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3D printing scaffolds with hydrogel materials for biomedical applications

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Review

Abstract: 3D printing has now been recognized as a very practical technique to create 3D structures with milli-/micron-scale resolution. In tissue engineering, particularly, people utilize 3D printing technique to integrate biodegradable polymers to tissue scaffolds. Hydrogel is highly potential material that provides aqua environment and enables nutrition and oxygen transportation, all of which are requirements for cells. A combination of hydrogel and 3D printing makes the developed platforms more biocompatible, being a benign niche for cells culture and study both in vitro and in vivo. Therefore, this review briefly introduces the background of 3D printing and the status of 3D printing and hydrogel scaffolds for application in biomedical research.

Key words: 3D printing; hydrogel; tissue engineering; scaffold.

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The History of 3D Printing

The first trial of 3D printing was emerged at late 1980's, and was initially called Rapid Prototyping (RP) technologies. They named it as RP because the technique was considered to be a quicker and more effective way to fabricate prototypes for product development in both industry and research area. At millimeter or micron scale, the RP technique is regarded as a revolution compared to the traditional cleanroom techniques (top-down), as it can significantly reduce the cost and time, realize more complicated 3D features, and realize in-situ fabrication (1). In 1980, Dr. Kodama first patented RP technique in Japan (2). In 1986, another patent issued for stereolithography was applied by Charles Hull. He invented his own stereolithography (SLA) machine in 1983. Then he initiated 3D system company to work on SLA technique, and so far the company is one of the three heads of 3D printing area (the other two are Stratasys and EnvisionTec.) (3). In 1992, Stratasys issued Fused Deposition Modelling (FDM) patent and made it to be a very successful technique (4). So far, more than 80% of the 3D printing machines are FDM based, and people have developed large amount kinds of materials that can be used in FDM, such as plastics, metals, gels, and so on. One of the most successful FDM 3D printing machines is Makerbot (5). It utilizes ABS or PLA as the printing materials and can give a very good result. Another successful 3D printing technique is based on photo-curable resin (6) Resin naturally forms as liquids, and can be solidified or cured by chemicals, lights, or heat, etc.(6). To photo-sensitive resin, a certain pattern of light or scanning of laser would form a real solidified piece in the resin and by printing this layer by layer, which eventually develops a 3D structure based on solidified resin materials. This technique becomes rather popular in the last 3 years, and it can be divided into 3 classes. Based on the difference of light source, it can be divided to be laser-based, LCD-based and DLP-based. Each of the specific technique has their advantages and disadvantages. However, normally DLP-based photocurable resin 3D printing system has higher resolution and higher printing speed (7).

Introduce 3d printing into bioapplications

Since 3D printing technique has the ability to fabricate 3D structures, people developed it for producing organs, bones, skulls and other body structures simply for showing purpose. As time goes on, 3D printing has been improved to implement more useful and complicated bio-structures, such as skins, blood vessels, cartilages, bones, and even more complicated organs (8), which cannot be achieved by using traditional lithography methods, such as photolithography (9,10) , soft-lithography (11), hot-embossing (12), self-assembly membrane (13,14) etc. Apparently, involving 3D printing into biomedical research demands much higher requirements, because the bio-systems are extremely complicated, including cells, extracellular matrix, crosslinked blood vessels, and the cells and ECM are highly ordered and packed. All these factors should be taken into account at the same time. Researchers tried to disentangle the problems from different aspects while with a variety of solutions. Firstly, a 3D bioprinting should incorporate cells into the printing materials. People found two ways, post-seeding and pre-seeding. These two concepts are named by the time cell seeded into the scaffolds (15). In post-seeding, a scaffold is previously printed, and then cells are seeded into the

Table 1. Comparison of different types of hydrogels for 3D bioprinting.

	Typical Materials	Advantages	Disadvantages	Example applications
Undegradable	HEMA	Cheap, high ratio of functional group, in vitro application	Not good for in vivo use	Guided cell growth (22), cell engineering (23)
Degradable	PEGDA	Cheap, high mechanical strength, photocurable, biocompatible	Low degradation rate, no cell adhesion motifs	Extracellular microenvironments (24)
	Gelatin and Composites	Contains RGD groups, thermo-reversible, good for in vivo and in vitro use	Extrusion based printing, low resolution	Multi-nozzles co-printing (25)
	GelMA	Contains RGD groups, good for in vivo and in vitro use, photocurable	Hard to produce, UV harmful to pre-seeded cells	Vasculature networks (26)
	Alginate	Easy jellification, tunable properties, cheap	No cell adhesion motifs, needs Ca^{2+} to keep gel-like	Tubing (27)

scaffold. Pre-seeding, in contrast, is processed in opposite way. Pre-seeding has the advantage of convenience than post-seeding, but it required more on the materials and the printing process (16). Pre-seeding needs more concern on pre-modification of cell adhesion motifs and growth factors, so the materials used in this way should be designed much more delicately. For post-seeding, since the printed scaffolds modification can be achieved before seeding, it is much easier to handle. However, post-seeding process always has lower seeding efficiency, which indicates how much the scaffold is occupied by cells. Therefore, to some extent, in tissue engineering, pre-seeding has higher applicability and efficiency.

3D Bio-printing Scaffolds with Hydrogel Biomaterials

3D bioprinting is a way to create scaffolds for cells differentiating, proliferating and growing. For both post-seeding and pre-seeding, the process should provide the main structures and cell-friendly properties. For different cells, requirements including growth factors, nutrition, environmental factors, etc. are varying. Therefore, selection of a specific material suitable for all purposes would be beneficial for scaffolds.

Hydrogel is a type of interesting material that is potentially suitable for fabricating 3D bio-printing scaffolds. Hydrogel is a gel in which the swelling agent is water. Normally the network of hydrogel is a polymer based. Hydrogel always can be controlled by changing environment (17). For example, a very common hydrogel—gelatin, is thermo-reversible, with the property of to be liquefied when heated and jellied when cooled. This property can be used for 3D printing. Some hydrogels can be jellied by reacting with ions, such as alginate (18). Some of them are not reversible, by strong covalent bonding, such as Matrigel® (19). This paper is focused the scaffolds produced by 3D bioprinting with hydrogel biomaterials. Two main methods are employed to realize 3D hydrogel printing, extruder-based and SLA-based. As mentioned above, the extruder based 3D printing is the main part in the industry, as the same trend in 3D bioprinting field. If the pre-extrude material is gel like, the extruder-based technique is easy to form 3D structure. Another way is to solidify the materials after extruding (20). By SLA-based technique, materials are solidified to be hydrogel by light or other initiations, to form desired 3D structures (21). Depending on the properties of bio-degradation, hydrogel can be classified into undegradable and biodegradable hydrogels. Table 1 compares the undegradable and biodegradable hydrogels scaffolds mentioned in this review.

Undegradable Hydrogels

Generally speaking, undegradable hydrogels are not good candidates for implantable scaffolds, but they are sometimes rather suitable for some biological research (16, 17). Lewis lab utilized the direct-writing technique to create 3D hydrogel scaffolds for guided cell growth. They used a very small tip (1-10 um) to form a very high resolution of porous hydrogel structure, as shown in Figure 1. In certain structure, cells can be guided to certain growth pattern. The high resolution printing technique can be used for many kinds of scaffolds preparing, since it can create pores with size same as cells, and the material it used is hydrogel, which could be functionalized by series of chemicals. It is especially suitable for bone and carti-

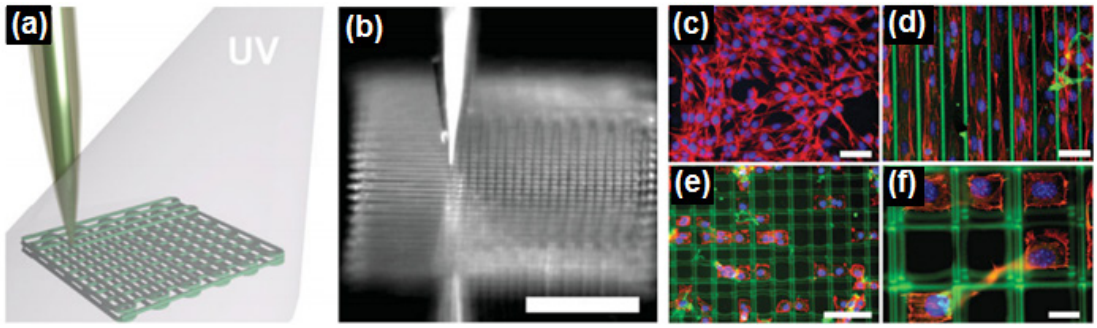

Figure 1. pHEMA scaffolds prepared by direct-writing 3D printing method and 3T3 fibroblasts culture on the scaffolds. (a) Schematic illustration of direct writing of a hydrogel-based ink; (b) Optical image of a 3D hydrogel scaffold acquired during direct ink writing, Scale bar: 200 μm; Optical fluorescence microscopy images of 3T3 fibroblasts plated on the (c) flat glass control, (d) 1D microperiodic hydrogel scaffold, and (e,f) 3D microperiodic hydrogel scaffolds (four layers). Scale bars are 100mm (c–e) and 20 mm (f), respectively. Reprinted with permission form ref (23). Copyright 2009 John Wiley and Sons.

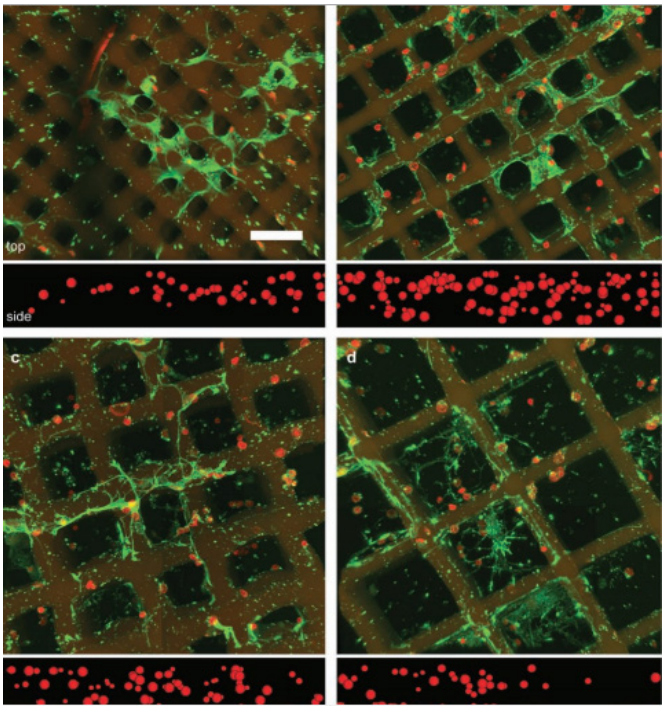

Figure 2. Confocal images (x-y scans, tiled) of primary rat hippocampal cells distributed within scaffolds of varying pitch: (a) 30 μ m, (b) 40 μ m, (c) 60 μ m, and (d) 80 μ m. Reprinted with permission form ref (22). Copyright 2011 John Wiley and Sons.

lage tissue regeneration. High porosity gives more capacity for cells and ECM and also provides easiness for their communications (23).

Lewis group also utilized the same method to print pHEMA. HEMA is a photocurable material, which has an acrylate group. The acrylate group can be polymerized by radical initiators. Normally UV light is used for breaking an initiator to generate radicals for polymerization. Once HEMA solution is extruded out of the nozzle, it can be solidified by UV light, then form very stable scaffolds. HEMA also has the ability to form hydrogel, due to its hydroxyl group on the end of branches. As a result, the 3D patterned pHEMA structure is quite suitable for robust neurons growth, and neurons have their fibers grown on the surface of pHEMA, then form connection with each other, as shown in Figure 2. (22)

Biodegradable hydrogels

In tissue engineering, the scaffolds are carriers for cells. As cells grow and proliferate, they would generate specific ECM to form stable and functional tissue or even organs. Scaffolds are normally not components of the ECM, so they need to be degradable in physiological conditions, and the degraded debris should be friendly to cells. Under physiological conditions, variety of bonds can be degraded, such as ether bond (30), ester bond (31), and peptide bond (32), and those are actually the key that people use to distinguish their biodegradability. Here we introduced several 3D bio-printed biodegradable hydrogels.

PEGDA

PEGDA is photocurable due to the double acrylate groups on the molecular chain terminals. The backbone of PEGDA is $–(CH_2CH_2O)–$, which enables slow in vivo biodegradation (33). Since it is photocurable, PEGDA can be 3D-printed to hydrogel with very high mechanical strength in its water solution (34). PEGDA is widely used for pre-seeding scaffold preparation. However, cells do not attach on pure PEGDA, since the PEG chains have very similar surface tension and cell membranes. That is because the main component of cell membrane is PC, and PC has all $–CH_3$ terminals, which are quite similar to $–(CH_2CH_2O)–$ of PEGDA, preventing cells from attaching on its surface. A normal way to solve this problem is to introduce one or several types of cell adhesion motifs which can be incorporated into the hydrogel backbones (35).

Wicker et al. utilized the SLA method to build a PEGDA hydrogel-based scaffold. The cell

attach motif is RGDS, conjugated to acryloyl-PEG by an NHS induced reaction, and then RGDS-PEG complex also has acrylate groups which can be modified into the PEGDA hydrogel. The scaffold is prepared with cells pre-seeded, eliminated the seeding process, which makes the seeding efficiency to be 100%. The laser-based SLA scanned on the surface of PEGDA and cell solution, to form a desired 3D structure, as shown in Figure 3 A and D. After 24 hours culturing, the percentage of viable cells is at least 87%. This paper also discussed the potential damaging caused by UV laser, since the wavelength used in the paper is 365 nm, which belongs to the UVA range. It turns out that the dosage of applied laser does not remarkably kill cells (36). Actually in photocurable hydrogel printing process, since the polymerization always happens in aqua solution, the initiator should be water-based. The most suitable initiator is the Irgacure 2959. It can be dissolved in water very well and creates minimal toxicity to cells. The problem for Irgacure 2959 is it only absorbs light with wavelength less than 390 nm. So to effectively cure a photocurable hydrogel, a less than 380 nm light source should be introduced (37).

Zhang et al. reported another (Digital Light Processing) DLP-based PEGDA hydrogel scaffold. As they claimed, they can rapidly fabricate complex 3D extracellular microenvironments for 3D cell culturing and modeling. DLP utilizes (digital micromirror device) DMD-based projector as light source and pattern generator, which can cure a layer of ink at one time, dramatically enhancing the efficiency. They also modified RGD onto the hydrogel to assist cell attachment, as shown in Figure 3 a-i (24). In addition, PEGDA hydrogel scaffolds provides good mechanical and biological customization, but it is limited to compression and tension, which means it is not suitable

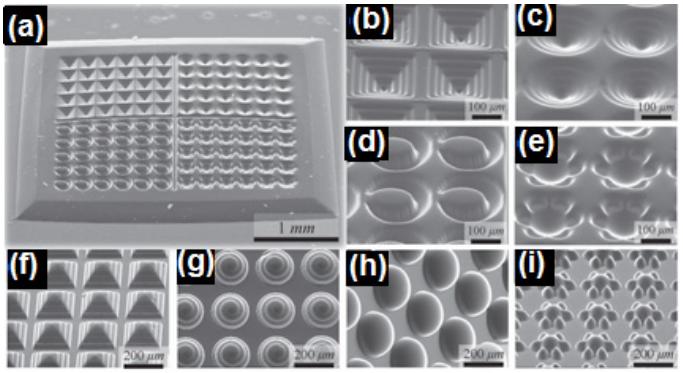

Figure 3. (a-i) SEM Images of the Fabricated 100%-PEGDA Microwells and Microarchitectures. Reprinted with permission form ref (24). Copyright 2012 John Wiley and Sons.

for blood vessel, aortic or heart valves applications(35, 36).

Gelatin and gelatin-composites

Gelatin is a thermal-reversible hydrogel once it is dissolved in water. In wide range of concentrations, gelatin remains liquid form in >30°C environment and becomes gel under 20°C. Gelatin has plenty of RGD groups on its peptide chains, which means gelatin has its own cell attach motif. It eliminates the need for other motif modification (40). The thermal-reversible property makes it very suitable for 3D printing. Landers et al. developed a method to make gelatin to be 3D printable. A thermostat is to stabilize the temperature of a cartridge, which holds a liquid form of printing ink—the hydrogel. A compressed air is used for generating a pulse to squeeze the solution out of the nozzle. After the liquid is squeezed out of the nozzle, it solidifies as it cools down. This method can also be called a direct-writing technique. With multiple layers of writing, it can form porous and cell-friendly scaffolds.

Aortic valves have two remarkably different parts: the wall and the valve leaflet, which are composed with different cells, smooth muscle cells (SMC) and aortic valve leaflet interstitial cells (VIC) respectively. To build a model or even tissue engineered aortic valve, at least two print heads should be employed. Butcher et al. designed a dual print-head extruder-based 3D printer to print alginate/gelatin hydrogel composites to create aortic valves. One reservoir of the printer is filled with SMC and the other filled with VIC. By properly distributing the volume of wall part and the leaflet part onto the two print-head, it forms a very delicate structure of aortic valve with two types of live cells. After 7 days of culturing, more than 80% of both cell types remain viable. The introduction of alginate helps the hydrogel exhibit linear stress–strain behavior, with higher extensibility and mechanical strength (25). In 3D bioprinting, dual print-head is sometimes not sufficient, so some researchers developed multi-head printers. Sun et al. developed a multi-nozzle machine for different materials 3D bio-printing. The key point for this technique is introduction of multi-cartridge and multi-nozzles. Each nozzle is connected to a cartridge separately and each of them is controlled separately by microvalves which are hooked up inside of nozzles. When applying a printing, the controllers for microvalves and roller systems work together to produce a better product. Depends on the pressure and material viscosity, the liquid out of nozzles can be either droplets or continuous flow (41). This method is quite suitable for simultaneously printing multiple hydrogels with multiple types of cells.

Gelatin Methacrylate (GelMA)

Gelatin can also be modified to be photocurable, in which way it would be easier to control and 3D printing. Gelatin has lysine on its peptide chains, and lysine terminal amino group can react with methacrylamide to form a photocurable methacrylate group (42). The advantages of formed GelMA hydrogel include 3D printable and almost all the advantages of gelatin, such as cell adhesion motif, biocompatible, biodegradable etc. GelMA can be degraded in vivo in near 2 months (43). Therefore, people

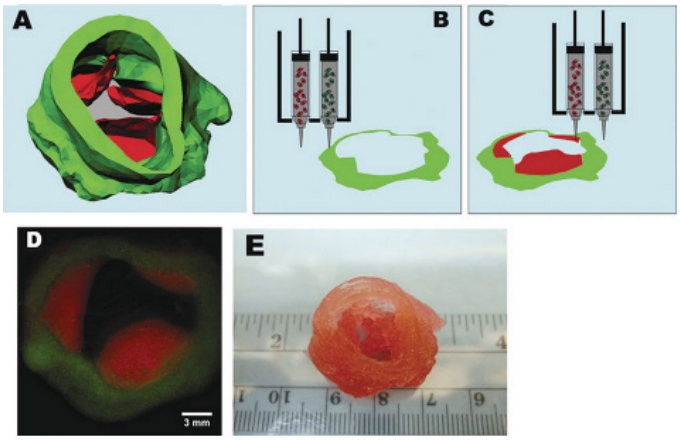

Figure 4. Bioprinting of aortic valve conduit. (A) Aortic valve model reconstructed from micro-CT images; (B, C) scheme bioprinting process with dual syringes; (D) fluorescent image of first printed two layers of aortic valve conduit; (E) as-printed aortic valve conduit. Reprinted with permission form Ref (25). Copyright 2002 John Wiley and Sons.

widely studied this material for scaffold fabrication.

Dubruel et al. printed a 3D mesh-like scaffold with GelMA. Cold jelly GelMA is extruded out of a nozzle with temperature control, and the cold gel keeps the printed shape and the whole 3D structure for a short time. After printing finished, a UV light source is introduced to cure the printed object. Cells are pre-seeded in GelMA and extruded during the printing process. GelMA provides enough biocompatible microenvironment for cells during printing and culturing, as well as it facilitates the printing process. They also discussed the viability of HepG2 cells under different extrusion pressure. It turns out conical needles are more suitable than cylindrical needles (44).

A main problem for tissue engineering is lack of vessel networks to transport nutrition and wastes. Ali group tactfully designed a 3D printing method to form functional human vascular networks with GelMA. Firstly, an extrusion-based printer prints agarose to form a vessel-like structure. The agarose hydrogel keeps jelly in cold environment and acts as sacrificial templates. Then GelMA solution with SMC cells is poured into the tank with agarose wires. UV light is introduced to cure the bulk of GelMA and agarose templates are eventually removed by water flushing to form hollow vessels. Finally, endothelial cells are seeded onto the surface of hollow vessels, forming an infusible live blood vessel model. It turns out the hollow channels dramatically enhance the cell viability (26). Actually according to literatures, cells would lack of nutrition and oxygen when cells are far away to supplies (45). So this method has potential application in the development of vascularized constructs and hydrogel microfluidics.

Alginate

Alginate is an anionic polysaccharide refined from cell walls of brown algae. Alginate can absorb as much as 200-300 times its weight water to form a high strength hydrogel. A more interesting property is sodium alginate has high solubility in water, but Calcium alginate does not, showing a hydrogel form. That means sodium alginate can be extruded out of a nozzle into a Calcium ion solution, alginate would turn to hydrogel. This makes alginate quite controllable for 3D manufacturing. Huang et al. utilized

Inkjet technology to print alginate hydrogel zig-zag tubes, which are filled with cells in the hydrogel and have potential applications in tissue engineering blood vessels (27).

Coaxial nozzle is a well-defined tool to create core-shell structures. It has been widely used for electrospinning fibers with different coatings. When filling in the inner nozzle with removable or even liquid, and the outer nozzle with hydrogel, a tubing-like structure would be obtained. However, normally hydrogel has very high viscosity and cannot be easily extruded out of the extreme small coaxial nozzles. Alginate has the perfect ability to form hydrogel with Calcium ion. Before it forms hydrogel, it characterizes as a liquid. So alginate is an ideal material for tubing hydrogel making. A delicate design is reported by Yu et al. Alginate solution with cells is filled into outer nozzle cartridge and inner cartridge is filled with cross-linker, Calcium ion solution, respectively. Before they mix at the outlet of the nozzle, both are liquid, which means it is more easily to be extruded. The obtained cell-laden tubing can be functionalized as blood vessel to transport liquid. Therefore, as they claimed, the results of the research can help with optimization and modification of bioprinting parameters as well as post-printing incubation for future functional 3D organ fabrication and maturation (46).

Conclusion

Hydrogels, especially the biodegradable hydrogels, have massive potential on tissue engineering scaffolds applications. They provide aqua environment and enable nutrition and oxygen transportation, all of which are requirements of cells. However, normally hydrogels are not easily to form a specific structure. Introduction of 3D printing technique allows hydrogels more easily to form desired structures and more suitable for tissue engineering applications. Since biological environment is complex, oncoming 3D-bioprinting technologies should pay more attention to precise controlling and handling multiple biomaterials.

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