Chinese Journal of Traumatology 19 (2016) 187–192

Contents lists available at ScienceDirect

Chinese Journal of Traumatology

journal homepage: http://www.elsevier.com/locate/CJTEE

Review article

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

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ARTICLE INFO

Article history:
Received 26 April 2016
Received in revised form
16 June 2016
Accepted 20 June 2016
Available online 16 July 2016

Keywords:
3D bioprinting
Hydrogels
Vascularization
Multiple cell lines

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.

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 coatings[7]; 2) de-cellular components with growth factors[2]; 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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Peer review under responsibility of Daping Hospital and the Research Institute of Surgery of the Third Military Medical University.

http://dx.doi.org/10.1016/j.cjtee.2016.06.007

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 ironic 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 regents or UV (or known as photocrosslinking) or other crosslinking method has been applied to form hydrogels.[16–18] Skardal et al[15] 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 al[18] coupled silk-gelatin crosslinked with mushroom tyrosinase and ultrasound crosslinking afterwards *in situ* to study the differentiation of MSCs.

Duan et al[4] 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 modules was only about 13 kPa. Kesti et al[19] 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 al[20] 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 al[21] 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 al[26] developed a thermoplastic polymer blend of poly(hydroxymethylglycolide-co-e-caprolactone)/poly(e-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]

$$A = \frac{Ec}{Eg} \tag{1}$$

where *Ec* and *Eg* 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 al[7] gave a more comprehensive model to evaluate the fiber reinforcement of hydrogels and made a prediction for the construct stiffness, *C*.

$$C = \frac{\rho^2 E N_f}{2R^2(1-\lambda)^{3/2}}. \tag{2}$$