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REVIEW ARTICLE

Hydrogels for 3D bioprinting in tissue engineering and regenerative medicine: Current progress and challenges

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Abstract

Three-dimensional (3D) bioprinting is a promising and innovative biomanufacturing technology, which can achieve precise position controlling of cells and extracellular matrix components, and further create complex and functional multi-cellular tissues or organs in a 3D environment. Bioink in the form of the cell-loaded hydrogel is most commonly used in bioprinting, and it is vital to the process of bioprinting. The bionic scaffold should possess suitable mechanical strength, biocompatibility, cell proliferation, survival, and other biological characteristics. The disadvantages of natural polymer hydrogel materials include poor mechanical properties as well as low printing performance and shape fidelity. Over the past years, a series of synthetic, modified, and nanocomposite hydrogels have been developed, which can interact through physical interactions, chemical covalent bond crosslinking, and bioconjugation reactions to change the characteristics to satisfy the requirements. In this review, a comprehensive summary is provided on recent research regarding the unique properties of hydrogel bioinks for bioprinting, with optimized methods and technologies highlighted, which have both high-value research significance and potential clinical applications. A critical analysis of the strengths and weaknesses of each hydrogel-based biomaterial ink is presented at the beginning or end of each section, alongside the latest improvement strategies employed by current researchers to address their respective shortcomings. Furthermore, we propose potential repair sites for each hydrogel-based ink based on their distinctive repair features, while reflecting on current research limitations. Finally, we synthesize and analyze expert opinions on the future of these hydrogel-based bioinks in the broader context of tissue engineering and regenerative medicine, offering valuable insights for future investigations.

Keywords: 3D bioprinting; Hydrogel; Bioink; Tissue engineering; Bionic scaffold

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Abbreviations

3D	Three dimensional	PGA	Polyglycolic acid
3DP	3D printing	PLGA	Poly–dl–lactide–coglycolide
3DBP	3D bioprinting	PEG	Polyethylene glycol
ECM	Extracellular matrix	PAOXA	Poly (2–alkyl–2–oxazoline)
dECM	Decellularized extracellular matrix	PVA	Polyvinyl alcohol
SLA	Stereolithography	PEGDA	Polyethylene glycol diacrylate
2PP	Two–photon polymerization	PEGDMA	Polyethylene glycol dimethacrylate
DLP	Digital light processing	PAAM	Polyacrylamide
DMD	Digital micromirror device	PU	Polyurethane
CAL	Computed axial lithography	PPO	Polypropylene oxide
PBS	Phosphate buffer saline	PEO	Polyethylene oxide
TG	Transglutaminase	PACG	Poly (N–acryloyl 2–glycine)
LAP	Lithium phenyl–2, 4, 6–trimethyl–benzoyl phosphinate	PCL	Poly (ε–caprolactone)
GP	Genipin	PLCL	Poly (lactide–caprolactone–cocaprolactone)
GTA	Glutaraldehyde	SA	Sodium alginate
EDC	1–ethyl–3 –(3–Dimethylaminopropyl) carbo-diimide	CS/CH	Chitosan
NHS	N–hydroxysuccinimide	SF	Silk fibrin
mTG	Microbial transglutaminase	HA	Hyaluronic acid
MBA	N–N'–methylenebis(acrylamide)	HAMA	Hyaluronic acid methacrylate
GMA	Glycidyl methacrylate	Gel	Gelatin
MFCs	Meniscus fibrochondrocytes	GelMA	Gelatin methacrylate
MSCs	Mesenchymal stem cells	MC	Methylcellulose
hMSCs	Human mesenchymal stem	BG	Bioactive glass
hBMSCs	Human bone marrow mesenchymal stem cells	HAP	Hydroxyapatite
HUVECs	Human umbilical vein endothelial cells	GO	Graphene oxide
BMP–2	Bone morphogenetic protein–2	rGO	Reduced graphene oxide
hTert–MSCs	Human mesenchymal stem cells	CNTs	Carbon nanotubes
HEK	Human embryonic kidney cells	MWCNT	Multi–wall carbon nanotubes
hADMSCs	Human adipose tissue mesenchymal stem cells	CNCs	Cellulose nanocrystals
CMs	Cardiomyocyte	CNFs	Cellulose nanofibrils
CFs	Cardiac fibroblasts	BNC	Bacterial nanocellulose
SMCs	Smooth muscle cells	GNRs	Gold nanorods
UCs	Urothelial cells	ESCs	Embryonic stem cells

1. Introduction

The rapid development of current medical technology allows for a high success rate in organ transplantation and tissue repair nowadays. Organ transplantation is a hopeful solution to patients who are suffering from diseases such as diabetes, liver failure, and heart failure. A great progress has also been achieved in the field of tissue repairs, especially in cartilage regeneration, wound healing, and adipose tissue reconstruction. However, only a few percentages of patients can be successfully treated due to the limited number of organs from donors, which is the biggest constraint in organ transplantation and tissue repair[1]. In recent years, considerable efforts on tissue engineering and regenerative medicine have been made by researchers aiming at solving problems such as insufficient donors and immune rejection. As a future alternative therapy, tissue engineering has great potential in the medical field, which mainly utilizes cells and biological materials to create autologous tissue or organ transplantation.

Regenerative medicine employs methods to repair the functions of cells, tissues, or organs, which are diseased or damaged, through substitution or regeneration[2,3]. Tissue engineering and regenerative medicine are two highly interrelated and multi-disciplinary research fields. However, in practice, the definitions of these two concepts are not identical. Regenerative medicine is a broader definition that not only includes tissue engineering, but also includes self-healing of the body's tissue systems. Tissue engineering and regenerative medicine are now widely conflated, mainly because the ultimate goal of tissue engineering is to replace or enhance the self-healing of tissues. Many researchers have used biological and engineering approaches to achieve the goal of promoting the repair and regeneration of tissues or organs, or even giving them a normal structure or function. Magalhaes *et al.*[4] used autologous primary uterine cells attached to a semi-circular poly-dl-lactide-coglycolide (PLGA) and polyglycolic acid (PGA) biodegradable scaffold to repair and reconstruct uterine defects in larger animal models (i.e., rabbits), thus enabling the complete process of pregnancy to term births. However, repairing and regenerating larger animal models or human uterine defects by the same method failed[5, 6]. Although *in vitro* biomimetic tissues and organs can be made using traditional methods that include electrospinning[7,8], rapid prototyping[9], and freeze-drying[10], the designed biomimetic tissues or/and scaffolds are difficult to form the three-dimensional (3D) structures compared with natural tissues or/and organs. Cells cannot be uniformly attached to traditional scaffolds and cannot proliferate and differentiate. Therefore, the scaffold without physiological activity will cause cell death and further cause tissue or/and organ necrosis if cells are directly seeded[11].

3D printing is an emerging technology that has been widely applied in the field of tissue engineering. Compared with traditional manufacturing technologies, it has made great progress, but there are still problems, such as low cell seeding efficiency, uneven distribution, and low spatial resolution. Therefore, 3D bioprinting which combines cells and biological materials has become a more attractive technology[12]. 3D bioprinting can construct complex and cell-loaded 3D structures, which are helpful for the application of scaffold-based or scaffold-free tissue and organ structures, microorganisms, and single-chip organ model systems. Generating functional human organs and tissues like the heart, liver, skin, and cartilage on the upcoming large-scale human body will play an essential role. With the increasing development, 3D bioprinting technology is leading a global revolution in the medical field, and enormous changes will take place in the treatment of diseases in the near future[2,13].

Applying suitable bioinks is the fundamental of 3D bioprinting. The general definition of bioinks is a formula containing biomaterials and bioactive components that can be processed by automated manufacturing technology[14]. The types of bioinks include hydrogels, cell aggregates, acellular matrix, etc.[15,16]. The ideal bioinks containing cells should be smoothly squeezed out of the printing needle without damaging the cells due to shear stress[14,17,18]. The hydrogels are high-molecular-weight polymer with a 3D-crosslinked network structure and water content equivalent to soft tissue, which is a commonly type of bioinks applied in 3D bioprinting owing to its excellent characteristics of simulating extracellular matrix (ECM) and printability. Hence, the general conventional definition of bioinks refers to a cell-loaded hydrogel formulation that can be processed by automated biomanufacturing technologies[14]. For a proper 3D bioprinting process, it is necessary to have both hydrogel bioinks capable of producing cell-loaded bioinks and a multi-scale spatial resolution that can mimic the native ECM[19]. The ideal hydrogel materials should have a series of good physical, chemical, and biological characteristics, such as printability, biocompatibility, degradability, mechanical properties, stability, non-toxic, and non-immunogenic properties, and should be able to promote cell adhesion proliferation and differentiation[15,19,20]. First of all, from the aspect of printability, hydrogel bioinks for printing should possess several characteristics: (i) Shear-thinning. It is expected that the viscosity of the hydrogel is lower at high shear rates. Because high shear thinning is beneficial to improve cell viability, it can also maintain the shape fidelity of the scaffold after printing. (ii) Yield stress; to prevent the spread of bioinks on the surface. (iii) Self-healing. The viscosity of the hydrogel will change during the extrusion printing process, and the original viscosity is expected to restore after printing. (v) Crosslinking ability, that is, the process of gelation has stability in physical, chemical, or biological media crosslinking agent. (v) Degradability. The hydrogel and the by-products produced should be biocompatible when degraded, with less toxicity or even non-toxicity[21-25]. Therefore, in this review, we discuss and summarize the researches of 3D bioprinting using hydrogel as bioinks in recent years. According to the commonly used classification methods of hydrogels, the preparation methods such as mixing, chemical modification, and nanodoping are analyzed from the perspective of different printing technologies. At the same time, we discuss the advantages and disadvantages of several common hydrogel bioinks available today, and present some ways to avoid the shortcomings so that these hydrogels can be better matched with 3D bioprinting for tissue engineering applications. We expect to discover the optimization and improvement of 3D bioprinting technologies to realize

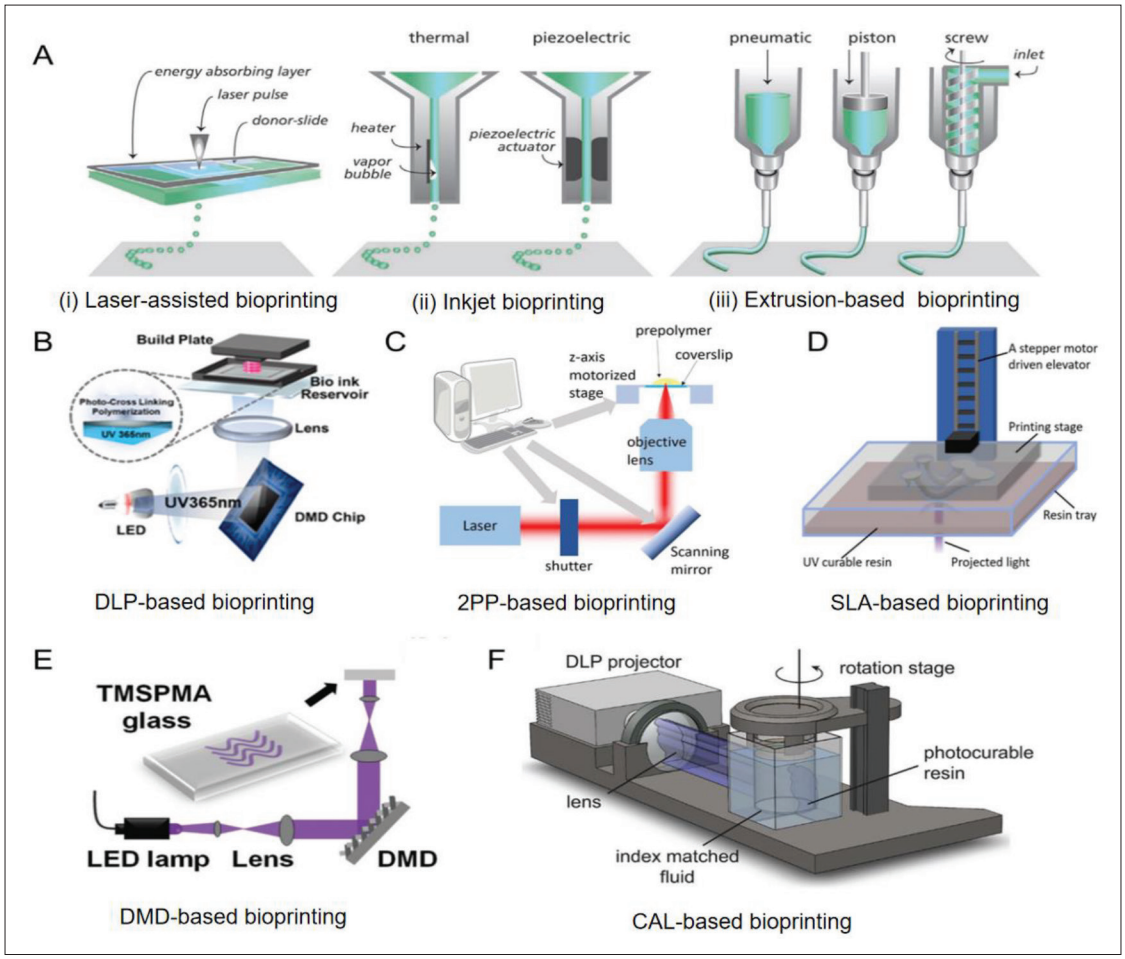


Figure 1. Schematic diagram of the principle of 3D bioprinting. (A) (i) Laser-assisted bioprinting; (ii) inkjet bioprinting; (iii) extrusion bioprinting. Reproduced with permission[26]. (B) Digital light processing (DLP) bioprinting. Reproduced with permission[34]. (C) Two-photon polymerization (2PP) bioprinting. Reproduced with permission[33]. (D) Stereolithography bioprinting. Reproduced with permission[32]. (E) Stereolithographic bioprinting is based on the digital micromirror device (DMD). Reproduced with permission[35]. (F) Computed axial lithography (CAL) bioprinting. Reproduced with permission[38].

the ideal performance and the formulas of useful hydrogel bioinks. At the same time, we also review the latest research progresses of bioprinted tissues/organs before clinical applications, such as skin, cartilage, heart, and kidney.

2. Research progresses of 3D bioprinting technology

As early as 2013, an article was published, detailing the 3D printing methods of extrusion-based bioprinting, inkjet bioprinting, and laser-assisted bioprinting in conjunction with the 25th anniversary of biomanufacturing hydrogels (Figure 1A)[26]. In other articles, the authors further analyzed the influence of 3D printing process parameters in detail on the structure of the printed scaffolds. The 3D bioprinting has become a relatively mature method. Based on these three types of printing, some other printing technologies are derived, such as nano-bioprinting, acoustic bioprinting, magnetic bioprinting, etc.[27,28] Jentsch *et al.*[29] achieved precise 3D bioprinting of cell-filled hydrogel structures by using the acoustic droplet ejection (ADE) method in combination with a 3D assembly technique of solidified droplets. The technique reduces the shear stress on the cells during printing so that the cells inside the hydrogel neither lose their biological activity nor exhibit long-term negative effects. In addition, the ADE technique can generate variable droplet sizes more than three length scales and tailor droplet formation by adjusting frequency, amplitude, and signal duration, all of which make this method of great potential in tissue engineering and regenerative medicine. Adine *et al.*[30] used the magnetic bioprinting technique to differentiate mesenchymal stem cells (MSCs) into an innervated secretory epithelial organ and labeled the cells with magnetic nanoparticles. This technique allows 3D

spatial arrangement of cells and formation of spheroids in a short period of time using magnetic interactions, which facilitates the cells to form their own 3D microenvironment and ECM, and promotes their mutual responses. Moreover, the magnetic nanoparticles used in this study can support cell proliferation and regulate their metabolism without triggering inflammation and oxidative stress. It was found that extrusion-based bioprinting is not only simple to operate and low cost, but also suitable for most biomaterials, making this method the most widely applied in 3D bioprinting. However, the preparation of non-synthetic bioinks with rheology and biocompatibility is also one of the main challenges of extrusion bioprinting[31].

In addition to the above-mentioned technologies, photocrosslinking-based 3D bioprinting technologies are also gradually being adopted for extensive use, including stereolithography, two-photon polymerization (2PP), and digital light processing (DLP)[32-34] (Figure 1B–D). Besides, another approach of stereolithography is the digital micromirror device (DMD) bioprinting (Figure 1E)[35,36]. Ying *et al.*[35] used this technique for bioprinting GelMA-PEO emulsion bioinks. They bioprinted the cell-filled construct using a pre-designed serpentine pattern, while the uncrosslinked bioink along with PEO droplets was washed off with PBS immediately after bioprinting. DMD bioprinting is on the basis of layer-by-layer photocrosslinking of the bioink in the reservoir, which avoids subjecting the cells to the shear stress associated with the extrusion process that leads to cell fragmentation[37]. However, DMD bioprinting technology also has some minor drawbacks, such as its layer-by-layer photocrosslinking technology, which may inevitably reduce printing efficiency. To solve the problem, Kelly *et al.*[38] proposed the computed axial lithography (CAL) technique in a volume accumulation method for target formation through photopolymerization, which is several ranks of magnitude faster than layer-by-layer printing (Figure 1F). The technique gives them the ability to synthesize 3D structures of arbitrary geometry through photopolymerization. The CAL method presents several strengths over traditional layer-based printing methods; for example, they can be used for circumventing support structures because it can print highly viscous liquids or even solids. It is also possible to use this technology to print 3D structures around pre-existing solid parts. In addition, CAL technology allows for larger print volumes along with faster print speeds. Compared to traditional extrusion-based bioprinting, the light-curing printing method has shown many advantages and will play a crucial role in the development of bioprinting. At present, particularly several natural and synthetic hydrogel polymers such as gelatin, chitosan, hyaluronic acid (HA), polyethylene glycol (PEG), etc. have been modified by combining methacrylate. Remarkable hydrogel materials are limited to the application of light-based 3D bioprinting technologies[39-41]. Nevertheless, as a key factor of light-curing printing, obtaining light-curing hydrogel materials is an important research direction, and it is also a challenge we must face in the future simultaneously.

3. Polymer-based hydrogel bioink

According to the sources and properties of hydrogels generally used in 3D bioprinting, they can be divided into three main categories: natural polymer-based hydrogels, synthetic hydrogels, and modified natural hydrogels.

3.1. Natural polymer-based hydrogels

Natural hydrogels can more effectively mimic the biopolymers that exist in natural ECM, which have the advantages of good biocompatibility, easy biodegradability, and low toxicity[33]. Natural hydrogels include sodium alginate (SA), gelatin, silk fibrin (SF), collagen, fibrin, and hyaluronic acid[42-44]. Here, we mainly review the natural polymer hydrogels that are most used in recent years.

3.1.1. Sodium alginate

In 3D bioprinting, SA is one of the most studied and broadly applied cell-loaded hydrogel materials. Because it can be gelled through simple ionic crosslinking and has good biocompatibility, it has better mechanical properties than other protein hydrogels[45]. However, the disadvantages of SA include poor printing performance, low mechanical strength, and poor structural stability of printing, and it cannot promote cell proliferation and differentiation. Increasing the viscosity of pure SA can meet the conditions such as the rheology of printing, but the fidelity of shape is too bad after printing[46]. Therefore, the current researches mainly focus on the composite of SA and other biological materials or the modification of SA and other materials[47-49]. When mixing with other materials, it is generally considered that the optimal concentration range of SA is 1% to 5%. When the concentration is lower than 1%, although it is easy to dissolve and mix, the shape fidelity becomes very bad after printing, and it can easily collapse. When the concentration is higher than 5%, the SA solution decreases cell viability, which is too viscous to be used as a bioink for extrusion printing. The concentration of the commonly used crosslinking agent calcium chloride is generally 0.5 M[31]. Sodium alginate-gelatin (SA-Gel) hydrogels have been extensively applied for extrusion bioprinting, which is the most common mixture form. Because the optimization of bioinks is essential for printing and cell adhesion and survival, many researchers have made some attempts to optimize the SA-Gel hydrogels. Liu *et al.*[50] investigated the effect of different concentrations of nano-

attapulgite (nano-ATP) on the printability and mechanical properties of SA-Gel bioinks. They fabricated sodium alginate (SA)/gelatin (Gel) hydrogel scaffolds doped with different contents of nano-ATP via 3D printing. It was found that the compressive strengths and compressive modulus of the composite hydrogel increased significantly with the increase of nano-ATP concentration. In addition, as the nano-ATP amount increased, the swollen scaffolds is able to better retain its shape and mechanical support. Thus, nano-ATP makes bioink more effective in inducing bone regeneration with the potential to repair bone defects. Chen *et al.*[51] adjusted the physicochemical and biochemical properties of the hydrogel by changing the concentration and crosslinking sequence of SA-Gel. They used Ca^{2+}-crosslinked SA molecules and transglutaminase (TG)-crosslinked gelatin molecules to construct the SA-Gel interpenetrating polymer network (IPN), which provides the best interior for cell survival microstructure and environment. Ionic and covalently crosslinked SA-Gel hydrogel is a material with great potential, not only in tissue/organ printing but also in the field of drug screening models and pathological mechanism analysis. Moreover, SA is usually mixed with other different biological materials, such as collagen, agarose, PEG, and carboxymethylcellulose (CMC)[31,49,52,53]. Geevarghese *et al.*[54] used a mixture of gelatin, CMC, and SA as a printing bioink to prepare scaffolds for cartilage tissue engineering. As the concentration of CMC in the mixture increased, the viscosity of the bioink also increased. Among them, 2% CMC has excellent printability and mechanical stability, while the bioink containing 4% CMC is highly viscous and not extrudable.

3.1.2. Gelatin

Gelatin (Gel) has antigenicity and low immunogenicity, and cell adhesion motifs (RGD peptides). Therefore, gelatin can be used as cell adhesion and metalloprotease-driven degradation sites, which can be effectively absorbed in the body without toxic degradation. Additionally, the raw materials of gelatin products are easily available and inexpensive[55]. In adipose tissue regeneration, gelatin is one of the most commonly used natural polymer materials for soft tissue repair[56]. Yet, the fundamental problem that needs to be solved urgently is maintaining the stability of the gelatin scaffold structure after printing. Currently, there are usually three strategies to resolve this problem. The first strategy is to mix the gelatin solution with other polymers. To form a stable 3D structure after printing, researchers use the properties of other materials to crosslink. In this case, since gelatin does not participate in the crosslinking reaction, it is unstable and easily degradable. The second strategy is what we will introduce in detail in section 3.3. The gelatin is modified, grafted with methacrylate, and then crosslinked under ultraviolet (UV) light with the help of a photoinitiator. The third strategy is the crosslinking of gelatin, which is divided into two situations: (i) the prepared gelatin hydrogel can usually be mixed with a crosslinking agent for reaction, and (ii) the gelatin used for printing is used to immerse the printed stent in a crosslinking agent solution for curing.

Yang *et al.*[57] described several crosslinking agents for gelatin. Commonly used crosslinking agents include chemical agents and enzymes, such as genipin (GP), glutaraldehyde (GTA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/n-hydroxysuccinimide (NHS), microbial transglutaminase (mTG), etc. They mixed a 4% gelatin solution with four crosslinking agents respectively, and after gelation at 37°C, they were frozen at -20°C for 8 h and then freeze-dried for 48 h to obtain gelatin sponge scaffolds. Based on the different properties of crosslinking agents, they found that (i) GTA was one of the most commonly used crosslinking agents with certain cytotoxicity; (ii) EDC/NHS had low toxicity and good biocompatibility, but the degradation rate is excessively quick; (iii) the mechanical strength of gelatin scaffolds is weak after crosslinking. Hence, the scaffolds are insufficient to provide a suitable living environment for the cells, which in turn leads to cell death. Genipin has a slighter toxicity compared to glutaraldehyde. But its crosslinking effect is over powerful, which results in lower swelling and high hardness of the gelatin sponges. Gelatin scaffolds crosslinked by genipin are not suitable for soft tissue repair, while it is suitable for hard tissue repair. The authors reported gelatin sponges crosslinked with transglutaminase for the first time, and found that mTG sponge had the best performance compared to the other three crosslinkers: good internal and external biocompatibility, uniform pores, and resistance to degradation. Compared with the other three crosslinking agents, it is the most suitable for soft tissue repair. The required properties of soft tissues are different, so the corresponding concentration of gelatin and the crosslinking agent are also different. Besides, as a bioink for 3D bioprinting, it is necessary to consider factors such as the temperature and viscosity of gelatin. Most importantly, the crosslinking agents are not limited to the list above, and there are some other chemical crosslinking agents. Negrini *et al.*[58] mixed gelatin with concentrations of 15% w/v and 25% w/v with different ratios of N-N'-methylenebis(acrylamide) (MBA)/gelatin amino crosslinkers. Gelatin does not need to be mixed with other polymers or chemically modified, and the crosslinking reaction can be initiated by adding MBA crosslinking agent. They consider that the suitable concentration of gelatin is 15% w/v in terms of mimicking the mechanical properties of adipose tissue. The concentration of MBA is 0.4% w/v,

and the optimal crosslinking temperature of the gelatin scaffold is 20°C after printing. Chemically crosslinking gelatin hydrogels is a simple method. *In vitro* cytotoxicity experiments using 3T3-L1 pre-adipocytes showed that the bioprinted scaffolds will not produce indirect cytotoxicity to cells. Finally, they also tested that the scaffold can promote the adhesion, proliferation, and adipogenic differentiation of human primary pre-adipocytes[56].

3.1.3. Silk fibroin

Silk fibrin (SF) is a natural fiber polymer extracted from silk. It not only has the characteristics of a natural hydrogel material but also has high tensile strength, excellent biological properties, and low inflammation. Because of the above characteristics, it has a wide range of applications in skin regeneration and wound healing[59,60]. SF is modified by grafting, coupling reaction, and amino acid modification compared with fibrin, hyaluronic acid, and collagen[61]. Silk fibroin-gelatin (SF-Gel) hydrogel bioink is one of the most commonly used combinations. This is because of their inherent biocompatibility, bioactive signatures, binding affinity for cells, and tunable mechanical properties. Castilho *et al.*[62] fabricated a new type of photocrosslinkable bioinks based on proteinaceous polymers, namely gelatin and silk fibroin, and allowed the 3D writing of microscale, cell-laden fibers through a cell electro-writing process (CEW). They were found to have good mechanical properties, reduced cell-filled fiber size (5–40 μm), and wonderful resolution and patterning accuracy compared to conventional extrusion bioprinting. These significant features of the new photosensitive hydrogel bioinks and CEW processes will allow the creation of micro-structured scaffolds that can better mimic the cellular microenvironment of regenerative medicine (RM) (e.g., muscle fibers, tendons, and neural networks) and organ-on-a-chip models.

3.1.4. Decellularized extracellular matrix

While a variety of hydrogels are already being used by many researchers today to configure bioinks for 3D bioprinting, decellularized extracellular matrix (dECM)-based bioinks with tissue specificity are gaining popularity. The dECM of chemically- and physically-removed cells resembles the ECM of target tissues in terms of chemical composition and structural complexity in an idiosyncratic environment[63]. However, it is difficult to use dECM alone as a bioprinting ink due to its poor mechanical stability[64]. Therefore, dissolved dECM is often mixed with other polymers or printed with other structural support materials. Zhuang *et al.*[65] mixed dECM and modified gelatin with nanoclay to make a new bioink. The concentration of dECM was as high as 75%, and the composite bioink maintained good printing performance while achieving high hepatocyte activity. Khati *et al.*[66] blended decellularized liver matrix (dLM) with gelatin and PEG for 3D bioprinting. The addition of gelatin and PEG improved the rheology, printability, and mechanical stability of the bioink, and the 3D-printed structure and dLM-rich growth factors supported the growth of HepG2 cells and improved the cytocompatibility of the hydrogel. The highly crosslinked dLM-G-PEG laid the foundation for subsequent toxicological studies on HepG2 cells.

3.1.5. Collagen

As one of the major components of dECM, collagen is the most widely distributed protein in the body. Its presence in all connective tissues makes it the most studied biomolecule in the ECM[67]. Collagen is widely studied and applied in tissue engineering and regenerative medicine because of its biodegradability, biocompatibility, low immunogenicity, and easy availability. However, after applying them to 3D bioprinting, researchers found that, similar to dECM, collagen often exhibits poor mechanical properties and structural stability[68], so exogenous crosslinking methods have been introduced to increase the crosslink density to improve its mechanical properties and printability. Chemical methods often include the introduction of chemical crosslinking agents such as aldehydes[69], genipin[70], and carbodiimide[71] to enhance the mechanical properties of collagen. Physical methods such as UV irradiation[72] and dehydrogenation heat can eliminate the negative effects of using chemical crosslinking agents. Serna *et al.*[73] introduced a photo-reactive agent, riboflavin, which was used in conjunction with UV irradiation to enhance the mechanical properties of collagen. However, physical methods inevitably cause conformational changes in the polypeptide chains as well as collagen denaturation[74]. In addition to the above-mentioned exogenous crosslinking methods, combining collagen with some polymeric materials (e.g., sodium alginate) can also improve its mechanical properties and printability. Clark *et al.*[75] modified collagen and mixed it with different concentrations of thiolated HA 3:1 and loaded HepG2 and patient-derived glioblastoma multiforme (GBM) cells. The concentration of HA was finally optimized to 15 mg/mL to match the bioprinting, and the printed organoid had excellent mechanical properties and could be used in subsequent drug screening applications.

In summary, most of the above-mentioned natural polymer-based hydrogels as bioinks for 3D bioprinting have good biocompatibility, low immunogenicity, and low inflammation as well as suitable biodegradability, and these advantages make them widely used in tissue engineering and regenerative medicine. However, as they mostly exhibit weak mechanical properties and poor structural stability, it

is difficult to use them alone as bioprinting inks, so many researchers introduced exogenous crosslinking methods or mixed natural polymer-based hydrogels with other polymers or printed them with other structural support materials, and the hydrogels can be tissue-specific adjusted to the specific repair site and repair modality. For example, genipin crosslinking of gelatin will lead to reduced swelling and increased hardness of gelatin due to the powerful crosslinking effect of genipin. Therefore, it is more suitable for the repair of hard areas such as bone tissue. In contrast, crosslinking of gelatin with mTG provides gelatin with uniform pore space and higher swelling properties, making it more suitable for repair of soft tissue sites and endowing it with the ability to load or encapsulate other substances such as drugs or cytokines. These approaches provide useful assistance for people to use natural polymer-based hydrogels as bioinks for 3D bioprinting in the future.

3.2. Synthetic polymer-based hydrogel

The most common synthetic hydrogels are alcohols, acrylates, and their derivatives, such as PEG and PEGDA, polyacrylamide (PAAM), and polyurethane (PU)[39,53,76]. Besides, Pluronic is also a common sacrificial material. We can process and modify natural hydrogels according to requirements.

3.2.1. Polyethylene glycol

PEG has good biocompatibility, non-immunogenicity, and non-toxicity, but due to a lack of cell adhesion sites on the surface, it cannot provide a suitable growth environment for cells. Therefore, it is often modified by blending, grafting, and interpenetrating with natural polymer hydrogels to optimize the performance of hydrogels[39,77]. PEG and SA can form an interpenetrating network to stably keep the 3D structure of the hydrogels in the form of covalent crosslinking. Different molecular weights of PEG and different crosslinking agents have a tremendous impact on the elasticity of the hydrogel. The application of Ca^{2+}-containing crosslinking agents in PEG-SA hydrogels can significantly increase its fracture energy. Conversely, with the molecular weight of PEG increasing, the length of the polymer chains also increases, resulting in higher tensile properties and higher fracture energy of the hydrogel[53]. PEG is usually used as a light-curing material after modification. PEG derivatives are mainly PEG acrylates, which include PEGDA, PEG dimethacrylate (PEGDMA), and multi-arm PEG acrylates[39,78]. As a bioink for 3D bioprinting, a blended solution of chitosan and PEG acrylic hydrogel is a potential candidate material. Morris *et al.*[39] used chitosan and PEGDA as bioinks to print scaffolds through stereolithography technology, and its mechanical strength met the requirements of cartilage tissue engineering. Because the molecular weight of chitosan affects the stability of the scaffolds, they first tested the high-molecular-weight and low-molecular-weight chitosan materials. Then, the ratio of the materials was optimized. Under low ratios (1:5 and 1:10) and high ratios (1:10 and 1:15), the results showed that high-molecular-weight chitosan has lower mechanical properties and smaller cell adhesion. However, low-molecular-weight chitosan exhibits a good outcome. In the end, they chose low-molecular-weight chitosan and PEGDA to study the optimal ratio, which is 1:7.5.

The bioink of this ratio is suitable for the printing of complex shapes. They created a human ear-shaped scaffold that showed interconnected porous structures after freeze-drying. Human bone marrow mesenchymal stem cells (hBMSCs) can adhere to the scaffold and proliferate. Jia *et al.*[78] designed a hybrid bioink composed of 4-arm poly (ethylene glycol)-tetraacrylate (PEGTA) and other hydrogels combined with a multi-layer coaxial extrusion system. Through ionic bonding and covalent photocrosslinking, a highly organized perfusion vascular structure is formed. The addition of PEGTA enhances the mechanical properties of the scaffold. Compared with linear PEG molecules, PEGTA scaffold allows better cell growth.

3.2.2. Pluronic

Pluronic, which is the trade name of poloxamer, is a synthetic block polymer composed of a hydrophobic polypropylene oxide (PPO) block and two hydrophilic polyethylene oxide (PEO) blocks. The Pluronic gel is a temperature-sensitive polymer with reversible gel properties; the gelation temperature of it depends on its type and concentration. Unlike gelatin and SA hydrogel, Pluronic is liquid at low temperatures (usually 4°C), forms a physical gel at high temperature (37°C), and can be dissolved in deionized water. Therefore, after printing scaffolds, lowering the temperature can remove Pluronic smoothly[79-81].

Pluronic materials show better results in cartilage tissue engineering. The high concentration of Pluronic can meet the rheological and gelling conditions required for extrusion printing. For example, Müller *et al.*[80] proposed a nanostructured method that can meet the performance requirements of Pluronic F127 gels during the entire bioprinting process. The pure Pluronic F127 hydrogel cannot culture cells for a long time. But after acrylate modification, a high concentration of Pluronic F127 is used in the printing process, and then it is eluted for chondrocyte culture. The results showed that cell viability increased from 62% to 86%. Besides, to improve the mechanical properties of Pluronic F127 hydrogel, a photocrosslinked hyaluronic acid methacrylate (HAMA) is mixed to form a stable network structure. Pluronic F127 is usually used as

a sacrificial material for constructing hollow blood vessel channels to simulate bionic blood vessels in the bioprinting of tissue engineering[82,83]. Karyappa *et al.*[84] used a low-viscosity, commercial polysiloxane resin (Ecoflex 10) as shell inks in conjunction with a coaxially extruded core fluid (Pluronic F127) for core–shell 3D printing in a Bingham plastic microparticle gels (ethanol gel). They wisely selected appropriate rheological properties and flow rates of the three phases, which allowed the formation of droplets composed of a core liquid distributed along the printed filament. The versatility of eCS3DP provides a simple way to fabricate 3D structures of a soft elastomeric matrix with embedded channels and paves the way for future fabrication of 3D structures with internal channels.

Compared to natural polymer-based hydrogels, although some synthetic polymer-based hydrogels have been widely used in tissue engineering and regenerative medicine, their safety and long-term effects still need to be more rigorously evaluated and monitored. In addition, the preparation of synthetic polymer-based hydrogels is more complex, requiring sophisticated instrumentation and technical support, and is therefore more costly, limiting its popularity in large-scale applications. However, synthetic polymer-based hydrogels also have some unique advantages, e.g., they are often highly tunable, and their physicochemical properties can be controlled by adjusting parameters such as composition, concentration, and degree of crosslinking of the material to give different mechanical properties, pore structure, and bioactivity[22]. In addition, synthetic polymer-based hydrogels can be precisely positioned and molded by 3D printing technology, allowing the preparation of tissue engineering constructs with complex structures and fine morphology, achieving high 3D printing accuracy[39,77]. Researchers can also provide cell adhesion sites for scaffolds through a customized approach to promote cell growth and differentiation, contributing to tissue regeneration and repair. Overall, finding some natural polymer-based hydrogel bioinks with better biocompatibility and lower immunogenicity, as well as reducing printing and fabrication costs, are top priorities for the future of natural polymer-based hydrogels as 3D bioprinting inks for wider use in tissue engineering and regenerative medicine.

3.3. Modified natural hydrogel

The modified natural hydrogels are obtained by chemically modifying the functional groups. The modified hydrogels still maintain the excellent biocompatibility of natural hydrogels and also has properties such as tunable mechanical strength. Generally, natural hydrogels are modified with methacrylic anhydride reagents. Such materials include gelatin, chitosan, and silk fibroin.

3.3.1. Modified gelatin

Among the numerous modified hydrogels, modified gelatin is one of the most widely used modified natural hydrogels, namely methacryloyl gelatin. Gelatin is a commonly used soft tissue repair material, but gelatin only physically crosslinks at low temperatures to form a hydrogel network and dissolves at 37°C. Therefore, after methacrylic anhydride modified gelatin, it becomes a photosensitive hydrogel material triggered by a photoinitiator. The reaction time under UV-visible light is 3 to 5 s, and the properties are stable. So far, the most widespread printing method for gelatin methacrylate (GelMA) hydrogels is extrusion bioprinting[85,86].

Although GelMA can form a chemical bonding hydrogel under UV light with the help of a photoinitiator, direct bioprinting is still somewhat difficult, because the shear-thinning behavior at 37°C is insufficient, and the viscosity is not enough to support printing, resulting in a low resolution[87]. The commonly used solution is to compound the GelMA hydrogel with other biological materials to improve its defects. Rastin *et al.*[22] summarized four different additives, i.e., polymers, fillers, particles, and fibers, which are often introduced into hydrogels to improve hydrogel bioinks with different functions. They added methylcellulose (MC), a water-soluble polymer, to improve the printability of GelMA. At the same time, GelMA hydrogel as a tackifier improves the thixotropic behavior of MC during the printing process and slows down the degradation rate of MC. Figure 2B(a) shows the complete process of printing and UV crosslinking. The rheological properties and mechanical properties of different ratios of MC/GelMA hydrogels are shown in Figure 2B(b). The addition of MC makes the hydrogel's stress larger, and the compression modulus (15 ± 1.2 KPa) of MC8/GelMA5 is three time higher than pure GelMA5 (4.5 ± 0.2 KPa). Moreover, the increase in GelMA composition also increases the compressive modulus. Pure GelMA hydrogel extrusion is a droplet whose viscosity is too low for printing. After adding MC, at a lower shear rate, different ratios of MC/GelMA bioinks have higher shape fidelity and viscosity after printing; at a higher shear rate, there is a similar viscosity, which shows shear thinning. Compared with pure MC, the addition of GelMA will increase the pressure during extrusion printing and improve the resolution of printing. However, when the concentration of GelMA is very high, the yield stress will be higher, and the hydraulic pressure of the extrusion printing will also be higher. The GelMA hydrogel becomes a solid filament and will maintain this structure that is difficult for the crosslinking reaction. Therefore, they chose MC8/GelMA5 as the optimal formulation for printing a four-layer grid structure, and 100-layer cylindrical and hexagonal two-

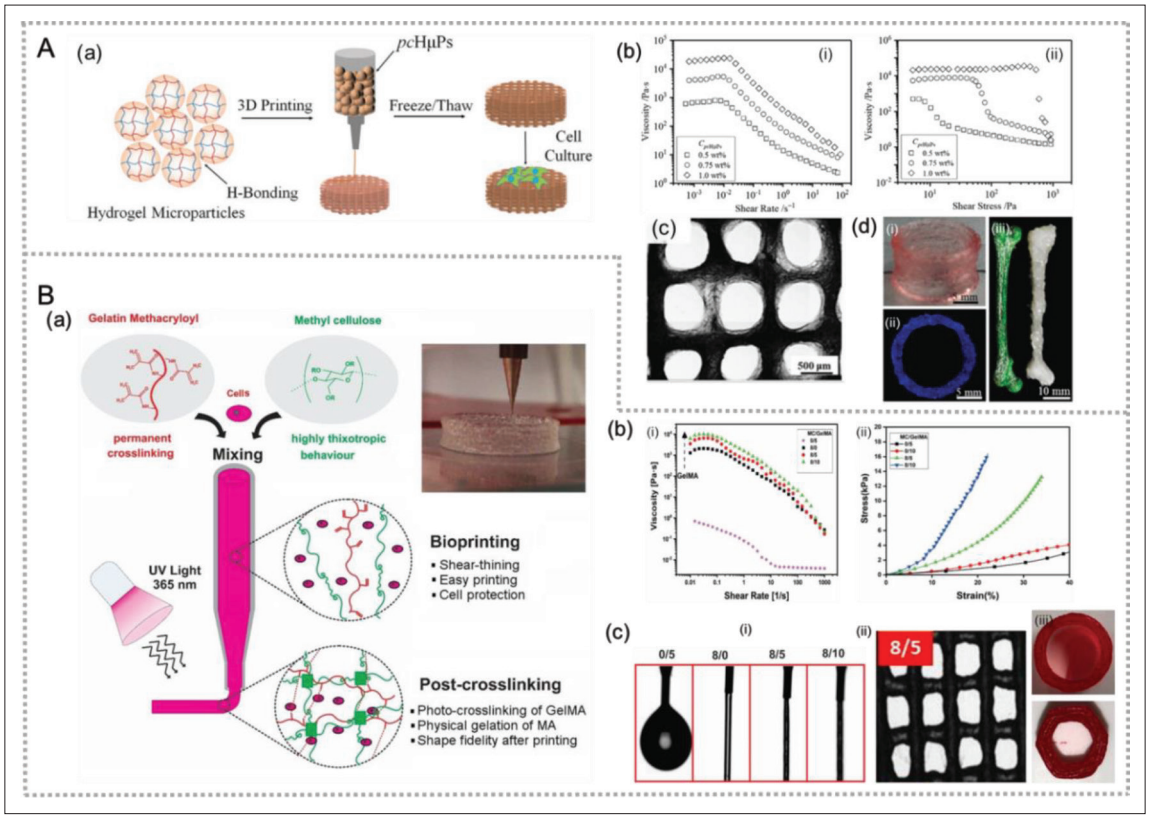


Figure 2. Bioprinting of modified chitosan and gelatin. (A) (a) Schematic diagram of DLP-printed chitosan hydrogel particles (CHI-MA) scaffold. (b) Rheological characteristic curve. (c) Single-layer grid structure. (d) 3D-printed bionic structure showing shape integrity. Reproduced with permission[88]. (B) (a) Schematic diagram showing extrusion-printed cell-loaded MC/GelMA hydrogel and crosslinking. (b) Rheological properties and stress–strain curve. (c) (i) Different ratios of MC/GelMA extrusion state; (ii) the mesh structure of MC8/GelMA5; (iii) the shape integrity of cylinders and hexagonal prisms. Reproduced with permission[22].

dimensional (2D) and 3D structures exhibit high shape integrity and sufficient strength to withstand the pressure (Figure 2B). It is a meaningful modification to the printing of GelMA hydrogel, which improves its functionality in 3D bioprinting.

To improve the printing performance of GelMA hydrogel, Ying *et al.*[35] mixed GelMA hydrogel and PEO to form two incompatible aqueous phases and used this new type of aqueous two-phase emulsion for extrusion bioprinting. Then, crosslinking was carried out to prepare porous hydrogel scaffolds through *in situ* UV light for 15 s. Compared with scaffolds printed by pure GelMA hydrogel, the scaffolds printed using this emulsion bioinks show a high degree of interconnection and integrity of the pore structures and have good printability. They optimized and tested different ratios of bioinks, and the results showed that the scaffold prepared with the ratio of 10 % w/v GelMA and 1.6 % w/v PEO is more suitable. When the volume of GelMA-PEO emulsion is 4:1, the pore size is smaller and the uniformity is higher. When the temperature is lower, the viscosity of the emulsion is higher, which is not the suitable bioinks for printing (the temperature of pure GelMA hydrogel suitable for extrusion printing is generally 15°C). Therefore, they selected the volume ratio of 1:1 for GelMA-PEO two-phase emulsion for testing cell culture and printing. GelMA-PEO hydrogel scaffold with porous structures exceedingly promotes the growth and diffusion of encapsulated cells and demonstrates high biocompatibility (Figure 3).

After improving the printing performance of GelMA hydrogel, researchers will also manage to improve the high fidelity and the shape integrity of the printed structure of GelMA bioinks. Liu *et al.*[85] obtained GelMA bioinks, namely GelMA physical gels (GPGs), by a simple physical cooling process. The GPGs bioink is directly printed based on extrusion printing. The printing and preparation process of GelMA hydrogel bioink containing human umbilical vein endothelial cells (HUVECs) is shown in Figure 4(a). After optimizing the concentration of hydrogel, GPGs bioink has shear thinning characteristics and self-healing ability at low concentration (<3 %). It forms a soft overall structure and maintains its structure during extrusion

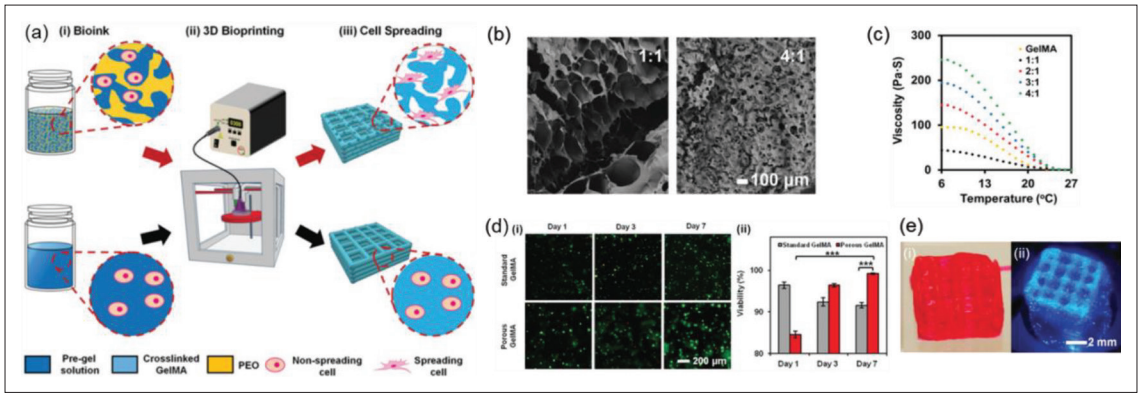


Figure 3. (a) A schematic diagram of the 3D bioprinting of the porous hydrogel structure of the two-phase emulsion bioink (top) and the conventional hydrogel structure (bottom). (b) SEM showing GelMA and PEO porous GelMA hydrogel with a volume ratio of 1:1 (left) and 4:1 (right). (c) The viscosity of different proportions of GelMA-PEO emulsion changes with temperature, and the viscosity of 5% pure GelMA is used as the control group. (d) Fluorescence micrograph showing the viability of HepG2 cells (human liver cancer cell) encapsulated on day 1, day 3, and day 7. The control group is the same as the above. (e) The printed scaffold structure: (i) pure GelMA and (ii) GelMA-PEO hydrogel. Reproduced with permission[35].

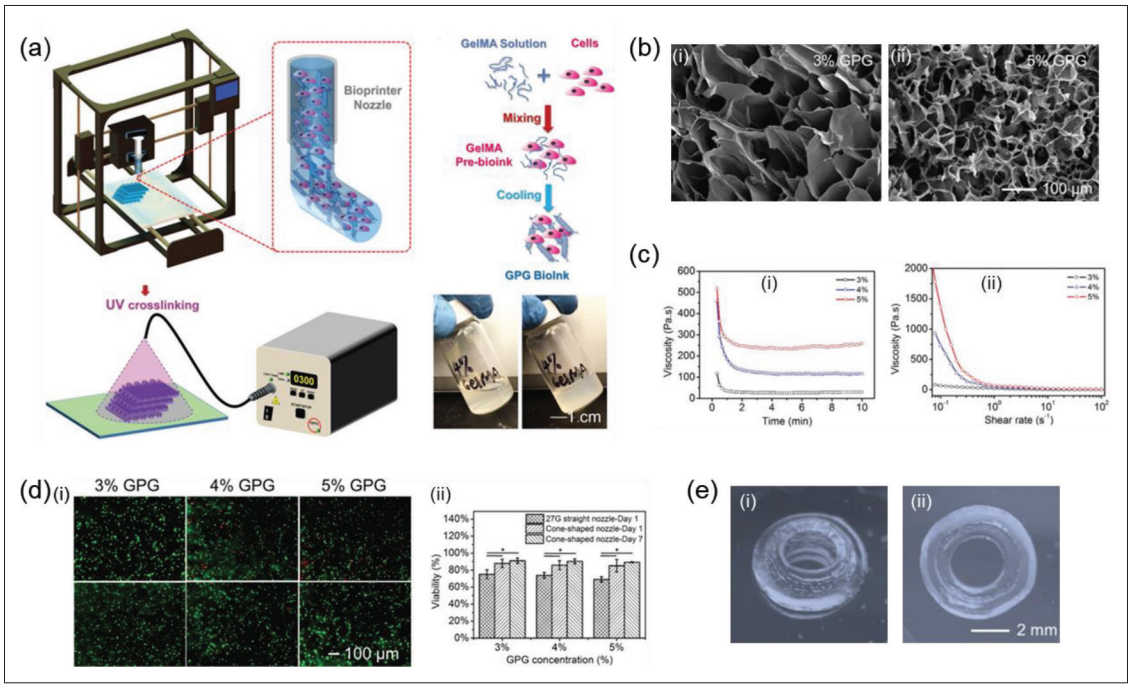


Figure 4. (a) Schematic diagram of GelMA physical gels (GPGs) bioprinting. (b) SEM showing porous structure with 3% and 5% GPGs concentration. (c) Rheological characteristic curves of different concentrations of GPGs. (d) Cell live/dead staining: straight nozzle (top) and tapered nozzle (bottom) to print cell viability test in different concentrations of GPGs hydrogel scaffold. (e) A tapered tube printed with 4% GPG bioink maintaining a complete shape. Reproduced with permission[85].

printing and deposition. Then UV crosslinking is permanently stable. They found that the scaffold structure printed with low-concentration GPGs bioink has a smaller pore size, higher porosity, and lower stiffness (compression modulus of 1.8 KPa). Among the 3%, 4%, and 5% GPGs bioinks containing cells, low concentrations of GPGs can achieve better cell viability and promote cell proliferation and differentiation. They used 4% GPGs bioink to print a tapered tube with a layer height of 16 layers and a wall thickness of 0.4 mm. The structure has good fidelity and will not deform (Figure 4). This new strategy for preparing GelMA physical gels is promising to develop the scaffolds with high-fidelity structure and high cell activity to improve some of its previous shortcomings.

3.3.2. Modified chitosan

Chitosan (CH/CS) has good biocompatibility, biodegradability, strong hydrophilicity, and antibacterial

properties. In addition, chitosan is also polycationic, and its positive charge can generate electrostatic interactions with negatively charged ECM molecules. Chitosan interacts with the negatively charged cell membrane of microorganisms, causing the death of bacteria. Therefore, this antibacterial property has expanded its application in the medical field[89,90].

After the chitosan is modified, its performance will be greatly improved. After being methacrylated, chitosan can be used as a light-curable material for printing and crosslinking. Chang *et al.*[91] synthesized a water-soluble methacrylated glycol chitosan (MeGC) and produced an MeGC-based bioink loaded with MG-63 cells using a visible light curing system with 12 μM riboflavin as a photoinitiator. They made the MeGC solution by adjusting the pH of the solution in the first step; in the second step, MG-63 cells were suspended in a 3% MeGC solution containing 12 μM riboflavin and cured at 430–485 nm visible light for 30 to 90 s using a visible light irradiator, and finally the hydrogel containing the cells would be 3D-bioprinted. The results showed that the survival and value-added of MeGC-70 were high, and the rheological properties of the bioink aqueous solution were optimized. CH materials can not only be printed in the form of liquid bioink, but can also be extruded in the form of solid bioink. Zhang *et al.*[88] used hydrogel particles as the bioink for extrusion-based 3D bioprinting. The particles are composed of chitosan methacrylate (CHMA) and freeze-thawed polyvinyl alcohol (PVA). Under the action of chemical crosslinking and physical crosslinking, the hydrogel was manufactured with fast self-healing and adjustable mechanical properties (Figure 2A). The CHMA/PVA composite hydrogels are broken into particles during extrusion and then a printable hydrogel is formed through the hydrogen bonding between chitosan and PVA chains. The innovative self-healing hydrogel particles exhibit excellent shear thinning, gel-sol transition, and good yield strength during extrusion printing. Besides, the self-supporting scaffold can adequately induce the growth, proliferation, and differentiation of bone marrow-derived MSCs. In addition to the above modification methods, mixing chitosan with other polymers, such as PEG, to prepare bioinks is also an effective way[39].

3.3.3. Modified silk fibroin

In addition to gelatin and chitosan, many researchers also have modified silk fibroin to improve their mechanical properties and to modulate their degradation rates. It is usually modified with glycidyl methacrylate (GMA) as a photocrosslinked hydrogel for DLP-based printing[34,92]. Kim *et al.*[92] prepared SF-based bioink (Sil-MA) and adjusted the rheology of the hydrogel by changing the ratio of Sil-MA to adapt to DLP-based printing. Among them, 30% of Sil-MA has suitable printability and good shape recovery. They fabricated a cricoid trachea by DLP-based printing for *in vitro* testing. Sil-MA hydrogel provides a suitable environment for the growth of chondrocytes and the formation of cartilage *in vitro*. The scaffold exists *in vitro* for up to 4 weeks and degrades up to 50%.

4. Nanocomposite hydrogel bioink

Despite the continuous investigations on natural and synthetic hydrogels in recent decades, it is still challenging to prepare tissue engineering scaffolds using single or mixed hydrogel materials, and some of the problems include weak mechanical properties, low cell activity, and poor processability. These difficulties have prompted researchers to find some suitable nanomaterials combined with hydrogels to improve the properties of hydrogels. Nanocomposite hydrogels have a wide range of applications in the fields of tissue engineering and regenerative medicine[93]. Hassan[12] *et al.* summarized the methods and applications of nanomaterials in compounding hydrogel biopolymers. They found that the main natural biopolymers are SA and collagen derivatives. This part reviews the commonly used nanomaterial composite hydrogels, such as inorganic nanomaterials, carbon-based nanomaterials, and nanofiber-based materials. A few researchers also use gold nanomaterials mixed with hydrogels as printed bioinks[94,95].

4.1. Inorganic nanocomposite hydrogel

Bioactive glass (BG), hydroxyapatite (HA), and Laponite are common inorganic nanomaterials with osteoinductive properties. Therefore, inorganic nanomaterials are usually mixed with hydrogels to repair bone and cartilage. According to related reports, the bioactive ions released by the BG during degradation can promote cell adhesion, proliferation, and differentiation, and accelerate tissue vascularization and increase expansion force. It is currently the only material that can be combined with bone tissues and connected with soft tissues[96]. Tissue engineering has developed rapidly in the field of regeneration of hard tissues, such as bone and cartilage, but bone graft therapy is very limited in clinical practice. Bones have strong self-regeneration ability, and self-recovery is more successful for small-scale bone defects. However, for large-scale bone defects that cannot be cured on their own, external bone scaffolds are needed to help bone regeneration and healing. Bone scaffolds need to meet some specific requirements such as bone conduction, controllable pore size, mechanical properties, and biocompatibility similar to natural bone, biodegradability, and adsorption capacity[97].

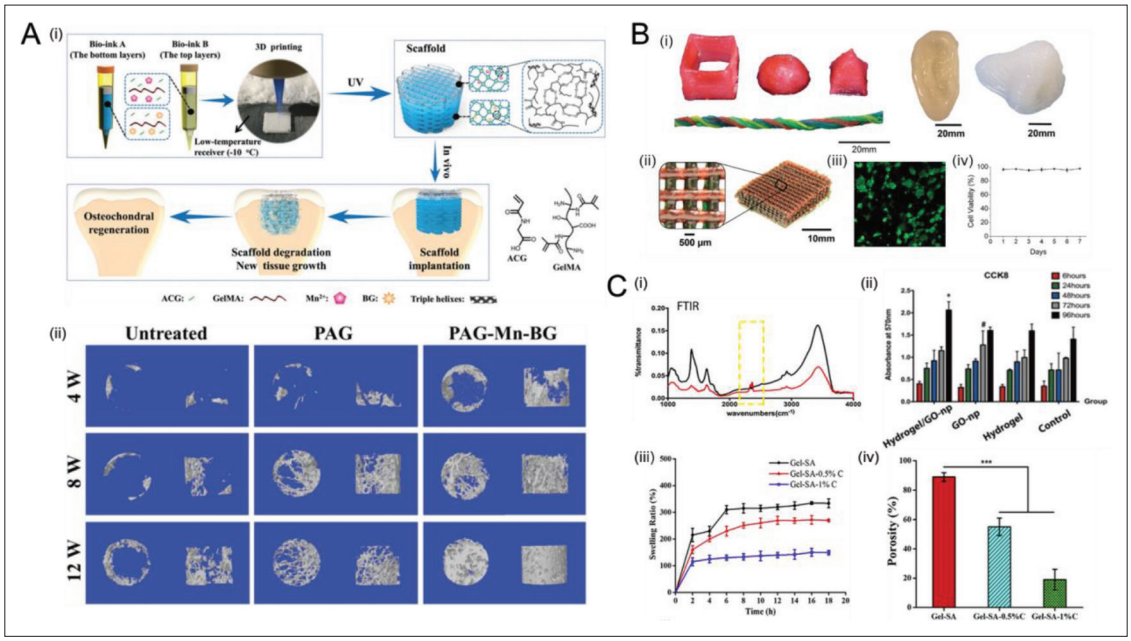


Figure 5. (A) (i) Bioprinting a scaffold with PACG-GelMA hydrogel-Mn^{2+} as the top layer and PACG-GelMA hydrogel-BG as the bottom layers; (ii) schematic diagram of repairing osteochondral defects. Reproduced with permission[100]. (B) 3D bioprinting of PEG-SA-nano clay hydrogel. (i) 3D bioprinting of different shapes: hollow cubes, hemispheres, pyramids, twisted ear shapes, and noses; (ii) meshes with tough and biocompatible hydrogels; (iii) live and dead staining of HEK cells; (iv) HEK cell viability at 7 day. Reproduced with permission[53]. (C) (i) Infrared spectrum: hydrogel (black line) and hydrogel attached to GO-np (red line); (ii) chondrocytes in hydrogel, GO-np, hydrogel/GO–vitality test of np, and blank group. Reproduced with permission[105]. (iii) Swelling ratio of printing and (iv) porosity of Gel-SA hydrogel scaffold containing CNTs. Reproduced with permission[106].

4.1.1. Bioactive glass

Bioactive glass (BG) is a silicate glass composed of SiO_2, Na_2O, CaO, etc. It has good biocompatibility, bioactivity, and degradability. The elements released during degradation can form a hydroxyapatite (HA) layer on the surface of the material similar to natural bone, which then forms a strong chemical bond with the adjacent bone surface and can promote the formation of new bone[98]. Ding *et al.*[99] fabricated a framework-filled structure consisting of polylactic acid (PLA), BG, and bone cement. The results showed that the scaffold provides instant and stable fixation of the bone defect. It is also characterized by continuous osteogenesis induction. The sustained degradation of PLA + 1% BG composite scaffold (PBG) provides space for bone growth. Gao *et al.*[100] used the 3D bioprinting of poly (N-acryloyl 2-glycine) (PACG) and GelMA hydrogel to obtain a biodegradable and supramolecular hydrogen bond-enhanced crosslinked gelatin hydrogel scaffold. To better promote osteochondral regeneration, they added biologically active manganese ions (Mn^{2+}) and BG to the scaffold materials, forming PACG-GelMA hydrogel-Mn^{2+} as the top layer while PACG-GelMA hydrogel-BG is the bottom support. The scaffold was applied to living cartilage repair, and it was found that the incorporation of BG improved the proliferation and differentiation of hBMSCs. Loaded Mn^{2+} can promote the chondrogenesis and differentiation of hBMSCs. After being implanted in the body for a period of time, it shows excellent repair performance for osteochondral defects. Also, regenerated cartilage and subchondral bone were apparently observed in the rat model. Therefore, in the process of bone repair and regeneration, BG showed obvious proliferation and differentiation effects on hBMSCs cells (Figure 5A).

4.1.2. Hydroxyapatite (HA)

HA is the main inorganic component of animal bones, so it is widely applied in hard tissue engineering such as bones and teeth. Aihemaiti *et al.*[101] optimized the construction parameters of 3D PLA/HA composite bone plates through many experimental tests and analyzed the effect of HA content on the flexural strength of the specimens. When the HA content is 20%, the cross-section is rough compared to pure PLA (0% HA) and 10% HA specimens. In addition, it contains a large amount of dents and pores, which reduces the bending properties of the specimens. Ergul *et al.* bioprinted CH/PVA scaffolds containing HA and tested the performance of HA scaffolds with different ratios[102]. They found that 15 wt% HA and CH/PVA hydrogels have obvious advantages as bioinks. The elastic modulus of the scaffold can reach 91.14 MPa, which is close to the elastic modulus of natural bone. Then, bone morphogenetic protein-2 (BMP-2) was inoculated onto

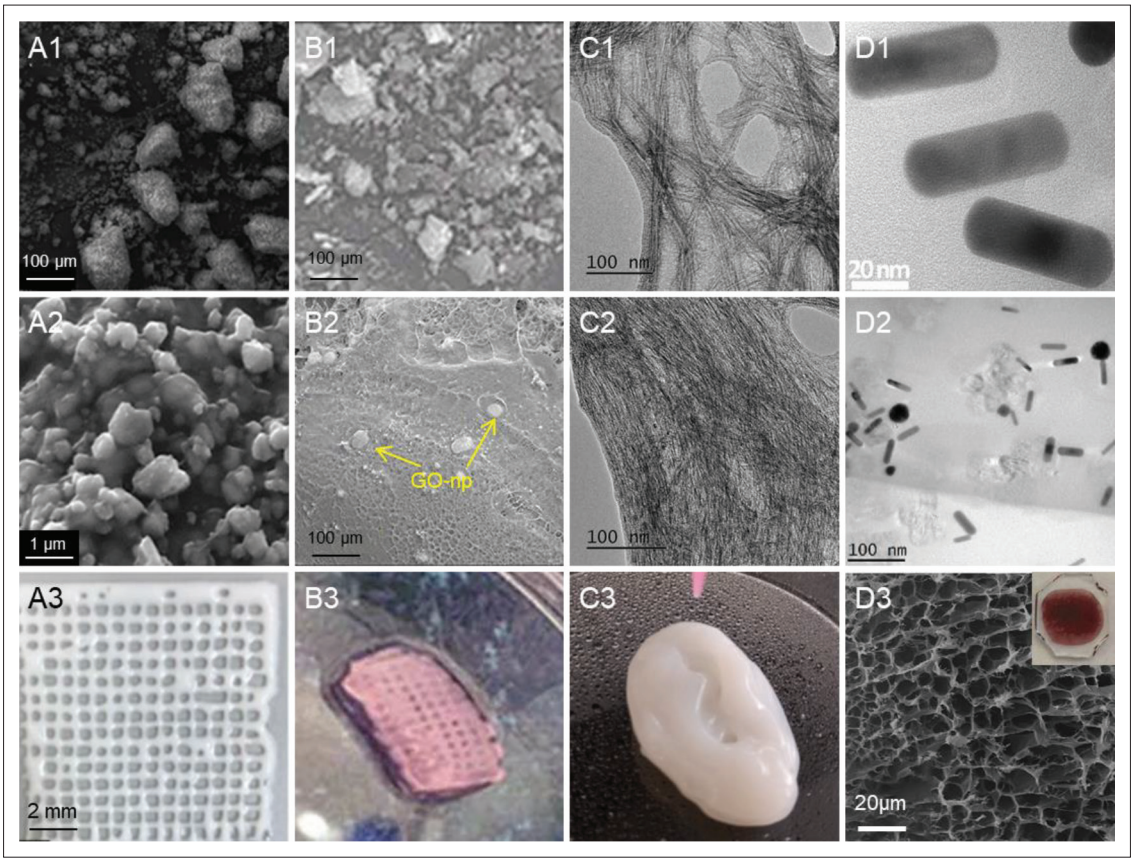


Figure 6. The images of nanoparticles. SEM showing (A) the topography (A1, A2) and the printed grid structure (A3) of HA at low and high magnification. Reproduced with permission[122]. (B) GO-np particles and GO-np loaded in the hydrogel (B1, B2) and printed grid structure (B3). Reproduced with permission[123]. TEM showing (C) nanocellulose (NCB) before (C1) and after crosslinking (C2) and printed human ear shape (C3). Reproduced with permission[28]. (D) Gold nanorods (GNRs) and the cross-sectional pore structure of the GNRs and the scaffold in the hydrogel. Reproduced with permission[124].

the scaffold. The CH/PVA/HA (15 wt%)/BMP-2 scaffold shows good biocompatibility and promotes the attachment and proliferation of human MSCs[102] (Figure 6A). Hydroxyapatite is an extensive additive component of bone grafts, and the scaffold mixed with hydrogel for printing is a promising treatment method in the field of bone tissue engineering.

4.1.3. Laponite (clay)

Among the scaffold materials used in bone and cartilage tissue engineering, nano-Laponite is one of the most promising materials due to its excellent biocompatibility, biodegradability, and non-toxic degradation products. Because its surface has positive and negative charges, it can form gels with other materials through electrostatic interactions, meeting the shear-thinning properties of bioinks in bioprinting, with larger specific surface areas and lower production costs[21,103]. Laponite is nanosized lithium–magnesium–sodium silicate of the smectite group, which is a synthetic material. Mixing with biofriendly polymer materials can change its mechanical and rheological properties. Laponite nanoparticles are used as a physical crosslinking agent, which is an ideal crosslinking method. Reacting with hydrogels will not produce toxic by-products[104].

Laponite silicate clay, as filler in the SA and MC hydrogels, has shown good experimental results in 3D bioprinting and drug delivery. Ahlfeld *et al.* used squeeze printing to achieve the high fidelity of the printing bracket and improve the printability of the hydrogel[21]. Human mesenchymal stem cells (hTert-MSCs) encapsulated in hydrogels showed high cell viability after 21 days of culture. Besides, bovine serum albumin and vascular endothelial growth factor (VEGF), which is an angiogenesis-related growth factor, are two model proteins. The sustained release phenomenon of loading in hydrogel proves the biological function advantage of Laponite in hydrogel[21]. As we mentioned earlier, the core of successfully printing hydrogels into a 3D structure is the viscosity of the

hydrogel solution and its high shear thinning. In 2015, Hong *et al.*[53] demonstrated that adding nanoclay to a SA-PEG-blended hydrogel solution can adjust its viscosity and improve rheological properties for the first time. They soaked the printed SA-PEG-nanoclay grid scaffold in a collagen solution containing human embryonic kidney cells (HEK). Then, the collagen solution formed gels in the pores of the scaffold, and the cells maintained high viability during the 7-day culture process. The nanocomposite hydrogel is tougher than natural cartilage and has the ability to encapsulate cells. It can be used to print some bionic tissues, such as human ears and noses (Figure 5B).

Overall, compared with the polymer-based hydrogels mentioned above, inorganic nanocomposite hydrogels as 3D bioprinting inks can facilitate repair not only by forming solid chemical bonds with adjacent tissue surfaces through the elements released during their degradation, but also by forming gels with other materials through electrostatic interactions to anchor cells in 3D structures, thus enabling high-fidelity printing[21,103]. However, their more complex preparation process and potential immunogenicity are one of the main reasons why they are currently not widely used in clinical repair.

4.2. Carbon-based nanocomposite hydrogels

4.2.1. Graphene and its derivatives

As the basic structure of graphitized materials, graphene is considered one of the most powerful materials so far. Graphene oxide (GO) and reduced graphene oxide (rGO) are common derivatives. Because graphene has unique physicochemical, biological, and electronic properties, the applications of graphene and its derivatives in the field of biomedicine are mainly in tissue engineering, biosensors, drug delivery, gene therapy, bioimaging, etc.[107,108] In recent years, 2D graphene has been introduced into the hydrogels to form composite materials, which were used as a bioink to obtain a 3D structure through 3D bioprinting technology. This is an innovative and revolutionary technological change, which has broad application prospects in tissue engineering[109,110].

GO can be obtained by oxidative exfoliation of graphite, which is several nanometers to several micrometers in size. It has a variety of chemical functional groups such as carboxyl groups, hydroxyl groups, and epoxy groups, which can combine with various molecules to show strong interaction. Therefore, GO can stably exist in an aqueous solution[111,112]. The nanocomposite formed by GO and the hydrogel exhibits enhanced mechanical properties. Besides, it interacts with the polymer with hydrogen donor/acceptor functional groups in the hydrogel to act as a physical crosslinker through hydrogen bonding. Therefore, the hydrogel can form a stable network structure[113]. Li *et al.*[114] used a combination of 3D-printed GO with SA and gelatin as the basis for a novel bioink to support human adipose-derived stem cells (ADSCs). After investigating the effects of different GO concentrations on cell affinity and viability, they found that GO concentrations in the range of 0.05% to 0.5% (w/w) were widely distributed in the SA/gelatin scaffold and could promote the growth and differentiation of human ADSCs. Cheng *et al.*[115] loaded GO nanoparticles (GO-np) into the hydrogel to protect cartilage tissue through the Rank/Rankl/OPG signaling pathway (Figure 6B). At a wavenumber of 2400 cm^{-1}, it proved that C≡C in GO-np is involved in the adsorption process. CCK8 test shows that GO-np nanocomposite hydrogel is beneficial to improve cell viability (Figure 5C(i) and (ii)). The results showed that GO-np may be used as a carrier for drug delivery to control its release to achieve the purpose of protecting cartilage tissue. GO has certain advantages as a drug carrier, and it can be widely used in the field of biomedicine as an intelligent nanomaterial in future.

GO can not only induce cartilage differentiation, but also has obvious osteogenic differentiation effects on bone regeneration. The composite material of GO combined with SA hydrogel shows a good performance. The bioinks mixed with 3% SA and 0.5 mg/mL GO combined with MSCs were printed into a 3D scaffold. MSCs showed good proliferation and high survival rate in an oxidative stress environment. The addition of GO overcomes the disadvantages of low printing quality and poor structural stability of SA hydrogel to a certain extent, which could enhance the mechanical properties of the hydrogel scaffold, promote cell proliferation, and induce osteogenic differentiation[116]. It can be seen that the nanocomposite of GO and hydrogel polymer has the potential to become a candidate material in bone tissue and cartilage tissue engineering. Besides, in the field of neural tissue engineering, graphene and GO are suitable for printing neural tissue structures containing stem cells. It has been proven that an extremely lower content of graphene or GO (25 ppm) mixed with biodegradable PU hydrogel can be used for the bioprinting of neural stem cells. To reduce the toxic effects of graphene on cells, a layer of Pluronic was coated on the surface. This research proposes a successful solution to the major cytotoxicity problem of graphene-based materials. The rheological properties of this graphene-based composite nanomaterial provide a suitable living environment for cell survival, increase cell oxygen metabolism, and have a significant neurological differentiation phenomenon[117].

4.2.2. Carbon nanotubes

Like graphene and its derivatives, carbon nanotubes (CNTs) also have excellent electrical conductivity, optical

properties, mechanical properties, and other physical and chemical properties and biological properties, and have a wide range of applications in the fields of biomedicine and tissue engineering[12,118]. CNTs include single-wall carbon nanotubes (SWCNTs) and multi-wall carbon nanotubes (MWCNTs). Sanjuan-Alberte *et al.*[119] combined the conductive properties of MWCNTs with the excellent biochemical properties of dECM for the first time. The results after applying certain electrical stimuli to the scaffold show that the combination of conductive material with external electrical stimuli can drive contractile behavior similar to physiological conditions. This suggests that this material has the potential to be used in the future to develop smart scaffolds for biosensing/actuation applications. Li *et al.*[120] used the rotating axis method to print CNT-doped SA-Gel hydrogel artificial blood vessel stents. They proved that the introduction of CNTs enhanced the mechanical properties and deformation recovery ability of SA-Gel hydrogel (Figure 5C(iii) and (iv)). Ho *et al.*[121] studied the potential of bioprinted poly (ε-caprolactone) (PCL)-CNTs composite scaffolds in cardiac tissue engineering. Because the incorporation of CNTs increases the degree of the arrangement of the PCL polymer molecular chains, resulting in better crystallinity, the hardness, elastic modulus, and maximum peak load of the PCL-CNTs composite material are all improved. In the cell viability experiment, by adjusting different concentrations of CNTs, the results showed that the 1 wt% CNT composite material has a proliferation effect on H9c2 cardiac cells. Besides, by adjusting the enzyme concentrations, the degradation rates can be controlled. CNTs can modify the surface of the scaffold to enhance the interaction between nerve cells and biological scaffold materials. The hydrogel mixed with amine-functionalized MWCNTs and a porous structure of nerve scaffold was prepared by stereolithography printing technology[118].

PCL-based polymer materials as scaffolds are widely used in tissue engineering. However, the high hydrophobicity and non-biological activity of the PCL surface will lead to a decrease in cell affinity and further prevent cells from attaching to the surface of the scaffolds. Composite with nanomaterials is one of the ways to solve these problems. The nanomaterials could be graphene, CNTs, nanoclays, and so on. Although graphene and its derivatives have many applications in biomedicine, its potential toxicity has gradually been revealed and attracted people's attention. However, the detailed mechanism behind its toxicity has not been fully discovered. It is worth noting that some researchers have proposed possible mechanisms[110]. For example, the currently widely accepted mechanism of graphene-induced toxicity is the physical interaction with cell membranes. The sharp edges of graphene sheets can damage cell membranes and cause leakage of intracellular substances[109]. Although some studies have reported the osteogenic ability of GO, its osteogenic mechanism is still unclear.

Carbon-based nanocomposite hydrogel materials have been widely used in biomedical applications, including drug delivery and cellular sensors, due to their unique advantages such as excellent optical properties, electrical and thermal conductivity, high mechanical strength, and large surface area. However, it has been demonstrated that carbon-based nanocomposite hydrogel materials lead to an increased production of reactive oxygen species[125], and the concentration increases with the higher concentration of the material. ROS may induce oxidative stress and inflammatory responses, which lead to damage to proteins and cell membranes, and even affect DNA in the nucleus. In addition to the increase in Reactive Oxygen Species (ROS), the aforementioned CNTs and GO also cause an increase in autophagosomes in macrophages[126]. The accumulated autophagosomes will cause cellular autophagy and lysosomal dysfunction, which will further promote ROS synthesis and lead to apoptosis. To mitigate the cytotoxicity of carbon-based nanocomposite hydrogel materials, researchers have found that the toxicity is related to their physicochemical properties, such as particle size, length, and structure[127]. Overall, although carbon-based nanomaterials have many advantages that other materials do not have, they should be used with care so as to reduce or even avoid cytotoxicity; for instance, by modifying the materials or changing their physical structure, they can safely be used in tissue engineering and regenerative medicine.

4.3. Nanofiber composite hydrogels

The typical characteristics of nanofiber materials are large surface area to volume ratio and large porosity. Electrospinning and sol-gel method are commonly used technologies for manufacturing nanofibers. Electrospinning is still the most effective technology for manufacturing nanofibers[128,129]. Nanocellulose belongs to polysaccharides, the novel type of natural nanomaterials, which can be extracted from plant or bacterial biosynthesis. It has good biocompatibility, water holding capacity, stability in a wide range of pH, a nanonetwork structure, and high stiffness and strength[130]. We have been emphasizing the optimization of the hydrogel bioinks formulation. The rheological properties are one of the fundamental factors in the 3D bioprinting process. It is extremely crucial to find a material that can improve the printability of the hydrogel and maintain the fidelity of the shape. This material can also be called a rheology modifier. In recent years, nanocellulose materials are mainly divided into three categories. The first category is

cellulose nanocrystals (CNCs), which are principally used as reinforcement materials for other hydrogels. The second category is cellulose nanofibrils (CNFs), which serve as potential carriers for functional ingredients like proteins. The third category is bacterial nanocellulose (BNC), which shows exceptional potential, but one of the biggest limitations is the technical problem of BNC production at present[131,132].

The addition of cellulose nanofibers to the SA hydrogel can improve the rheological properties of its printing[31,49]. Jessop *et al.*[31] used biomass-derived cellulose nanocrystals (CNCs, 3%), biomass-derived cellulose nanofibers (CNFs, 6%), and a unique mixture of the two (NCB, 3%) as extrusion. For the bioinks, they optimized the formula of the bioinks. By printing a single-layer square grid with a height of 1.7 mm, they tested the resolution of three different formulations of nanocellulose and SA, which showed high resolution. The results showed that the bioink has good shear thinning characteristics and great shape fidelity after printing. Among them, the transmission scanning electron microscope of NCB-AG (Figure 6C, (C1)) shows the entanglement state between the nanofibers before the calcium chloride crosslinking, forming a sparse and scattered structure. Moreover, the pores between nanofibers are very large, resulting in an unstable structure. Figure 6C (C2) is the state after crosslinking. It is obvious that the crosslinking effect of SA entangles nanofibers and CNCs together, forming a dense and firm structure, and the arrangement is orderly. Therefore, they used the NCB–AG combination of bioinks to print several complex shapes, such as hollow and solid cylinders, pyramids, and cubes, as well as human right ear models. They continued to test the compatibility of human wing chondrocytes. The results showed that bioinks provide a suitable environment for cell survival and differentiation while maintaining the shape and structure of the scaffold. Sultan *et al.*[133] used SA/gelatin hydrogel bioinks reinforced with CNCs to form an interpenetrating polymer network structure through a double crosslinking reaction of covalent and ionic crosslinking. Because the crystals are oriented, they found that when the orientation of CNCs is consistent with the printing direction, a scaffold with uniform pore size can be obtained. In short, CNCs not only improve the rheology of hydrogels but also make it easy to print controllable pore sizes and gradient pore structures. Besides, the scaffold is suitable for cell interaction, which once again proves that CNCs have great potential to be used in 3D bioprinting bioinks. Some studies on nanocellulose have found that nanocellulose has great potential as a bioink that can be used for bioprinting. In the future, more tests on printing performance, mechanical properties, and cell compatibility will be needed in this regard.

4.4. Gold nanoparticle composite hydrogels

In the construction of bone and heart tissues, the poor electrical conductivity of polymer-based hydrogel materials is one of the major challenges for their wide application, and this is where the addition of materials with electrical conductivity is needed to induce the formation of new tissues and promote intercellular signaling. There are several types of conductive nanomaterials such as graphene, GO, and CNTs mentioned in carbon-based nanomaterials. However, although CNT is a popular conductive material, its cytotoxicity is controversial. Currently, among various conductive nanomaterials, gold nanomaterials are emerging as the best candidates due to the fact that they often exhibit several attractive properties, including good cytocompatibility, no cytotoxicity, easy preparation and sizing, high reproducibility, and easy surface modification, as well as the ability to propagate electrical signals efficiently[131,132]. It binds to various thiol-containing biomolecules through gold–thiol bonds to promote cell proliferation and increase cell–cell signaling. Overall, gold nanostructures are extremely promising materials for biomedical research, and researchers often use conductive hydrogels containing gold nanorods (GNRs) for cardiac tissues because of their excellent electrical conductivity.

However, except for repair sites that are in need of biomaterials with electrical conductivity, gold nanoparticle composite hydrogels may not be as effective as other polymer-based hydrogels when applied to tissues such as the urethra and skin, due to their lack of bioactivity and relatively uncertain biostability. Therefore, researchers have often incorporated them into many cell-containing bioinks and biomaterials to enhance and expand their functionality and printability for tissue engineering and regenerative medicine applications[134].

In the construction of bone tissues and heart tissues, materials with conductive functions need to be added to induce the formation of new tissues and promote signal conduction between cells. There are several types of conductivity nanomaterials such as graphene, GO, and CNTs, which are mentioned in carbon-based nanomaterials. Besides, gold nanoparticles are also conductive and can transmit electrical signals[135,136]. Although CNT is a popular conductive material, its cytotoxicity is disputed. Gold nanomaterials exhibit some attractive properties, including good cell compatibility, non-cytotoxicity, easy preparation and size, high reproducibility, and easy surface modification. It combines with various thiol-containing biological molecules through gold–thiol bonds to promote cell proliferation and increase cell–cell signal transmission[36]. Gold nanostructures are exceedingly promising materials

in biomedical research, including nanowires, nanorods, and nanospheres[95].

Some researchers used conductive hydrogels containing GNRs in cardiac tissue. Navaei *et al.*[95] developed GNR-GelMA hybrid hydrogels as a functional cardiac patch. When GNRs were introduced, the mechanical and biological properties of the hydrogel were enhanced. High concentrations of GNRs (1 and 1.5 mg/mL) promote the electrical conductivity of the hydrogel, which is conducive to the conduction of electrical signals between cardiomyocytes (CMs) and improves the contractility of the tissue. Figure 6D shows the TEM image of GNRs incorporated into GelMA hydrogel. However, the functional heart patch is a 2D structure, which cannot meet the requirements of certain tissue and cell regeneration in the 3D environment in the body. In 2017, Zhu *et al.*[94] developed a gold nanocomposite bioink for 3D bioprinting. They firstly coated the surface of GNRs to obtain C-GNRs. GelMA molecules are then wrapped on the surface of C-GNRs to obtain G-GNRs, which can be evenly dispersed in water. Next, they mixed G-GNRs (with a concentration of 0.1–0.5 mg/mL) with GelMA and SA polymer solution to obtain gold nanocomposite bioink. They chose 0.1 mg/mL G-GNRs compound hydrogel bioinks to suspend and print the complex structure in the support medium of the gelatin solution. This spiral structure has a high shape fidelity. The CMs and cardiac fibroblasts (CFs) (the ratio of CMs and CFs is 1:1) obtained from newborn rats were jointly loaded into bioinks for printing. After a few days of culture, the cells fuse in the scaffold structure to form a new organizational layer. They also found that G-GNRs can promote signal transmission between heart cells, and the contraction behavior of 3D-bioprinted constructs has been improved, which could be due to the ability of GNRs to inhibit the excessive proliferation of CFs. Compared with the 2D manufacturing of heart patches, 3D-bioprinted heart structures have great advantages in terms of functionality, bionics, and simplicity.

5. Pre-clinical research on printed organs *in vitro*

Although the studies of tissue engineering have only been developed for only three decades, many basic theories have been proposed, and tissue construction and *in vivo* implantation in animals have been vastly conducted a great volume of basic theory, tissue construction and *in vivo* implantation in animals have been accomplished. With the rapid progress of cytology, molecular biology, and biomaterials research, the research and application of tissue engineering of various tissues in the clinic have also made great progress.

5.1. Flat tissue

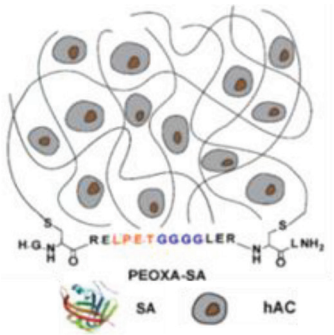
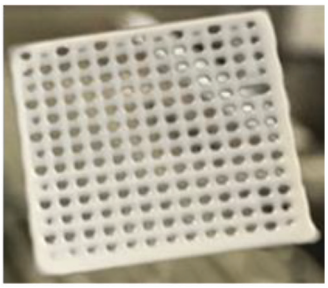
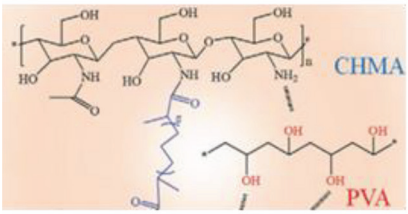
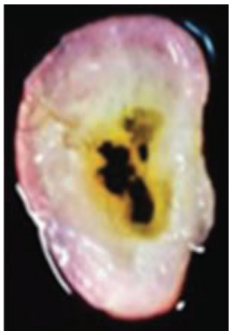
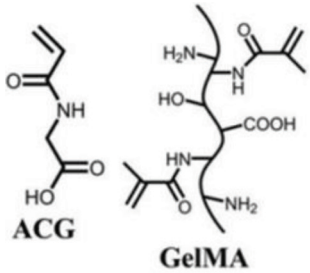
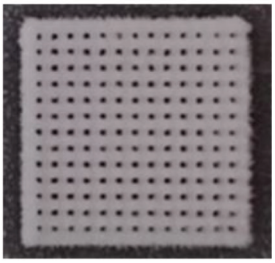
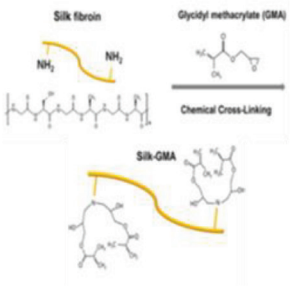
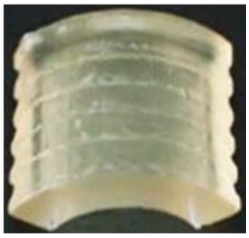
The skin is the largest tissue in the human body and has a complex multi-layer structure. The treatment to severe skin damage due to diabetes, ulcers, and other wounds that cannot heal by themselves is hampered by insufficient skin source for transplantation. Tissue-engineered skin provides a new way of treating skin damage[137,138]. Compared with traditional skin tissue engineering technologies, 3D bioprinting technology has the characteristics of accurate cell positioning and efficient layer-by-layer printing, which can greatly shorten the manufacturing cycle and increase efficiency[139,140]. Despite the considerable benefits in burns and chronic wounds, the current 3D-printed tissue-engineered skins still cannot satisfy the requirements of human skin's functions, as they are incapable of pigmentation and vascularization, and lack of hair follicles and sweat glands[60]. At present, there are still tremendous challenges in manufacturing skins with complete functions. However, bioprinting relying on highly automated devices is conducive to the construction of layered skin tissue composed of a variety of cells and materials, which can enhance the homology with human natural skin and also improve its barrier and complexity functions[141].

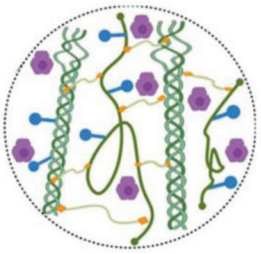
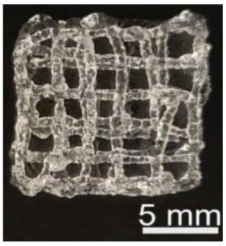
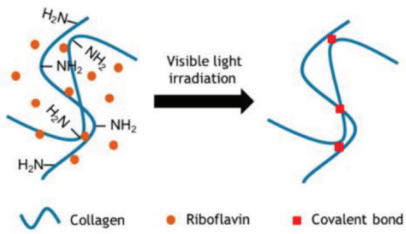
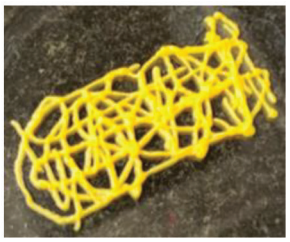
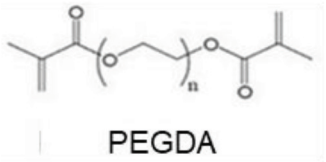
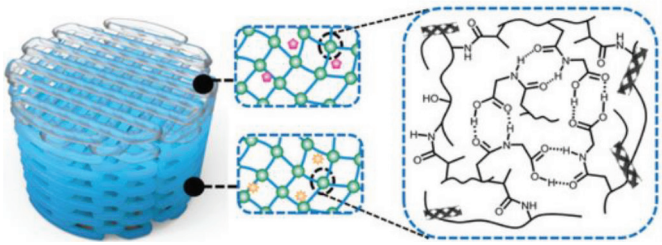
Compared with other printing methods, *in situ* bioprinting has attracted more and more attention from researchers and made great improvements. It can print on the skin and external damaged areas or previously exposed parts of surgery[142,143]. In 2018, the Wake Forest Institute of Regenerative Medicine made important progress in *in situ* bioprinting of autologous skin cells[144]. They used a new mobile skin printing system to quickly print large-scale wounds *in situ*, using autologous dermal fibroblasts and epidermal keratinocytes mixed with hydrogel to form cell therapy. The treatment effect of *in situ* bioprinting on wound defects of rats and pigs was observed. These two kinds of cells can be distributed layer by layer to form a double skin structure. Compared with the untreated control group, *in situ* printing of autologous cells can quickly promote wound closure, prevent wound contraction, and has a layered structure similar to healthy skin regeneration, which can accelerate the formation of normal skin functions (Figure 7A). In 2020, Urciuolo *et al.*[143] performed bioprinting in the tissues of living mice. Cells are loaded with photosensitive biopolymer hydrogels as bioinks, making it possible to create 3D structures and functional tissues in living animals for organ repair or reconstruction. They named this concept "3D bioprinting." In living 3D bioprinting, skin becomes the best target tissue. There is no need for open surgery, the complexity of the operation is minimized, and the success rate of 3D bioprinting is also improved. It is particularly noteworthy that they combined coumarin derivatives with the backbone

Table 1. Summary of hydrogel 3D bioprinting research

Hydrogel	Modified form	Other materials/nanomaterials		Bioprinting method	References
Sodium alginate	–	Nanocellulose or cellulose nanofibrils		Extrusion	[31][49][129]
	–	Gelatin	–	Extrusion	[18][51][157][159]
	–		CNTs	Extrusion	[120]
	–		cellulose nanocrystals	Extrusion	[130]
	–		Nano–ATP	Extrusion	[46]
	–		Xanthan gum	Extrusion	[151]
		Nanosilicate clay, methylcellulose		Extrusion	[21]
	–	Collagen type I, agarose		Extrusion	[52]
	–	–		Extrusion	[126]
Chitosan	CHI–MA	–		DLP	[40]
		Polyvinyl alcohol		Extrusion	[88]
	MeGC	–		DLP	[91]
	–	Hydroxyapatite		Extrusion	[102]
	–	Collagen type I, GO–np		Extrusion	[126]
Gelatin	–	–		Extrusion	[56]
	–	Poly (lactic–co–glycolic acid)		Electrospinning	[113]
	GelMA	Poly(ethylene oxide)		Extrusion	[35]
		Methylcellulose		Extrusion	[22]
		Gold nanorod, SA		Extrusion	[94][95]
		Poly (N–acryloyl 2–glycine), BG		Extrusion	[90]
HA	HAMA	Poly (N–isopropylacrylamide)		Extrusion	[42]
Silk	Silk–GMA or Sil–MA	–		DLP	[34][92]
MAAc	PAAm	MEDSAH		SLA	[32]
	–	NPAM		Extrusion	[76]
PEG	PEGDA	Chitosan		SLA	[39]
		SA, nanoclay		Extrusion	[53]
		MWCNT		SLA	[118]
	PEGTA	GelMA, SA		Extrusion	[78]
	Branched PEG	Gelatin		Injection	[139]
Pluronic F127	–	GelMA		Extrusion	[79][82]
	Acrylated PF127	HAMA		Extrusion	[80]
	–	dECM		Extrusion	[83]
dECM	–	GelMA, nanoclay		Extrusion	[65]
	–	Gelatin, PEG		Extrusion	[66]
Collagen	–	Riboflavin		Extrusion	[72][73]
	Methacrylated collagen	HA		Extrusion	[75]
BG	–	PACG, GelMA		Extrusion	[100]
	–	PLA		Extrusion	[99]
GO	–	Alginate, gelatin		Extrusion	[114]
	–	Collagen, chitosan		Extrusion	[115]
CNTs	–	dECM		Extrusion	[119]
	–	Gelatin, alginate		Extrusion	[120]
	–	PLC		Extrusion	[121]
GNRs	–	GelMA		Extrusion	[95]
	–	GelMA, SA		Extrusion	[94]

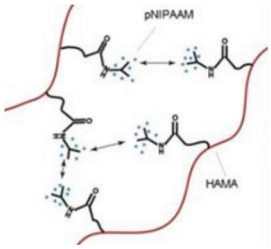
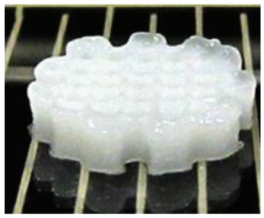
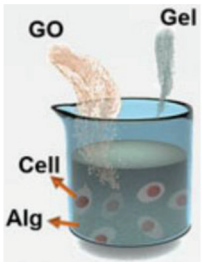
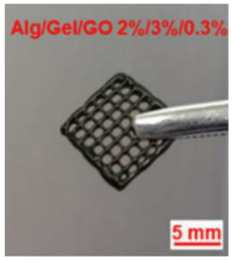
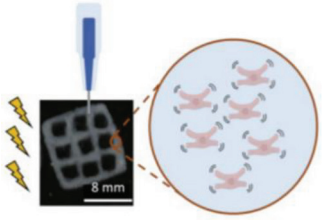
Table 2. Summary of hydrogel structural formulas (reaction mechanism), printing scaffolds, and the FDA approval status of the hydrogels applicable to tissue engineering and regenerative medicine

Hydrogel	For example		FDA approval status (Yes/No)
	Structural or reaction mechanism	Scaffolds	
Sodium alginate (SA)			Yes
Reproduced with permission[49].			
Chitosan (CS)			Yes
Reproduced with permission[88].			
Gelatin			Yes
Reproduced with permission[90].			
Silk fibrin (SF)			Yes
Reproduced with permission[34].			

Hydrogel	For example		FDA approval status (Yes/No)
	Structural or reaction mechanism	Scaffolds	
Decellularized extracellular matrix (dECM)			Yes
	Reproduced with permission[66].		
Collagen			Yes
	Reproduced with permission[73].		
Polyethylene glycol (PEG)			Yes
	Reproduced with permission[39].		
Pluronic F127	-		Yes
Bioactive glass (BG)			No
	Reproduced with permission[100].		

(*Continued*)

Table 2. (Continued)

Hydrogel	For example		FDA approval status (Yes/No)
	Structural or reaction mechanism	Scaffolds	
Hydroxyapatite (HA)	  Reproduced with permission[42].		Yes
Nanoclay	-		No
Graphene oxide (GO)	  Reproduced with permission[114].		No
Carbon nanotubes (CNTs)	 Reproduced with permission[119].		No
Gold nanorods (GNRs)	–		No

of GelMA and PEG polymers as photosensitive crosslinking groups and crosslinked under near-infrared light (λ = 850 nm) to form hydrogels. They have proved that the injection of this kind of hydrogel into the tissue has the potential to support the formation of new tissues, and it can also span tissues in different target organs, which can observe the conditions in the body through timely imaging.

5.2. Tubular organ

Tubular tissues such as the trachea, esophagus, urethra, and blood vessels are important organs of the human body. Among them, the highly complex, multi-tissue tubular structure is vascular tissue, which has the widest diameter and the ability to withstand high pressure[145-149]. Tubular organs are composed of various cells with different secretion and/or molecular absorption capabilities. Nutrients or metabolic wastes in the body are transported through tubular organs in the form of solid, gas, or liquid[150]. In clinical treatment involving trachea and urethra, long-segment defects (generally more than 50% of the length) account for about half of tracheal and urethral strictures[151,152]. Therefore, long-segment defects are the main problem in the treatment of tubular tissue reconstruction. In tissue engineering and regenerative medicine, the design of tubular scaffolds with the ideal structure and functional characteristics remains a huge challenge.

At present, the clinical treatment of tracheal stenosis is mainly through surgical operations, such as autograft, allograft, external materials, and so on. Due to the limited technology, the above methods will bring various complications, and it is difficult to repair long-segment defects. Huo *et al.*[153] used 3D bioprinting technology and photocrosslinkable tissue-specific bioinks to fabricate cartilage-vascularized fibrous tissue-integrated trachea (CVFIT). This multi-component bioink not only meets the basic requirements for 3D bioprinting based on

temperature-sensitive and high-viscosity properties, but also simulates the tissue-specific microenvironments of cartilage and vascularized fibrous tissue. Because the alternating soft and hard tissue structure of the stent is very close to the human body's own trachea, functional reconstruction of the mechanical and physiological properties of the trachea has been successfully achieved. Hong *et al.*[34] used glycidyl methacrylate (GMA) to modify the chondrocyte-loaded SF hydrogel (Silk-GMA) for DLP-based printing. Research and testing showed that the scaffold has a powerful mechanical advantage effect on the regeneration of defective tissues. At the same time, *in vivo* experiments were carried out on a rabbit model with a partially defective trachea, and new cartilage tissue and epithelium (Figure 7B) were found around the transplanted Silk-GMA hydrogel. The esophagus is next to the trachea, which also has strictures and inflammation. Currently, there is no direct treatment method for patients with radiation esophagitis. Ha *et al.*[148] proposed a pioneering direct treatment strategy. They developed an esophageal-derived dECM (EdECM) hydrogel and then used a rotating rod combined with 3D printing system to prepare an EdECM hydrogel stent. They verified that EdECM hydrogel has good rheological properties and biological functions. Besides, in a rat model of radiation esophagitis, the therapeutic effect of the stent was observed, proving that local treatment is feasible. However, this study failed to point out the mechanism by which ECM promotes local treatment response. But we can still get some enlightenment. By extracting the tissue-specific EdECM hydrogel from the esophageal ECM, it can produce local treatment without the use of drugs. That is, the dECM hydrogel obtained on a specific site is essential for tissue repair.

In urethral repair tissue engineering, its structure is similar to that of the trachea. The construction methods of 3D bioprinting technology can be used as reference methods. Zhang *et al.*[146] designed a "sandwich" tubular structure. Using PCL and poly (lactide-caprolactone-cocaprolactone) (PLCL) thermoplastic polymer and cell-loaded fibrin, gelatin, and other hydrogels realizes the 3D-bioprinted bionic urethral structure for the first time. They first obtained the structural index of the rabbit urethra and then used a spiral stent made of a blend of PCL and PLCL (50:50 ratio) as the intermediate layer. Three layers of the smooth muscle cells (SMCs)-loaded hydrogel are printed on the outer layer of the scaffold, and two layers of the urothelial cells (UCs)-loaded hydrogel are printed on the inner layer. The results showed that the 3D-bioprinted PCL/PLCL scaffold has mechanical properties comparable to those of the natural rabbit urethra. The fibrin hydrogel

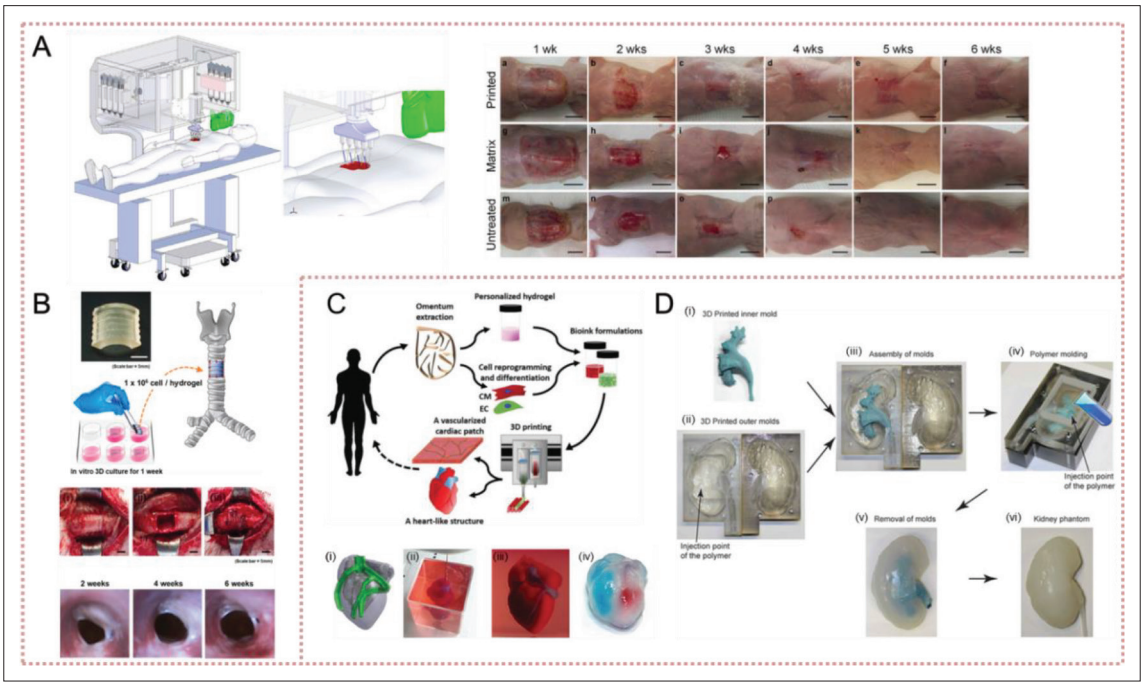


Figure 7. (A) Schematic diagram of *in situ* printing; experimental comparison of *in situ* printing on mouse skin. Reproduced with permission[158]. (B) DLP-printed silk-GMA hydrogel to prepare tracheal stents, which were used in rabbit models to promote the regeneration of cartilage tissue and epithelium. Reproduced with permission[31]. (C) The first ever printed organ was a heart printed in a suspended medium. Reproduced with permission[159]. (D) Flow chart of 3D printing kidney phantom. Reproduced with permission[160].

provides a suitable 3D microenvironment for cell growth. SMCs and UCs survive and proliferate well in the printed urethra.

5.3. Complex organ

At present, in many industrialized countries in the world, cardiovascular and cerebrovascular diseases become the most common non-communicable diseases that threaten human health and life. These diseases are the leading causes of death in the middle-aged and elderly, who are the most commonly affected by these diseases[154]. The cost of performing a heart transplant in patients with cardiovascular diseases is expensive, and there are not many donors. The emergence of tissue engineering provides a new method for the construction of heart tissues/organs. However, the most difficult part is to build a blood vessel network that matches the patient's blood vessel anatomy[155]. Based on the previously proposed strategy, printed bioink in the support medium can make it stably exist[156]. Noor *et al.*[155] developed a support medium mixed with SA and xanthan gum, which is a completely transparent and cell-friendly microparticle formulation. The medium supports the printing of large-size intact tissues and/or organs with thick vascularization and high complexity. Of course, bioinks are still the focus of our attention. They removed omentum tissue from the body and separated the cells from the matrix. Cell recoding can differentiate into CMs and endothelial cells, and the acellular matrix is processed into personalized hydrogels. Then, the two kinds of cells are mixed with hydrogel to prepare bioinks. This self-extracted material will not cause immune rejection in the patient. A huge breakthrough of 3D bioprinting was achieved when the world's first complete heart organ (height: 20 mm; diameter: 14 mm) containing a blood vessel network and perfusion was successfully printed (Figure 7C). Although the printed blood vessel network is limited, we can still learn from this personalized printing strategy. The construction of organ models is necessary for guiding the transplantation treatment of complex organs/tissues such as heart, kidney, and lung. Especially in urology, the phantom of the kidney *in vivo* provides detailed anatomical data for replicating the bionic model *in vitro*. Adams *et al.*[157] used a soft mold technology combining 3D wax printing and polymer molding to obtain modeling data through CT scanning of the anatomical structure of the human kidney. This design mimics the detailed anatomical structure of a real kidney, using soft materials with a tensile modulus of 0.8 to 1.5 MPa and biocompatible hydrogel to simulate human kidney tissue. The preparation method is low cost and has good robustness and high reproducibility of organs. It is a means to obtain a repeatable and robust model suitable for surgical simulation and training purposes (Figure 7D).

6. Conclusion and outlooks

In the field of 3D bioprinting, the design and improvement of biomaterials with better performance, vascularization simulation of organs/tissues, chip functionalization to simulate the physiological environment *in vivo*, and how to construct the culture conditions of organs/tissues, *etc.* are the directions of most research focus[82,161,162]. However, in recent years, more and more attention has been paid to various process parameters from 3D printers to hydrogels. Because the parameters have a great impact on the printing resolution and the fidelity of biological materials, some researchers have specifically studied the printability of biomaterials and the printing process parameters. With the help of a series of explorations on the printing of gelatin/SA hydrogel, they found that the most important factors affecting print quality are air pressure, squeeze rate, and print distance. Combined with the test results, a suitable printing process parameter scheme was determined[161]. Ouyang *et al.*[163] studied the effects of gelatin/SA hydrogel characteristics and printing parameters on the printability of the hydrogel and the viability of embryonic stem cells (ESCs). They evaluated the rheology of Gel-SA hydrogel to optimize the hydrogel formulation, printing temperature, gel time, and other parameters. They also proposed the influence of factors such as differences in cell types and printing time on cell-loaded bioink printing. Therefore, for a successful 3D bioprinting technology platform, it depends not only on the printing process and biological materials but also on the cells. Specifically, these factors include printing speed, shear stress, printing temperature, nozzle diameter, etc.; suitable biomaterials, concentration ratio, extrusion state, crosslinking method, etc.; cell source, density, and survival state; high-fidelity scaffold structure and ideal three-dimensional microenvironment, etc.[18,28,163-165]. A complete tissue construction process generally includes imaging, model design, selection of biological materials, selection of cells, determination of printing methods, and *in vivo* and *in vitro* applications[166]. Therefore, any factors can affect the success of complete tissue/organ construction. The bioinks' materials used for printing, rather than the bioprinting technologies, are usually the challenges in the development of tissue engineering. In other words, the current limited number of bioinks can meet the requirements of printing biophysics and at the same time provide an ideal 3D environment for cells[41,80,167]. Therefore, the development of new biomaterials and the design of new bioink formulations are currently the main focus areas, which are also the main challenges facing researchers in printing.

This article reviews the research progress of the performance of hydrogel bioinks for 3D bioprinting, as

well as the application of tissue engineering before the transformation into clinical practice. Hydrogels are a kind of biomaterials with great application potential, which are widely used in soft tissue and hard tissue regeneration. The application of hydrogel has many advantages as a scaffold material in tissue engineering. According to different hydrogel properties, using different 3D printing technologies prepares different tissue scaffolds. However, some shortcomings need to be overcome, such as weak mechanical properties, lower printability, unstable crosslinking, unfavorable effects on cell survival, and very fast or slow degradation. We found that several different hydrogel materials are often combined, and the mechanical properties, shear-thinning, and stability of the scaffold are improved by adding nanoparticles or nanofiber materials. What's more, the biocompatibility of the hydrogel and the acid–base environment which is suitable for cell growth are adjusted by releasing biologically active factors.

Another example is the composite surface modification of the hydrogel and nanoscale materials, which adjusts the properties of the hydrogel, increases its surface biological activity, enhances the interaction between cells and cells, cell and matrix, and performs functions on the surface of nanomaterials with chemical modification[28,168]. These research methods have achieved good results to a certain extent. Only a few printed scaffolds are used in human experiments for research, so there is still a long way before they will be applied in clinical settings. In short, we believe that hydrogels have broad prospects as bioinks for 3D bioprinting, and the tissues or organs used to construct will surely reach new heights in medical treatment.

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Conflict of interest

The authors declare no conflict of interests.

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