, vascularized tissues [7]. Some research shows printing with hollow fibres [58], which can be lined with endothelial cells, to create 3D constructs that are completely perfusable [59]. Another method to create perfusable channels in constructs is by printing with fugitive inks as discussed previously.

Hard tissue fabrication also benefits from 3D bioprinting [60]. In the fabrication of bone and cartilage an important factor is the mechanical strength of the construct, as mechanical stability is an important function of these tissues [20,61,62]. Kang et al. [63] were able to print mechanically stable constructs by co-printing cell-laden hydrogels with a biodegradable polymer. At the same time, the incorporation of microchannels in the design allows nutrients and oxygen to penetrate these constructs of a clinically relevant size. Wang et al. [64] showed the possibility of using Adipose derived Stem Cells (ASCs) to differentiate *in situ* towards the osteogenic lineage as a way to use a patient's own cells as a source for the bioprinting.

Conclusions

Besides the exciting developments, challenges remain to apply 3D bioprinting for tissue engineering applications. The novel use of fugutive inks have led to the creation of intricate, cell-laden structures including thick, vascularized tissues. However, it remains challenging to create vascularized tissues that can be sustained in *in-vivo* environments. For bioprinting without fugutive inks, fine-tuning mechanical properties for printable biomaterials have been the focus of the recent research. The more established applications appear to be hard tissue engineering where bioprinted bone and cartilage constructs have been in-vivo tested. On the other hand, bioprinted soft tissues including liver and tumour tissues have been demonstrated to create in-vitro models for drug testing applications, which is closer to reality than bioprinted tissues for medical transplantion. In the past few years, many new exciting developments in the bioprinting field has been enabled by the bioinks, we therefore envisage that the new breakthough in bioinks will remain the key highlights of the future developments in 3D bioprinting for tissue engineering.

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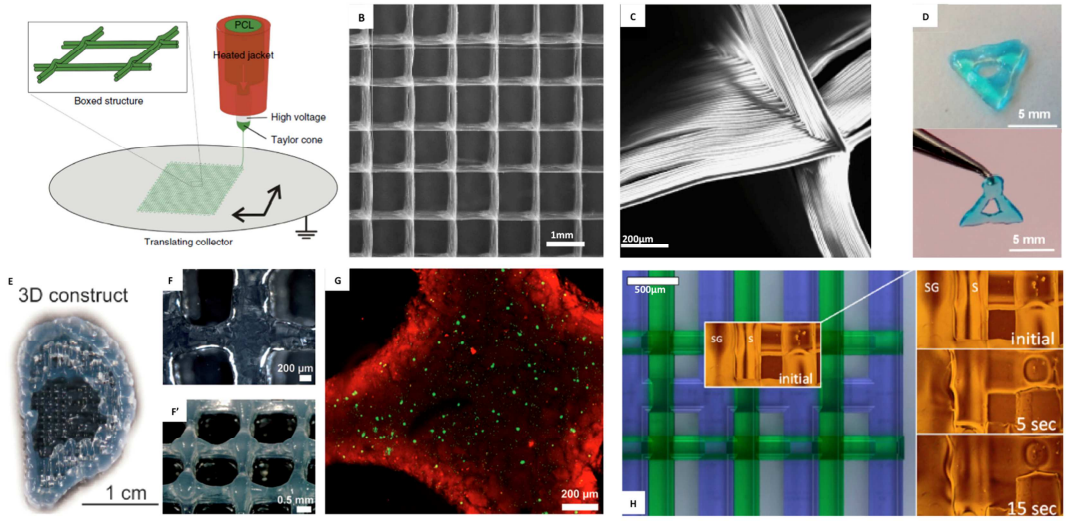

Fig. 1: Biomaterials for use in 3D bioprinting. (A-C) Highly organized microfibers of PCL are used to reinforce hydrogels for hard tissue engineering. (A)Thin PCL fibres are deposited by using melt-electrospinning in a direct writing mode. (B) fibres were stacked in a 0-90 orientation at a 1mm interval. (C) detailed image of fibres that fused at cross-sections. (D) 3D printed construct made from DNA-peptide hydrogel, strong enough to be picked up (E) Silk peptide hydrogels are mechanically strong enough to create large, intricate structures. (F) close up of cell encapsulated in silk peptide hydrogels, in 2 (F) or 8 (F') layers. A life dead staining of human fibroblasts in the gel (G) shows good cell survival. (H) Demonstration of solubility modification of silk by the addition of polyols. Blue strands were printed with insoluble Silk:Glycerol (SG) and green strands with silk (S). After the addition of water, the silk strands solve in roughly 15 seconds, leaving only the Silk:Glycerol strands.

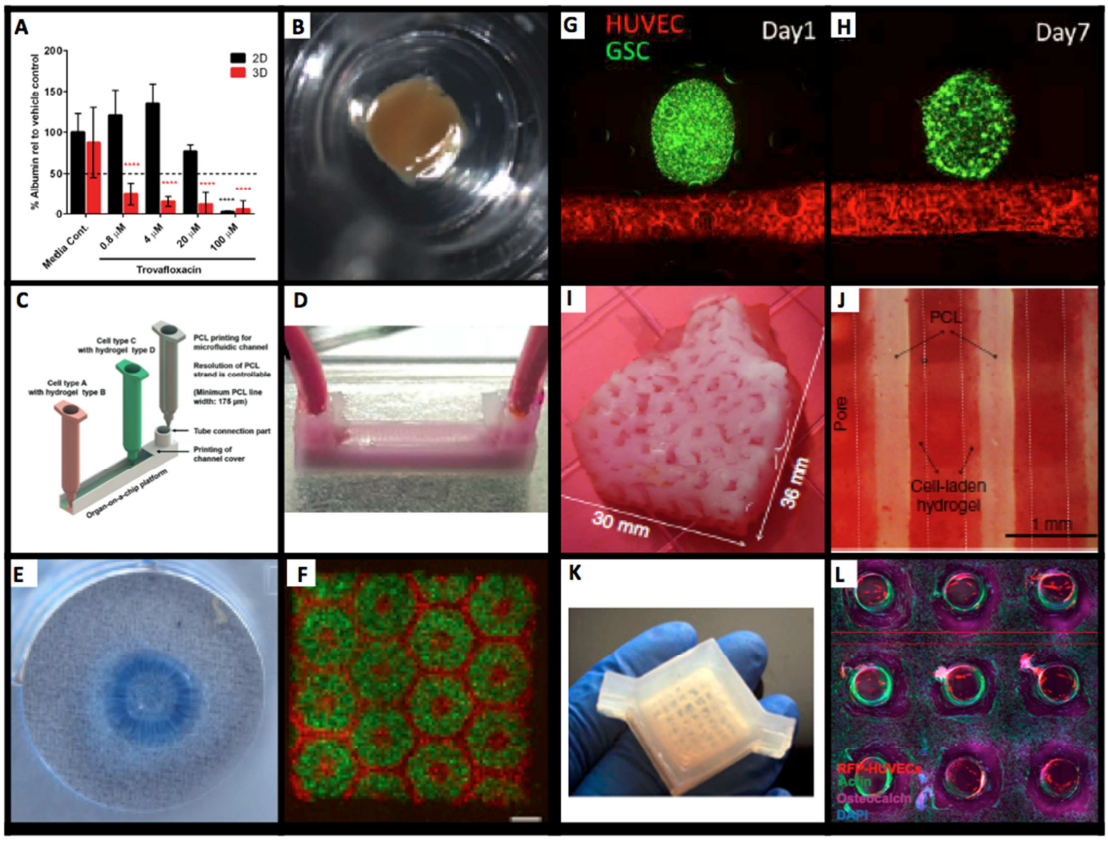

Fig. 2 Applications of bioprinting. (A,B) Bioprinted 3D Liver Tissue allows hepatotoxic effects to be discovered *in vitro*. The cytotoxic effects of trovafloxacin could be seen in concentrations as low as 0.8µM (A) using 3D bioprinted liver tissue(B), whereas a concentration of 100µM was necessary to see the effect in 2D culture. (C,D) one step fabrication of organ-on-a-chip can be realized using 3D Bioprinting. (C) A schematic overview of the bioprinting process that leads to a perfusable microchannel (D). (E,F) Induced Pluripotent Stem Cells (IPS-cells) can be used for personal drug testing. (E) A model of 3D printed alginate structures (40 layers) which were used to differentiate IPS-cells towards the hepatic lineage in 3D. IPS-derived hepatocyte-like cells(green) can very precisely be patterned with supporting cells (red) in a physiological relevant pattern. (G,H) Bioprinting has allowed us to investigate the cell-cell interaction between glioblastoma cells (Green) and endothelial cells (red). Over time, the glioblastoma cells proliferated and migrated towards a perfused channel, lined with endothelial cells. (I,J) Hard Tissue printing allows us to create structural stable constructs with clinically relevant sizes. (I) A 3D printed mandible bone defect construct, based on human CT image data. Cells printed in the construct were able to undergo osteogenic differentiation, as shown by the Alizarin red S staining (J). (K,L) By printing with fugitive materials, channels can be created in bioprinted constructs, which allows perfusion of bigger constructs. (K) A thick construct was perfused with medium and growth factors through micro-channels (L), allowing for in situ osteogenic differentiation of human Mesenchymal Stem Cells. Micro-channels were lined with Human Umbilical Vein Endothelial Cells (HUVECs, red). Osteocalcin was stained in purple, nuclei in blue using DAPI and actin in green.