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Bio-ink Materials for 3D Bio-printing

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3D printing is also known as additive manufacturing technique in which has been used in various commercial fields such as engineering, art, education, and medicine. The applications such as fabrication of tissues and organs, implants, drug delivery, creation surgical models using 3D printer in medical field are expanding. Recently, 3D printing has been developing for produce biomimetic 3D structure using biomaterials containing living cells and that is commonly called "3D bio-printing". The 3D bio-printing technologies are usually classified four upon printing methods: Laser-assisted printing, Inkjet, extrusion, and stereolithography. In the bio-printing, bio-inks (combined hydrogels and living cells) are as important components as bio-printing technologies. The presence of various types of bioinks, however, in this review, we focused on the bio-inks which enables bioprinting efficacy using hydrogels with living cells.

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Introduction

Tissue engineering and regenerative medicine has been highlighted for regeneration of tissues or organs to replace or repair damaged organs and tissues. To achieve the goal of tissue engineering, scientists defined three major technological components composing tissue engineering, which are cells, signaling molecules and scaffolds. Until now, various approaches have been explored to restore structures and functions of *in vivo* tissues and organs. Tissue engineering products requires various technological backgrounds such as life science, medical science, material science and mechanical engineering to take advantages of those major three components (3, 4).

Recently, among the three essential components, scaffold fabrication is profoundly affected by the new technology, additive manufacturing, in other words, 3D printing (1, 2). In addition,

3D printing technology can also be combined with cell seeding processes. Various types of cells now can be printed with three dimensional, locational accuracy. to have spatially tailored manner. Three dimensional control of cell location is one of the major advantages of 3D printing. In clinical point of view, damaged tissues are usually irregular in shape and many different types of cells reside in the tissues (5, 6). Tissues composed of single cells are rare, and most of the tissues have blood vessels, connective tissue components and other functional components. Further, blood vessels have endothelial layer, media, and adventitia in which different cell types are distributed. And connective tissues are composed of different types of cells for each specific tissue function. Conventional cell seeding techniques have been limited success in three dimensional control of cell location. In contrast, 3D cell printing (hereafter we call "bioprinting") has high spatial control mechanism with elec-

tronic and mechanical accuracy, can allocate cells in the programmed position. With the advantage of spatial accuracy, 3D printing also has the powerful function of locating extracellular matrix (ECM) materials at the predetermined position (7-9).

In the bio-printing, bio-inks are as important components as bio-printing technologies. The bio-ink is emerged as the use of ink-jet printing, including polymer and hydrogel for scaffolds, growth factors, cells in tissue engineering. In this review, we focus the hydrogel for scaffold (10), capable to provide cellular microenvironment (11, 12) and building blocks for 3D bio-printing (13). The hydrogels are the polymeric materials derived from naturally or synthetically, capable of embedding water in their three-dimensional network. Hydrogels is considered candidate for engineered tissue structure due to their compositional and structural similarities to the natural extracellular matrix. The key functions of hydrogel are deliver the embedded cells to the desired position in the 3D structure, promote cellular reactivity compared to other polymeric scaffold, and permit transport of nutrients and growth factors to cellular proliferation and differentiation. In this review, we discuss the

necessary properties of the hydrogels performing as bio-inks, and the principles of the bio-printing methods. In this review, 3D printing technology and bioink materials for bioprinting will be discussed for the ‘more useful’ outputs in tissue engineering and regenerative medicine (14).

Bioprinting Technologies

Laser-assisted bio-printing

Laser-assisted bio-printing method (LaBP) is based on the concept of laser-induced forward transfer (LIFT), and is a precise technology using laser (15). LaBP is consists of two layers. Upper glass slide named donor layer that is a glass cover with an energy absorbing layer and a layer of biological materials containing cells (16). The laser absorbing layer is received the pulsed laser and transferred heat so generated high gas pressure. Consequently, hydrogel precursor with cell ejected toward lower glass slide named collector layer (16) (Fig. 1A). LaBP enables printing the hydrogels with a wide range of viscosity (1–300 mPa/s). This printing method does not have negative

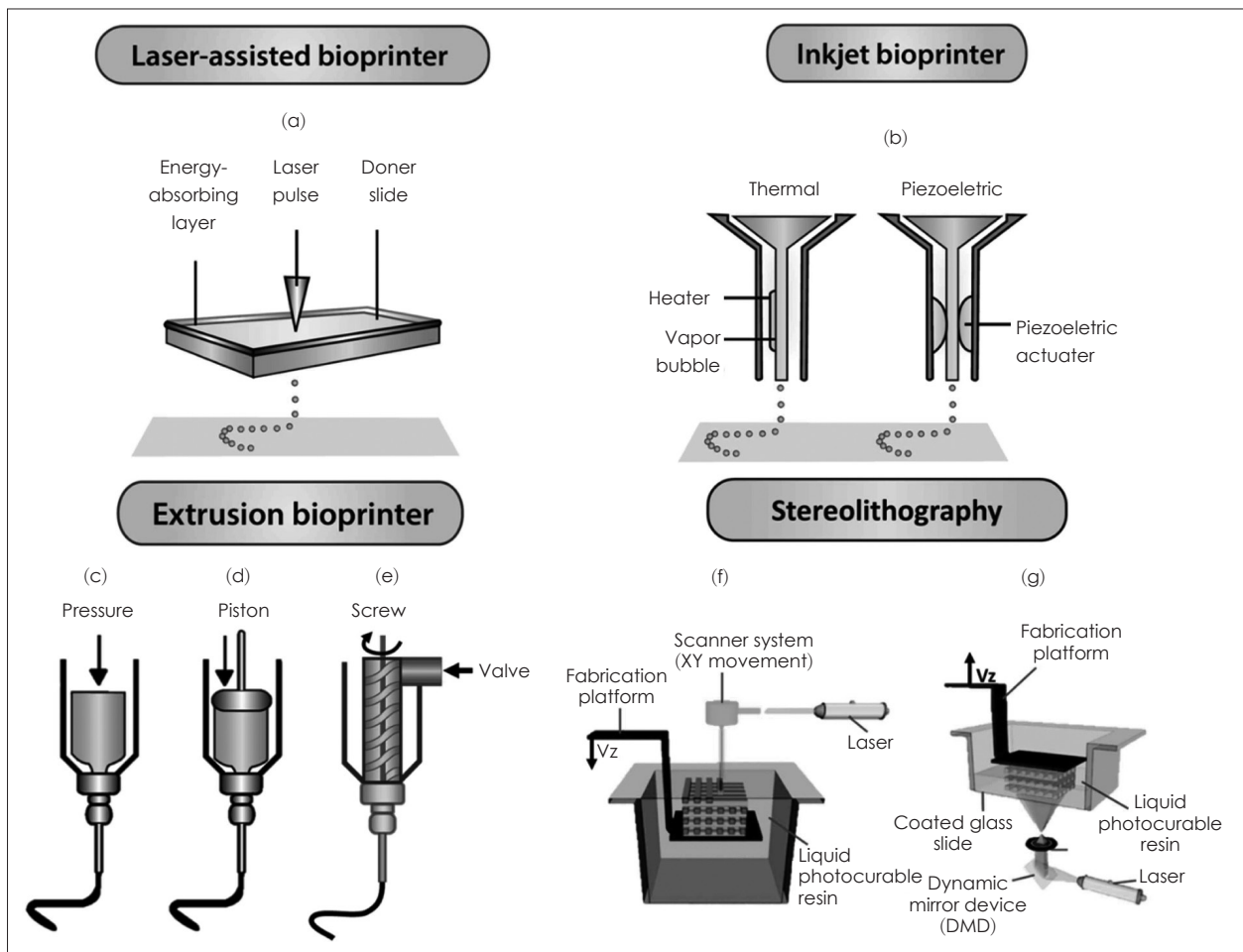


Fig. 1. Four classification for bio-printing systems.

affect the function of the embedded living cells (17), maintaining the high cell viability (4). The cost of the printer is high and the printing speed to fabricate three-dimensional structure is not fast against the inkjet printing system (4).

Inkjet printing

Inkjet printing technique is known as a most common printing technique and in other words it is called drop-on-demand printing method (DODP) (18, 19). Inkjet printing is very fast (1–104 drops/s) when compared to other printing technologies and the cost of the printer is not expensive. Inkjet printers can be sorted into thermal and piezoelectric methods. Thermal inkjet printer known as bubble jet method can eject ink from the print head or nozzle by heating to create the pulse that expels droplets (Fig. 1B) (19). The heating and evaporation lead expansion of vapor, and then ink extruded from the nozzle by pressure caused by bubble expansion. Piezoelectric inkjet printer have inkjet nozzle and piezoelectric actuator that create pulse by electric signal, which extruded droplets from the nozzle (20). This printing method have advantages that high resolution, reproducibility (21) and fast speed of printing. In order to avoid clogging, the hydrogel of high viscosity cannot use in this printing method (22). As the hydrogel viscosity increasing, it cannot squeeze out hydrogel and cells may be remain in the nozzle (23). It is critical drawback of inkjet printing method.

Extrusion printing

Extrusion printing is one of the most commonly used the printing methods for tissue engineering and it is modification printing method of inkjet printing (24). Depending on the operating principle be divided in three systems. The systems are pneumatic, piston, screw. Pneumatic system is dispensed pre-hydrogel solution containing living cells using compressed gases, but it is difficult to control the amount of the hydrogel that come out from the nozzle (25). Piston and screw are printed by mechanical forces without gases and pre-hydrogel solution containing cells are dispensed by pump (Fig. 1C, D, E) (2). Extrusion printing is possible to print almost the hydrogels of

various viscosities and it also can print the hydrogels of high cell density. However, cell viability is reduced when pre-hydrogel solution containing cells is printed because embedded cells in hydrogels are under massive stresses (2, 25).

Stereolithography

Stereolithography (SLA) is similar to laser-assisted bio-printing and widely used in tissue engineering field (Fig. 1F, 1G) (26). The pre-hydrogel solution is solidified by photo-initiated polymerization to produce intricate 3D structure (27). Generally, SLA is can be divided into two different types: single-photon and multiphoton methods. The single-photon method is occurred by single photon absorption and this process can be led to photo-initiator excitation (28). The multi-photon method is caused by solidification using two or more photons sequential or simultaneous absorption. The resolution of the SLA printing is superior to other printing methods (usually, 20 μm) (29). However, the embedded-cells' viability is reduced because of the fabrication process of the 3D structure using SLA printing is cytotoxic (30).

Hydrogel Properties for Bio-Ink

Cell-laden hydrogels are used the term bio-inks and they are play a crucial role to fabricate three dimensional structures in 3D bio-printing (52). Bio-inks are required for various properties because they provide chemically suitable microenvironment in order to cell proliferation, differentiation, and migration, and also gives mechanically structural support in 3D printed structures (53). While the cells are growing in the printed structure, the 3D printed tissue architecture should be maintained. The printed structure needs enough stiffness represented by high viscosity and crosslink density (54). Further, high biocompatibility of the printed structure provides many chances for medical applications.

Rheology

In scientific field, rheology is the deformation of the flow of

Table 1. Comparison of the most commonly used four bio-printing technologies

	Cost	Printing speed	Viscosity	Resolution	Cell viability	Printed tissue and organ	Ref.
Inkjet	Low	Fast	3.5–12 mPa/s	High	85%	Blood vessel, bone, cartilage, and neuron	(31-34)
LaBP	High	Medium	1–300 mPa/s	High (>20 μm)	95%	Blood vessel, bone, skin, and adipose	(35-38)
Extrusion	Medium	Slow	30 to >6x10 ⁷ mPa/s	Medium (>100 μm)	40–80%	Blood vessel, bone, cartilage, neuron, and muscle	(39-48)
Stereo-lithography	Low	Fast	Not limited	High	85%	Blood vessel and cartilage	(49-51)

materials when a force is applied into the materials from outside. When hydrogels are assessed as bio-inks in bio-printing, rheology is underestimated despite of its importance (55). Viscosity and shear thinning are basic concepts that should be considered in rheological properties. These two concepts are highly relevant to bio-printing especially significant to the extrusion printing methods (2). Viscosity is a property necessary to encapsulate uniform cells and it is determined by concentration and molecular weight of precursor solution of the bio-ink. As viscosity increased, collapse of printed 3D structures is delayed (56). However, there are limitations for cell proliferation and migration in high viscosity bioink materials. In bio-printing using low viscosity hydrogels, 3D structures cannot maintain the form and produce the site of cell adhesion (57). Shear thinning is the properties related to printability and it is defined to the inverse proportion between shear rate and viscosity (58). When shear stress applied to polymer solution, complicated polymers are stretched and aligned and then viscosity is decreased. It has high viscosity not only in the syringe before extrusion but also after deposition. Only polymer solution pass through the nozzle, it has low viscosity (59). Christopher et al. reported 3D printing of shear-thinning hydrogels using hyaluronan acid (HA) into 3D constructs having open channels with high resolution (60).

Gelation

A necessary property to maintain printed 3D structures. As change with gelation time, printing fidelity can also be varied. So, gelation time is an important component for physical and chemical aspect of scaffold materials. Gelation time is measured to combine polymer precursor solution and cross-linking agents using physical and chemical crosslinking methods. Short gelation time means good for shape stability. Also, viscosity and gelation time are related to printability. Gelation time has relevance with crosslinking and is influenced by crosslinking agents and materials of precursor polymer solution. Physically crosslinked hydrogel bioinks are allowed reversible interactions to keep uniform viscosity, and required good biocompatibility. However, physical-crosslinking systems are required post-crosslinking process and structures are mechanically weak. Chemically crosslinked hydrogels also have same advantages with physical crosslinking and conducted more rapidly gelation than physical crosslinking. But, crosslinking agents may affect embedded living cells with polymer solution.

Biocompatibility

Biocompatibility is related to various environments such as

biological or mechanical environments. The term refers to the capability or reaction of the biomaterials against the response of the host (61). When the printed 3D structure using hydrogels is transplanted into the host's bodies such as animals or humans, we must consider the biocompatibility of the hydrogel materials (r). Biocompatibility is determined by various experiments. For example, cytocompatibility.

Materials for Bio-Inks

Materials used to bio-inks generally categorized two types. Natural-derived polymers such as gelatin, collagen, alginate and fibrin are studied in tissue engineering and generative medicine and are used for materials of capsulated cells. Natural-derived polymers are widely renowned for materials of bio-inks and isolated animals (62) Meanwhile modified polymers are produced using synthesized or mixed different polymers. In this section, characteristics of natural polymers and modified polymers summarized. Hydrogels of natural-derived materials are employed in the field of tissue engineering and regenerative medicine because natural-derived materials are similar to that of native tissues or organs in the body (63).

Natural-based polymers

Collagen

Collagen is one of the natural polymers and main component in connective tissue that gives support (64). It is the most abundant protein in mammals including humans, which is make up approximately 30% of the whole protein possessed in the body (62). Collagen is consisted of proline, glycine, glycine and hydroxyproline (65). Collagen have various different shape in the body, collagen is commonly existed in skin, bone and cartilage (66). Collagen regulates cell behaviors containing migration, proliferation, adhesion, and differentiation (67). The collagen hydrogel precursor containing cells is used for bio-printing. Lee et al. printed multi-layered skin tissue using collagen hydrogel containing keratinocytes and fibroblasts and demonstrated that collagen hydrogel is potential material as a skin scaffold (68). But, collagen almost used with other polymers because collagen hydrogel is too weak to fabricate the scaffolds (69).

Gelatin

Gelatin is partially hydrolyzed form on collagen and it has high biocompatibility because it is obtained from collagen and structurally similar in both polymers (70). Gelatin has RGD sequences, which helps cell adhesion (71). It dissolves only when the temperature is higher than about 40°C. The gelatin solution

is changed into gel-like state while it is cooled below 30°C. Gelatin is widely versatile polymers in bio-printing because of its thermal-sensitive property (72). But it could not be used without other polymers because it undergoes a reversible reaction and also has difficult to optimize the temperature or viscosity of gelatin solution during bio-printing (72). Yan et al. have printed 3D structure using gelatin/chitosan hydrogel containing hepatocytes and cultured for 6 days (73) and then, the following research is used cell-laden gelatin hydrogel and printed using extrusion method. The structures are consisted of 30 layers. Hepatocytes are remained alive for more than 2 months and performed biological function in the structure (74).

Alginate

Alginate is polysaccharides derived from seaweeds which are found in many places all over the world (75). Dissolved alginate in the aqueous solution forms hydrocolloid. Hydrocolloid formed alginate is good dressing for wounds (76). For example, the alginate extracted from brown algae applied as alginate applied as material of wound dressing (77). Alginate is similar to natural extracellular (ECM) structurally and it has good biocompatibility (75). However, alginate hydrogel avoid to cell adhesion because it does not have cell adhesive site therefore it should be added to like RGD as cell-binding molecules (78). In addition,

the mechanical stiffness is gradually lost and the printed 3D structure degraded in the end due to continuous crosslinked ions within the media (79). As shown in the following structural formula, alginate consisted of a mixture of b-D-mannuronic acid (M) and L-guluronic acid residues (G) (80). To overcome this disadvantage, the ratio of M to G is needed to control. The ratios of G for M is higher, alginate hydrogel becomes more stiff gel (81). Dong-Woo Cho et al. printed three-dimensional PCL-alginate-chondrocytes scaffolds using layer by layer deposition printing for cartilage tissue engineering. Encapsulated chondrocytes were found to high cell viability (~85%) and frame was degraded 4 weeks after implementation (82).

Chitosan

Chitosan is a linear polysaccharide obtained from chitin and usually contained in squid bones or shells of crustacean (84) It is potential materials of hydrogels for long-term drug delivery and wound dressing (85). Chitosan is dissolved in acidic conditions as a pH of 5 or less (86) and the chitosan hydrogel can be gelled when the pH value increased (87) Ozan akkus et al. printed 3D scaffold with chitosan-PEGDA hybrid gel using stereolithography and chitosan and PEGDA compositions were varied with three conditions (Chitosan: PEGDA=1:5, 1:10, 1:15). Human mesenchymal stem cells (hMSCs) were observed high

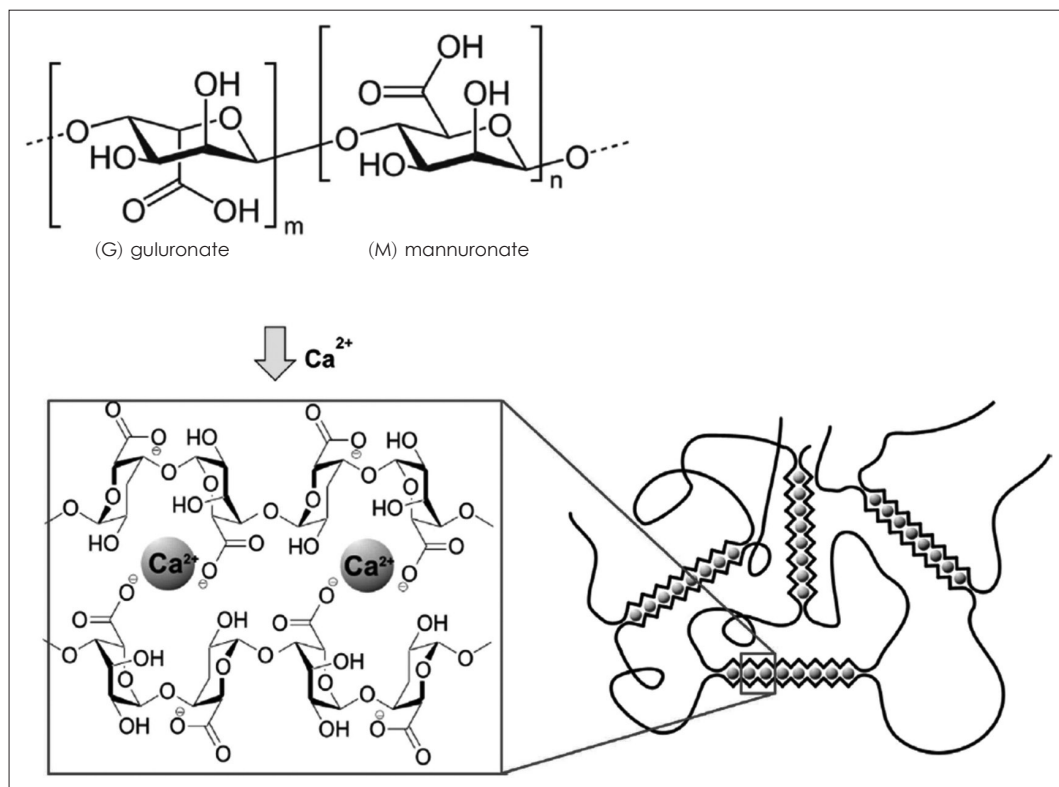


Fig. 2. Schematic crosslinking of the alginates and calcium cations in egg-box model (83).

cell viability about 90% in three conditions (88).

Hyaluronic acid

Hyaluronic acid is called as hyaluronan or hyaluronate and it is composed of D-glucuronic acid and N-acetyl-glucosamine units linear polysaccharide that is promising polymer materi-

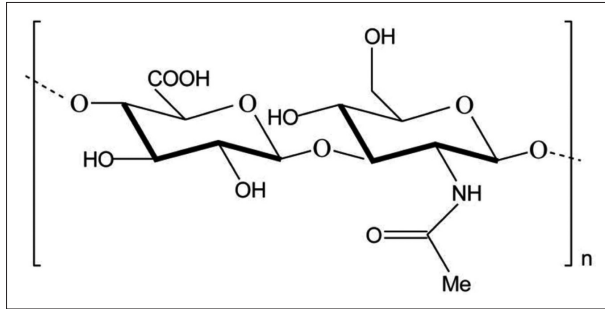


Fig. 3. Chemical structure of hyaluronic acid (93).

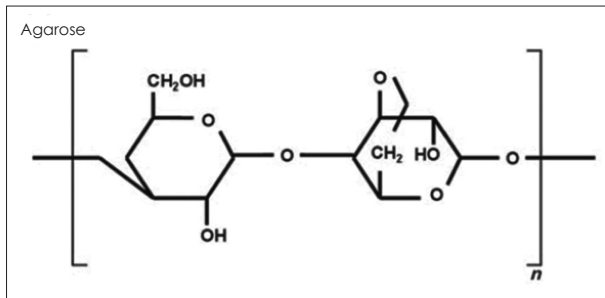


Fig. 4. Chemical structure of agarose (99).

als derived naturally as bio-inks (89). Hyaluronic acid is promising polymer as bio-ink for 3D bio-printing because its sustainly biodegradable, biocompatibility and non-immunogenic properties (90). However, Hyaluronic acid is not stable construct because of its high water solubility (91). Robert L. Mauck et al. have printed 3D structure using Hyaluronic acid gel containing mesenchymal stem cells (MSCs) and cultured for 12 weeks (92).

Fibrin

Fibrin is one of the ECM components and it is promising polymer for bio-printing (94). Fibrin is usually used as glue and it is spontaneously gelled by the reaction of fibrinogen and thrombin (95). Embedded cells are well spreads out and adhere to proper sites in the printed structure due to its abundant cell adhesive cites (96). Skardal A et al. have printed cell-laden fibrin/collagen hydrogel containing amniotic fluid-derived stem (AFS) and bone marrow-derived MSCs onto skin wounds cultured for 14 days (97).

Agarose

Agarose is polysaccharide extracted from seaweeds. Agarose is non-degradable natural polymer in human's body and it is not fit for mammalian cell types and it has poor printability due to agarose is derived from plant. The gelation of the agarose is occurred when the temperature of the dissolved agarose is cooling down to room temperature. The advantage of agarose is

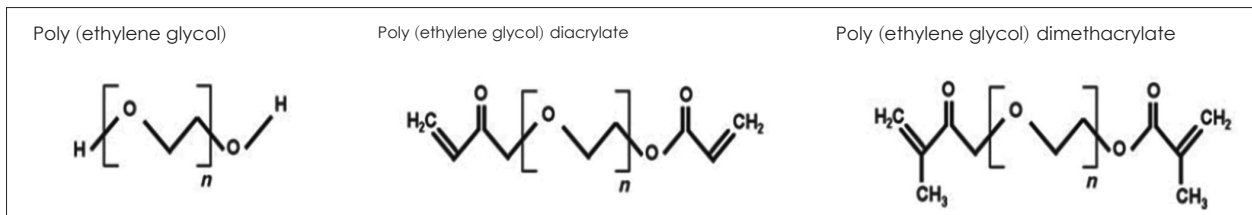


Table 2. Cross-linking methods and time of the materials of hydrogels commonly used for 3D bio-printing (105)

Material of hydrogel	Cross-linking method	Cross-linking time
Collagen	Hydrophobic bonding	0.5–60 min
Gelatin	Temperature	Minutes to hours
Gelatin	Glutaraldehyde	Hours
Alginate	CaCl ₂	Seconds
Chitosan	pH (Basic)	5-50min
Hyaluronic acid	Thiol group cross-link	15-30min
Hyaluronic acid	UV light	Seconds
Fibrin	Thrombin	Seconds
PEG	UV light	Minutes
PEGDA	UV light	Minutes
PEGMA	UV light	Minutes
PEGDMA	UV light	Minutes
GPT	Hydrogen peroxide	Seconds

thermal-sensitive property so cross-linking agents are not necessary to add. Daniela, F. Duarte Campos et al. have printed using agarose hydrogel with Human mesenchymal stem cells and MG-63 cells and cultured for 21 days (98).

Gelatin methacrylate (GelMA)

Gelatin methacrylate is versatile polymers for various tissue engineering applications and also has tunable and biocompatible properties. It is a photopolymerizable hydrogel. This is synthesized using gelatin and methacrylic anhydride (MA) and solidified under UV irradiation (100). GelMA has property that is similar to extracellular matrix (ECM) and it also has some peptides useful for cell adhesion and proliferation (101). Recently, GelMA hydrogels are used in biomedical applications to fabricate bones, cartilages, and vascular tissues. GelMa have

long term cell viability and 3D cell binding, and migration properties. Luiz et al. printed directly with cell-laden GelMa and cultured for 8 days (102).

Gelatin-PEG-Tyramine (GPT)

Gelatin-PEG-Tyramine hydrogels are rapidly formed, which are cross-linkable in situ via enzyme-mediated reaction using horseradish peroxidase (HRP), and hydrogen peroxides (H₂O₂). The gelatin and PEG are widely known to have biocompatible and biodegradable properties. It has relatively strong mechanical strength, but, Chemical crosslink agent like hydrogen peroxide is necessary component for hydrogel gelation process and crosslink agent affects to cell viability.

Table 3. Materials of cell-laden hydrogels (Bio-inks), printing technology, and types of cells in 3D bio-printing

Bio-ink materials	Printing technology	Cell encapsulation	Ref.
Collagen	Laser-assisted	NIH3T3 fibroblasts and HaCaT keratinocytes	(22, 106)
	Inkjet	C3H/10T1/2 cells	(107)
	Inkjet	HFF-1 fibroblasts and HaCaT keratinocytes	(108)
	Inkjet	rabbit articular chondrocytes	(63)
Gelatin/Alginate/Collagen	Extrusion	Bovine aortic endothelial cells (BAECs)	(109)
	Extrusion	Human corneal epithelial cells (HCECs)	(110)
Gelatin	Extrusion	Hepatocytes	(74)
Gelatin/Chitosan	Extrusion	Hepatocytes	(73)
Gelatin/Alginate	Extrusion	Aortic root sinus smooth muscle cells (SMC) and aortic valve leaflet interstitial cells (VIC)	(111)
Gelatin/Alginate	Extrusion	Hepatocytes	(112)
Alginate	Laser-assisted	MG63 cells	(113)
	Laser-assisted	Rabbit carcinoma cells (B16) and Human umbilical vein endothelial cells (Eahy926)	(114)
Alginate/PVA/HA	Inkjet	HeLa cells	(115)
Alginate/Collagen	Inkjet	Human amniotic fluid-derived stem cells (hAFSCs), canine smooth muscle cells (dSMCs), and bovine aortic endothelial cells (bECs)	(116)
Alginate	Extrusion	Human fetal cardiomyocyte progenitor cells (hCMPCs)	(117)
	Extrusion	Stromal vascular fraction cells (SVFs)	(118)
Alginate	Extrusion	Cartilage progenitor cells (CPCs)	(119)
Alginate/nano-HA	Laser-assisted	Human endothelial cells (Eahy926)	(120)
Fibrinogen/Alginate/Gelatin	Extrusion	Adipose-derived stromal (ADS)	(121)
Hyaluronic acid/GelMA	Extrusion	Chondrocytes	(122)
Chitosan/Gelatin	Extrusion	Hepatocytes	(73)
Agarose	Extrusion	Human mesenchymal stem cells and MG-63 cells	(123)
	Extrusion	Bone marrow stromal cells (BMSCs)	(124)
Agarose/Collagen	Inkjet	human umbilical artery smooth muscle cells (HUASMCs)	(125)
PEGDA/Alginate	Extrusion	Porcine aortic valve interstitial cells (PAVIC)	(126)
PEGDA/Chitosan	SLA	Human mesenchymal stem cells (hMSCs)	(88)
PEGDMA	Inkjet	Human chondrocytes	(127)
GelMA/PEGDA	Extrusion	human aortic valve interstitial cells (HAVIC) and human aortic valve sinus smooth muscle cells (HASSMC)	(128)
Matrigel	Laser-assisted	HUVECs	(129)

Synthetic bioinks

Poly (ethylene glycol) (PEG)

PEG has property of absorbing water. The solubility of PEG is fits for cell capsulation but, PEG is not strong to fabricate cell-laden 3D structure. Thus, PEG has to undergoes chemically modification process due to modified PEG is enable to form chemical networks. The PEG is approved polymer for human clinical applications because it is biocompatible and bioactive material and can form various synthetic polymers. When the PEGs are exposed to the light like UV, free radicals are produced by photo-initiators. The double carbon bonds of the PEGs are reactivated by free radicals and they also can produce PEGDA, PEGMA, and PEGDMA polymers. These three hydrogels of the polymers are all formed hydrogels that undergoes photopolymerization process. There are many derivations of poly (ethylene glycol) such as PEGDA, PEGMA, and PEGDMA. Di-acrylated PEG (PEGDA) is often slowly degradable polymers *in vitro* and *in vivo* studies. It is synthesized under the mild conditions. The formed esters of the PEGDA are not stable during acrylates of the PEGs. And this process is caused by poor biodegradability (103). PEGMA means methacrylated PEG. It is undergoes radical polymerization using the light such as UV. The gelation of PEGMA hydrogel occurs due to its alkene bond interactions in the one side of its PEG (104).

PEGDMA is abbreviation for dimethacrylated PEGs. The gelation of the PEGDMA is conducted by photo-initiators. The gelation of the PEGDMA polymer solution because of the presence of the alkene bonds in the PEGDMA (104).

Summary of bio-inks with cells

In bio-printing, the major goal is lager sized-structure with complex and functional architectures. In order to produce three dimensional structures, suitable bio-inks with chemical and physical properties are need (4). To date, researchers are used to naturally derived polymers such as collagen, gelatin and fibrin or synthetic derived polymers such as GelMA, PEGDA, PEGDMA, and Gelatin-poly (ethylene glycol)-Tyramine. Recently, natural-polymer combined other natural polymer or synthetic polymer. The lack of bio-ink materials for producing larger sized-structure is currently problems need to be solved.

Conclusion

Cell-laden hydrogels (bio-inks) are used for the fabrication of three-dimensional structure using bio-printing technique. This technique is advanced technique that can deposit cell-laden

hydrogel in constant volume to fabricate the 3D structure. The hydrogel are important component in bio-printing technique and hydrogels are derived from natural ECM components or synthesis of polymers. Hydrogels are needed several characteristics such as biocompatible and biodegradable. Many groups attempt to find ideal hydrogels that have beneficial properties for embedded cells.

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