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Review article

Three-dimensional bioprinting is not only about cell-laden structures

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ABSTRACT

In this review, we focused on a few obstacles that hinder three-dimensional (3D) bioprinting process in tissue engineering. One of the obstacles is the bioinks used to deliver cells. Hydrogels are the most widely used bioink materials; however, they are mechanically weak in nature and cannot meet the requirements for supporting structures, especially when the tissues, such as cartilage, require extracellular matrix to be mechanically strong. Secondly and more importantly, tissue regeneration is not only about building all the components in a way that mimics the structures of living tissues, but also about how to make the constructs function normally in the long term. One of the key issues is sufficient nutrient and oxygen supply to the engineered living constructs. The other is to coordinate the interplays between cells, bioactive agents and extracellular matrix in a natural way. This article reviews the approaches to improve the mechanical strength of hydrogels and their suitability for 3D bioprinting; moreover, the key issues of multiple cell lines coprinting with multiple growth factors, vascularization within engineered living constructs etc. were also reviewed.

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Introduction

Three-dimensional (3D) bioprinting has been on the spotlight recently due to its potential to deliver cells, biomaterials and bioactive agents to precise locations and form living structures. It is also known as cell-laden structures in a layer by layer fashion.^{1,2} To date, this technique has succeeded in fabricating tissues, such as bones, skins,³ complex mini tissues like liver and heart,⁴ even as a tool to study cell biology.⁵

The common bioprinting systems are based on ink jetting, extrusion and laser-induced printing. In ink jet printing, structures with precise control are limited due to low concentrations of bioinks. Laser-induced printing requires rapid gelation of hydrogels; therefore, the materials are limited. Extrusion-based 3D bioprinting is the most common system in fabricating living constructs.⁶

In 3D bioprinting, hydrogels not only serve as bioinks to deliver cells or support cell growth, but also provide cells with access to oxygen and nutrient which are essential for differentiation and

proliferation. Therefore, the hydrogels in 3D printing should possess a number of characteristics, namely, 1) porous structures that allow filtration of oxygen and nutrition. When the engineered constructs is thicker than 1 mm, oxygen and nutrients are difficult to perfuse into the construct, which may result in cell death; 2) mechanical support; 3) biocompatible; and 4) printable properties, such as adequate viscosity and shear thinning properties. Hydrogels are high molecular crosslinked structures suitable for cell growth and proliferation; however, they are weak in nature. Several methods have been utilized to achieve better elasticity and stiffness, such as combination with other materials, incorporation with inorganic nano-particles, multiple crosslinking methods, or with supporting (reinforcement) structures.^{7–10}

Tissue engineering is not only about cell-laden structures. During the process of cells growing into functional tissues, bioactive agents play an important role in cell differentiation and proliferation. There have been several attempts to deliver bioactive agents: 1) incorporation into scaffolds with controlled release profile, such as coatings⁷; 2) de-cellular components with growth factors²; and 3) active controlled release by microchannels.^{11–13}

In order to regenerate living constructs, which have the scale of human tissues, vascularization is the key step for cell-laden structures to survive in the long term, especially for thick tissues that

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normally span over 1 mm where oxygen and nutrition is difficult to guaranteed.¹¹ There have been a number of studies on creating vascular networks within the constructs,¹² with significant contribution by microfluidics platform.^{11,13}

Several cell types have been corprinted to mimic human tissues. However, engineered constructs of living tissues is an interdisciplinary area of research, which requires the advances from materials, engineering and biology as a whole. There is still a long way from complete organ printing. However, multi-disciplinary tissue engineering offers the potential to build functional tissues outside the body.

In this review, the approaches that build hydrogels with tailored mechanical strength and printability for 3D bioprinting were first discussed; then key issues, such as coprinting and coculturing of multiple cell lines, delivering of bioactive agents, vascularization network within living constructs, which are essential for a 3D bioprinted live construct to function normally in the long term, were also discussed in detail.

Tuned mechanical strength of hydrogels

Hydrogels can be formed using natural or synthesized materials by chemical, physical or biological crosslinking methods. For example, collagen can be chemically crosslinked by covalent bonding agents, which bind either free amine or carboxyl groups of collagen, or can be bound by dehydrothermal treatment (DHT) or UV irradiation, or biologically by transglutaminase. Each of these methods has demonstrated different degrees of mechanical strength which depends on the mechanism, concentration and exposure time. However, the mechanical strength by these methods is weak. Furthermore, hydrogels in 3D bioprinting are required to gel at a relatively fast speed in order to achieve high printing resolution. Although alginate hydrogels can be formed by ionic crosslinking with relatively fast gelation time, it is not an ideal biomaterial to fabricate living constructs due to its inadequate degradability *in vivo*. UV crosslinking has shown relatively fast gelation time, which may be promising for 3D bioprinting.^{14–17}

There are several approaches to improve mechanical strength; the crosslinking methods, gelation time, mechanical strength, cell viability and models in 3D bioprinting are summarized in Table 1.

Multiple materials/multistage crosslinking methods

Combination of covalent crosslinking method by chemical reagents or UV (or known as photocrosslinking) or other crosslinking method has been applied to form hydrogels.^{16–18} Skardal et al¹⁵ formed a hydrogel using thiol-modified HA/gelatin crosslinked with PEGDA before printing and 8-arm PEG alkynes with UV crosslinking method during 3D printing. The shear elastic modulus G' was increased from 0.1 kPa to 15–20 kPa. Human liver spheroids in the diameter of 250–350 μm were generated, and the albumin production from the liver constructs increased significantly from day 3 to day 10 from approximately 40 ng/ml to 80 ng/ml, but remained stable for the remaining days in culture. Das et al¹⁸ coupled silk-gelatin crosslinked with mushroom tyrosinase and ultrasound crosslinking afterwards *in situ* to study the differentiation of MSCs.

Duan et al⁴ developed photocrosslinkable hydrogel formulations based on methacrylated HA (here referred to as MA-HA) and GelMA to print heart valve conduits encapsulating human aortic valvular interstitial cells. The most promising polymer formulation (4% MA-HA/10% GelMA containing the photoinitiator of I2959) regarding to matrix stiffness, viscosity, cell spreading and printing accuracy was printed into a receiving platform to

produce a 3D cellular trileaflet heart valve model. After photocrosslinking with UV light, the constructs maintained structural integrity and supported high cell viability of 92.1% for up to 7 days of *in vitro* culture. In their work, it was found that higher polymer concentration of GelMA reduced the compressive modulus of the hybrid hydrogels due to high viscosity of the hydrogel that hindered the photocrosslinking process; however, the optimized compressive modulus was only about 13 kPa. Kesti et al¹⁹ blended the thermoresponsive polymer poly (*N*-isopropylacrylamide)-grafted hyaluronan (HA-pNIPAAm) with methacrylated hyaluronan (HAMA), and high-resolution scaffolds with good viability were printed. HA-pNIPAAm provided fast gelation and immediate post-printing structural fidelity, while HAMA ensured long-term mechanical stability upon photocrosslinking. Cooper et al²⁰ implemented tissue-penetrating double network and successfully restored the mechanical properties of degenerated articular cartilage *in situ*. This work shed the light in hydrogel-based cartilage repair.

Photoinitiators were also under intensive study to increase cell viability and biocompatibility.^{21–23} Billiet et al²¹ replaced the commonly used photoinitiator I2959 with VA-086. The viability of hepatocarcinoma cell line (HepG2) was 98% at printing pressure of 0.5 bar. Albumin, HNF4a, Ki67 and proliferating cell nuclear antigen (PCNA) expression was confirmed.

Supporting structures and reinforcement

Mechanical strength of hydrogels might be improved by polymer concentration, crosslinking density or the abovementioned multiple crosslinking methods, which, however might reduce the biological performance of the hydrogels. Supporting structures and reinforcement may be other options to improve the mechanical properties of scaffold. Strands made of synthetic polymers such as polycaprolactone (PCL) has been used as supporting structures.^{7,24,25} Cell-laden hierarchical scaffolds consisting of micro-sized PCL and electrospun PCL nanofibers/cell-laden alginate struts are created for tissue regeneration (as shown in Fig. 1).²⁴ PCL strands with much smaller size than that made of Fused Deposition Method (FDM) were produced using near field direct writing. It was found that PCL porosity and GelMA crosslinking degree has strong effects on the stiffness of the composites. The stiffness is increased up to 50 thresholds due to synergistic effects.⁷ Boere et al²⁶ developed a thermoplastic polymer blend of poly(hydroxymethylglycolide-co- ϵ -caprolactone)/poly(ϵ -caprolactone) (pHMGCL/PCL) functionalized with methacrylate groups and covalently grafted to GelMA hydrogel through photo polymerization. The grafting resulted in an at least fivefold increase in interface-binding strength between the hydrogel and thermoplastic polymer material.

A factor was introduced to evaluate the reinforcement effect of hydrogels.²⁷

$$A = \frac{E_c}{E_g} \quad (1)$$

where E_c and E_g are the compressive Young's modulus of the composite and the gel matrix, respectively. A is, therefore, a simple normalization of the reinforced modulus.

Visser et al⁷ gave a more comprehensive model to evaluate the fiber reinforcement of hydrogels and made a prediction for the construct stiffness, C .

$$C = \frac{\rho^2 E_n f}{2R^2 (1 - \lambda)^{3/2}} \quad (2)$$

Table 1
Literature review of living constructs by 3D bioprinting.

Materials	Crosslinking	Mechanical strength	Printing system and parameters	Cell viability	Application
Thiol-HA and Gelatin ¹⁵	Two stages: PEGDA (30 min) and PEG-alkyne UV crosslinking (2–4 s)	Shear elastic modulus G' of 15–20 kPa (similar to native liver tissue)	In-house design; Extrusion-based bioprinting with 20–30 gauge needle	Albumin and urea productions over 14 d were confirmed	Liver spheroids
Nanocellulose-Alginate ²⁸	Ionicly-crosslinked (10 min)	Compress stiffness of 70–240 kPa	Microvalve dispenser; Needle size of 300 μm ; dispensing pressure of 20–60 kPa; dosing distance of 50–70 μm	Human nasoseptal chondrocytes; Cell viability was 72.8% at 1 d after printing	Cartilage
Silk Gelatin ¹⁸	Tyrosinase and sonification	Unspecified	Height of each layer and printing temperature were optimized; Temperature of the syringe barrel was maintained at 28 °C and the printing chamber was maintained at 18 °C	Stem cells chondrocytes and osteocytes density was 2×10^6 – 5×10^6 cells/ml; Cell viability was 96% at 1 d after printing and 87% upto 30 d	Bone and cartilage
Alginate ⁴¹	Ionicly-crosslinked with CaCl_2 and barium chloride	Stiffness of 20 kPa	Nozzle ID of 0.33–0.45 mm; Printing speed of 0.45 ml/min and 0.65 ml/min	U87-MG cells at 2×10^6 cells/ml; Cell viabilities were 61.5%–93% at different crosslinking densities (88% at 1 d)	Unspecified
Gelatin methacrylamide ²¹	Photocrosslinking with VA-086 (UV-A light, 365 nm, 4 mW/cm ²)	Unspecified	Pressure at 0.5–4 bar	Hepatocarcinoma cell line; Viability was 98% at printing pressure of 0.5 bar; Albumin, HNF4a, Ki67 and PCNA expression was confirmed.	Liver
GelMA PEGDA ¹⁴	UV crosslinking GelMA and PEGDA 4.8 mW/cm ² (2 min)	Elastic modulus: 5 kPa	Digital micromirror device projection printing	Fibroblasts; Viability was 80% after 10 d	Unspecified
Alginate/HA ⁴²	Ionicly-crosslinked with EDC	Unspecified	Envision TEC 3D-bioplotter	Schwann cells	Unspecified
PU NPs PCL based ⁸	Thermal response amphiphilic PU gel	Young's modulus: 15–157 MPa	The syringe diameter was 260 μm ; gas pressure was 241–275 kPa, and the volume flow rate was 1.67 $\mu\text{l}/\text{min}$	MSCs; Viability was 100% after printing and 40% after 1 d	Unspecified
PU NPs PCL based ³¹	Thermal response at 37°	G' : 680–2400 Pa	Nozzle of 250 μm and pressure of 55 kPa; In house build printing system	Neural stem cells	Unspecified
Collagen ³³	Crosslinked with NaHCO_3	Unspecified	Droplet dispersion pressure at 1–3 psi; In house microvalve system	Keratinocytes and Fibroblasts	Skin
PEGX-gelatin PEGX-Fibrin ¹⁶	Crosslinked with EDC/NHS and UV light at the intensity of 15–20 mW/cm ² (10 min)	G' : 4–23 Pa G'' : 4–105 Pa	Diameter nozzles of 200 μm ; Envision TEC 3D-bioplotter; Pressure of 1–2.5 bar; Speed of 5 mm/s	Human umbilical vein endothelial cells, Human dermal fibroblasts and Human mesenchymal stem cells	Tissue and organ mimics
GelMA/Melt espining PCL ⁷	GelMA APS/TENED	Stiffness of hydrogel: 7.1–15.8 kPa, after incorporated with PCL: 405 kPa	PCL was extruded at a rate of 18 $\mu\text{l}/\text{h}$; Electrostatic field of 8–10 kV between the syringe needle tip (23G); BioScaffolder system	Chondrocytes cell viability was 82% at 1 d and 75% at 7 d	Cartilage
Glycosaminoglycans (GAGs) ²⁰	Ethylene glycol dimethacrylate as crosslinker pre-crosslinked; Photoinitiating system composed of eosin Y, triethanolamine and N-vinylpyrrolidone (visible laser light: 514 nm, 500 mW/cm ²)	Compressive stiffness: 0.78 MPa	Cylindrical bovine osteochondral explants (7 mm in diameter)	Articular cartilage	Cartilage

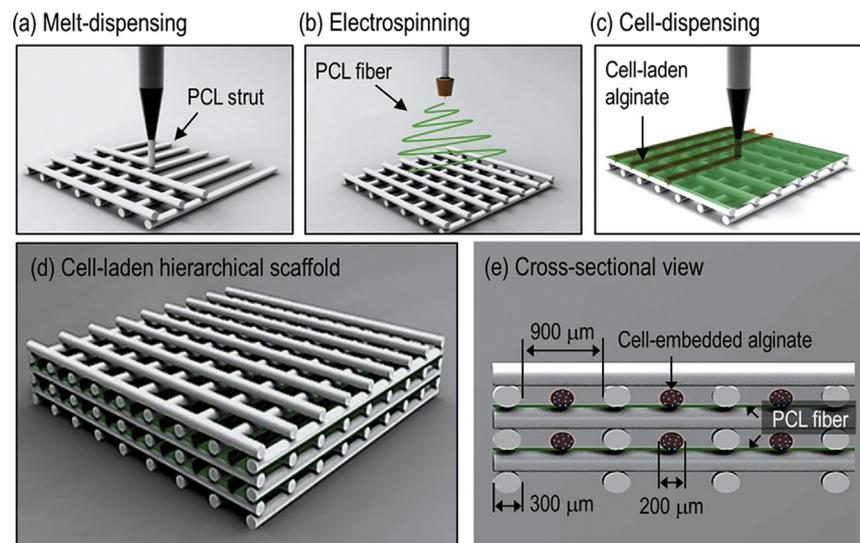


Fig. 1. PCL strands support cell-embedded alginate constructs. Figure has been reproduced with permission from Yeo.²⁴

where N_f denotes the number of fibers in the construct, E the Young's modulus of the reinforcing polymer, ρ the fiber radius, R the construct radius and λ the axial strain (expressed as a fraction of the initial height).

Moreover, hydrogels have been reinforced by nanoparticles, fibers, nano-whiskers to improve its mechanical strength.^{10,28–30} It was shown that 20% of nano-tempo-oxidized bacterial cellulose (TOBC) could increase the compressed modulus by 43%, which was about 162 kPa.²⁹ The CNT-GelMA hybrids were also photopatternable, allowing for easy fabrication of microscale structures without harsh processes.¹⁰ NIH-3T3 cells and human mesenchymal stem cells (hMSCs) readily spread and proliferated after encapsulation in CNT-GelMA hybrid microgels. By controlling the amount of CNTs incorporated into the GelMA hydrogel system, the mechanical properties of the hybrid material can be tuned, making it suitable for various tissue engineering applications.

Thermal response synthetic nanoparticles hydrogels as bioinks

Thermal-responded hydrogel can be printed at higher temperature with relatively low viscosity and solidified at 37 °C. Waterborne polyurethane (PU) nanoparticles (NPs) or dispersions based on PCL were synthesized by two-step chemical reaction specially for 3D printing.⁸ The PU NPs have relative low viscosity during printing process which can reduce cell damage and have a relatively high Young's module of 157 Mpa; however, the swelling ratio was larger than 200%. The viability of MSCs after one day of bioprinting was around 40%. The authors claimed that the different cell survival and cell growth rates were more likely due to the stiffness rather than swelling behavior of the hydrogels. Hsieh et al.³¹ has further improved the swelling of PUs based on poly(ϵ -caprolactone) diol (PCL diol), and poly(L-lactide) diol (PLLA diol) and poly(D, L-lactide) diol (PDLLA diol). The swelling ratio of these gels was less than 10%. Neural stem cells cultured in these gels showed that PUs based on PDLLA diol were more likely to support cell growth and proliferation with the cell surviving rate of around 70% after one day of printing.

Delivery of bioactive agents

Decellularized tissue matrix contains natural growth factors which is essential for cell growth and proliferation.^{15,32} A numerical tissue-specific factors, such as bFGF, BMP, VEGF, derived from decellularized tissue extracellular matrix, were incorporated into hydrolic acid (HA) and gelatin liver specific hydrogels¹⁵ while making living constructs by 3D printing. The results showed higher cell viabilities compared with gelatin-based hydrogels. Hoch et al.³² employed decellularized extracellular matrices (DMs) with alginate hydrogels and implanted them into a subcutaneous ectopic site, which showed that the persistence of mesenchymal stem cells (MSCs) increased 5-fold, vessel density increased 3-fold, and bone formation was 2-fold more than that of MSCs delivered without DMs. These results underscore the need for deploying MSCs using biomaterial platforms such as DMs to preserve the *in vitro*-acquired mineral-producing phenotype and accelerate the bone repair process.

Multiple cell lines

Lee et al.³³ created multi-layered engineered tissue composites consisting of human skin fibroblasts and keratinocytes which mimic skin layers by 3D bioprinting technique. The process was repeated in layer-by-layer fashion on a planar tissue culture dish, resulting in two distinct cell layers of inner fibroblasts and outer

keratinocytes, and multi-layered cell–hydrogel composites on a non-planar surface were also produced and cultured for potential applications including skin wound repair. PEGX–fibrinogen and PEGX–gelatin were successfully corprinted to demonstrate the ability to spatially organize multiple types of ECM within one 3D construct.¹⁶ hMSCs were seeded onto HUVEC-laden constructs. The addition of hMSCs also slowed the degradation of the gel by 2 d compared with HUVEC-laden constructs without hMSCs. Although the gel degraded, hMSCs maintained a grid-like pattern after 2 weeks and surprisingly, the cells deposited sufficient matrix that led to a robust and opaque skin-like tissue that was lifted and handled with forceps. This might also pave the way to study cell–cell signaling in 3D bioprinted living constructs.

Vascularization

Despite tremendous progress in fabricating complex tissue constructs in the past few years, approaches for controlled vascularization within hydrogel-based engineered tissue constructs have remained limited and yet hinder large-scale tissue regeneration. Most of the vascularization networks within cell-laden structures were made of water soluble thermoplastic materials, such as sugar, poly(vinyl alcohol) (PVA), etc, as sacrificial microchannels.^{25,34–36} Vascular network in fibrin gel was formed using 3D printed sugar as a sacrificial microchannel material.³⁷ A versatile sacrificial molding technique enabling the fabrication of bulk, cell-laden and porous scaffolds with embedded branched fluidic networks were reported by Tocchio et al.³⁶ Bertassoni et al.¹³ successfully embedded functional and perfusable microchannels inside a number of widely used hydrogels, such as methacrylated gelatin (GelMA), star poly(ethylene glycol-co-lactide) acrylate (SPELA), poly(ethylene glycol) dimethacrylate (PEGDMA) and poly(ethylene glycol) diacrylate (PEGDA). In particular, GelMA hydrogels were used as a model to demonstrate the functionality of the fabricated vascular networks in improving mass transport, cellular viability and differentiation within the cell-laden tissue constructs. In addition, successful formation of endothelial monolayers within the fabricated channels was confirmed.

3D bioprinting combined with microfluidic technique bears the great potential for the future off-the-shelf engineering of thick and complex tissues with a fully functional vasculature.³⁸ Kolesky et al.³⁹ created prevascularized tissue constructs by combined bioprinting of microvessels, multiple cell types and extracellular matrix. Kang et al.²⁵ fabricated human-scale tissue constructs incorporated with microchannels that facilitate perfusion of nutrients to printed cells, hereby to overcome the diffusion limit of 100–200 μm for cell survival in engineered tissues using an Integrated Tissue–Organ Printer (ITOP). A facile approach to fabricate branched microfluidic network with circular cross-sections in gelatin hydrogels by combining micromolding and enzymatically-crosslinking method was reported by He et al.⁴⁰ Their work might provide a simple way to fabricate circular microfluidic network in biologically-relevant hydrogels to advance various applications of *in vitro* tissue models, organ-on-a-chip systems and vascularization studies.

Challenges

The viscosity of bioinks should be in a range that allows them to be printable and structurally stable; however, it is difficult to obtain a homogeneous distribution of cells within a viscous hydrogel and subsequently into the scaffolds; therefore, how to distribute cells within the hydrogels without damaging cells

should be considered. Printing with high precision is important in 3D bioprinting to achieve accurate spatial distribution of materials and cells; therefore, swelling behavior of hydrogels needs to be closely controlled in order to obtain structures with high resolution. Long term effects of 3D bioprinting and crosslinking process on cell differentiation and proliferation, and more importantly, the functioning of living constructs need to be evaluated. The stability of a vascularization network within hydrogels also needs evaluation for the thick engineered tissues to survive a long time.

Conclusions

3D bioprinting is promising in tissue regeneration and repair; however, tissue engineering is not only about structurally mimicking living tissues, but also the living constructs with normal functioning in the long term. There are a number of obstacles in 3D bioprinting technique: bioinks to deliver cells and form stable structures with adequate mechanical and biological properties; vascularization within the living structures to supply oxygen and nutrient for human-size constructs; successful coprinting and co-culture of multiple-cell lines; satisfaction of the coordinate interplays between cells, scaffolds and bioactive agents. In this review, hydrogel reinforcement methods are given in detail. Microfiber reinforcement and multi-crosslinking methods are robust in improving mechanical strength of hydrogels. Photocrosslinking and thermoresponsive offer fast gelation, which are required by 3D printing process. Microfluidics coupled with 3D bioprinting show promising results in creating vascularization networks within living constructs. Multiple cell line co-culturing might play a key role in successful tissue regeneration with normal functions in the long term.

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