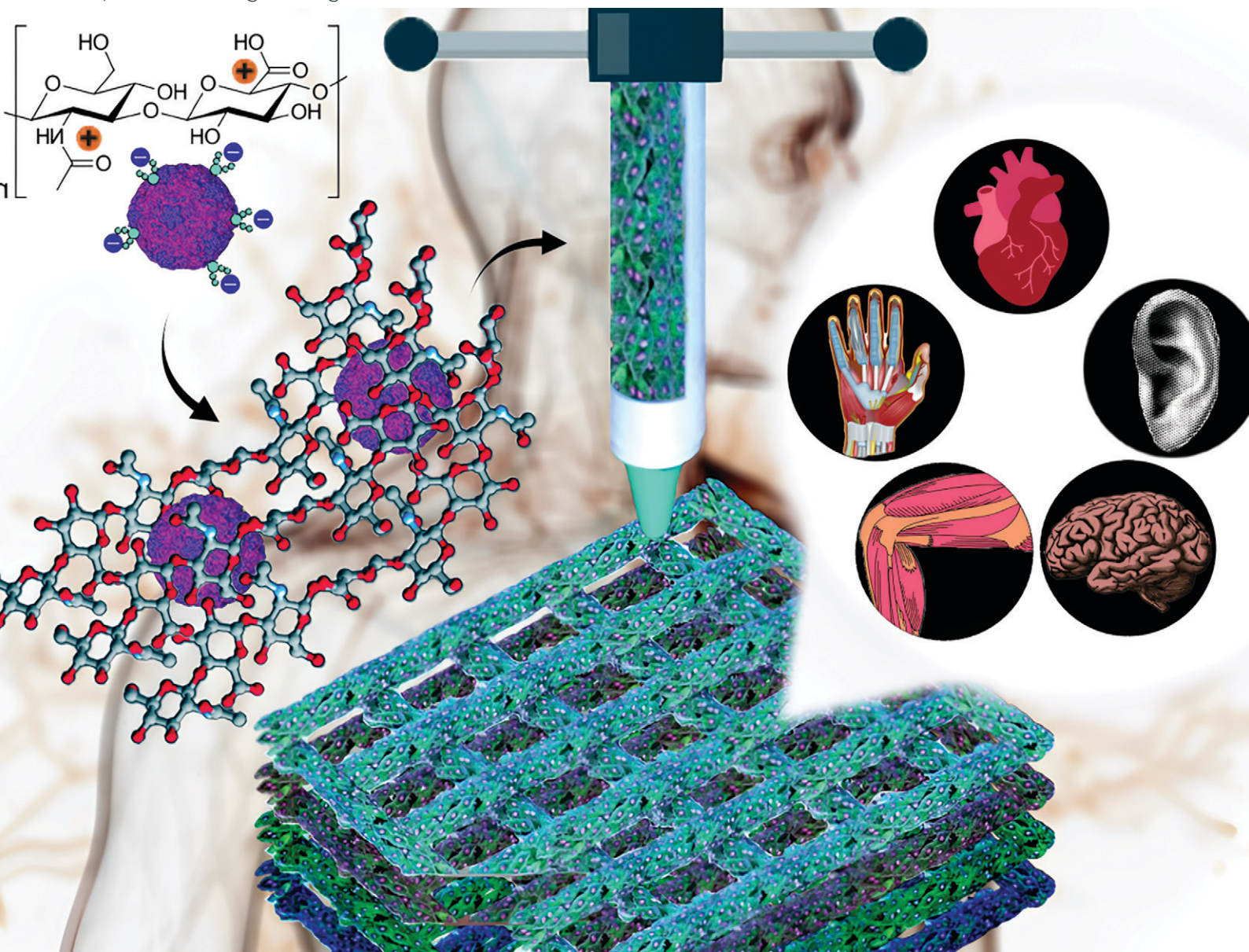


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# Recent advances in 3D bioprinting of polysaccharide-based bioinks for fabrication of bioengineered tissues

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Bioink in three-dimensional (3D) bioprinting of biomimetic tissue scaffolds has emerged as a key factor for the success of tissue engineering and regenerative medicine. The bioinks used for extrusion 3D bioprinting have hydrogel matrices with different kinds of polymeric biomaterials such as proteins, peptides, polysaccharides, hydrophilic synthetic polymers, and others. Natural polysaccharides such as alginate, chitosan, and hyaluronic acid have garnered significant attention as bioink materials due to their excellent biocompatibility, extracellular matrix mimetic properties, biodegradability, injectability, bioprintability and structural versatility among their many advantages, even though many research groups focus on the study of protein-based bioinks to utilize their high potential of cell adhesiveness. This review encompasses recent advancements of polysaccharide-based hydrogels and bioinks for bioengineered tissue regeneration and reconstruction, especially by focusing on fabrication of multilayered complex structures for biomimetic tissue engineering applications.

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## Design, System, Application

A bioprinting technology is a layer-by-layer 3D deposition of cell-laden hydrogel, *i.e.* a bioink, by extrusion-based printing technology to fabricate 3D constructs for its applications to regeneration of diseased tissues such as cartilage, bones, skin, skeletal muscle, and neural tissues. Even though this technology has attracted much attention in tissue engineering and regenerative medicine, the design and fabrication of tissue engineering scaffolds have yet to be solved by overcoming the difficulties of multilayered 3D scaffold construction in stable 3D structures. We here report the design of polysaccharide-based hydrogels, bioinks and 3D bioprinting scaffolds, targeting tissue engineering applications. For the 3D bioprinting and regeneration of diverse damaged tissues, the issues such as cell sources, biocompatibility, bioink and bioprinting properties, polymeric biomaterial selection, bioprintability performance, scaffold fabrication, and their applications to tissue regeneration have been described. Furthermore, the benefits, difficulties, and future directions of 3D bioprinting of polysaccharide-based bioinks for multilayered tissue engineering are discussed.

## 1. Introduction

Complex and multilayered tissues represent a fascinating frontier in the fields of biomaterials, 3D bioprinting, regenerative medicine, and biomedical engineering.<sup>1–4</sup> These tissues are composed of various cell types and extracellular matrices (ECM) intricately arranged in three-dimensional molecular structures, mimicking the natural complexity of

human tissues and organs. 3D bioprinting allows the molecular, structural systems to be designed as a tailored scaffold construction of multilayered tissues, with the potential to revolutionize regenerative medicine. This cutting-edge technique leverages the precision of 3D bioprinting to create intricate, multilayered constructs that closely mimic the native architecture of human tissues and organs.<sup>5–7</sup> By combining various natural biomaterials, including polysaccharides, with living cells and bioactive factors, 3D bioprinting allows the bioengineering of multilayered, functional and biocompatible tissue scaffolds. Within native tissues, diverse cell types like endothelial, epithelial, muscular, and adipose cells exist or co-exist in an intricate ECM in different tissues, intricately woven with interconnected networks.<sup>8,9</sup> The goal of tissue engineering is to emulate this natural milieu, thereby fostering the creation and revitalization of fully functional tissues and organs

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through designed biodegradable scaffolds. Recent technological advancements like bioprinting and advanced manufacturing have illuminated a promising trajectory for tissue engineering and reproductive medicine, profoundly shaping their future.<sup>10,11</sup>

Polysaccharides serve a variety of purposes, such as structural properties, tissue regeneration,<sup>12</sup> antibacterial,<sup>13</sup> antiviral,<sup>14</sup> antitumor,<sup>15,16</sup> and immune regulatory properties in tissue engineering.<sup>17,18</sup> Hyaluronic acid and chitosan, derived from human connective tissue and arthropod exoskeletons respectively, along with alginic acid from brown algae, are key polysaccharides used in tissue engineering. These materials are processed into hydrogels and bioinks using various synthesis technologies. Hydrogels, known for their ability to retain significant amounts of water in a 3D polymeric network, differ from bioinks, which incorporate living cells within their polymer structure. Additionally, these materials can be enhanced with bioactive molecules like growth factors, nanoparticles, and drugs to tailor their properties for specific applications.<sup>19</sup> The bioink is employed as a biomaterial for the fabrication of tissue engineering scaffolds. Thus, hydrogels are composed of highly hydrated 3D environments that resemble the ECM of tissues. Furthermore, because of their special architecture, which is porous and flexible, cells can migrate and communicate with each other, as well as receive nutrients, oxygen, and other water-soluble substances in a specific tissue.<sup>19</sup> Different techniques can be used to design a hydrogel, based on the intended attributes and purpose in tissue engineering. Physically cross-linked hydrogels are created through molecular interactions.<sup>20</sup> The interactions can be influenced by changing the surrounding environment or applying mechanical forces. They are reversible and regenerated (associations break and continuously reform).<sup>21</sup> For instance, ionic interactions with calcium ions ( $\text{Ca}^{2+}$ ) can cross-link alginate to create a hydrophilic 3D network that resembles an egg-box model structure.<sup>22</sup> Polymer chains joined by covalent bonds make up chemically cross-linked hydrogels.<sup>23</sup> There are two general methods for preparing these hydrogels, the first involves using a cross-linking agent to polymerize a hydrophilic monomer; the second method involves directly cross-linking water-soluble polymers.<sup>24</sup> Due to the strong covalent connections that form between polymeric chains, chemical crosslinking is typically more persistent than physical cross linking and has stronger mechanical qualities, making it appropriate for long-term use.<sup>21</sup> Chemical hydrogels are formed by reacting polysaccharide functional groups ( $-\text{COOH}$ ,  $-\text{OH}$ ,  $-\text{NH}_2$ ) with cross-linkers and other chemicals, ensuring that these groups network to create the gel. This process enhances the hydrogel's physical properties like viscosity, elasticity, and stability by forming a durable 3D structure with improved mechanical qualities.<sup>25</sup> These crosslinking induces formation of a hydrogel and makes layer-by-layer additive manufacturing of tissue engineering scaffolds by 3D bioprinting technology.

Proteins and their domain, *i.e.* peptides and oligopeptides, are essential for constructing hydrogel/bioink scaffolds in tissue engineering, thanks to their biocompatibility and ability to foster cell adhesion and then tissue regeneration. Proteins like collagen, fibronectin, gelatin, fibrin, and silk are crucial for their roles in enhancing cell interactions and scaffold functionality in tissue engineering.<sup>26</sup> Collagen, which is an abundant component in the ECM, supports cell adhesion and tissue regeneration, while its denatured form, gelatin, and its modified form (GelMA) have been employed as typical bioink hydrogels and allow for precise scaffold structures through photo cross-linking while maintaining biodegradability.<sup>26</sup> Fibrin is valued for its natural ability to form scaffolds that promote cell growth, especially in wound healing,<sup>27</sup> while silk fibroin's strength makes it suitable for structural repairs in bone and ligament contexts.<sup>28</sup> Incorporating peptides such as RGD, YIGSR, and IVAK have been employed as alternative biomaterials for proteins to prevent the issue of complex handling in synthesis by higher molecular weight. Peptides are the specific interaction domains of cell-adhesive proteins.<sup>26</sup> Even though this strategic application underscores their pivotal role in developing advanced, functional scaffolds for tissue engineering, they have limitations in their bioink applications, such as difficulty in gel synthesis compared to polysaccharide polymers.

Polysaccharides, with their diverse biophysical and chemical properties, are crucial in tissue engineering and 3D bioprinting. Classified as anionic, cationic, or neutral based on their natural charges, these polysaccharides such as alginate (ALG), chitosan, HA, gelatin (GEG), pectin, guar gum, and chondroitin sulfate are transformed into hydrogels through chemical and physical modifications. They are essential in various tissues within animal bodies, performing key biological, physiological, and structural functions influenced by their chemical composition, molecular weight, shape, and hydrophilicity.<sup>29,30</sup> For example, sulfated marine polysaccharides like fucoidan and chondroitin sulfate have anti-inflammatory and antiviral properties, potentially blocking viruses from attaching to host cells. Chitosan, deacetylated from chitin, shows antibacterial effects by interfering with anions on bacterial cell walls, which can disrupt vital processes and kill the bacteria. Meanwhile, neutral dextran, due to its water solubility, resists protein adsorption.<sup>31,32</sup>

The development of 3D bioprinting has been emphasized as a particular solution for creating unique 3D matrices (fabrication) similar to native tissues and organs, the regulated deposition of different biomaterials in layer-by-layer forms, and the shortcomings of the traditional approach. It is undoubtedly a groundbreaking method with several uses in tissue engineering, regenerative medicine, food industry, drug delivery and other bioengineering fields.<sup>33,34</sup> 3D bioprinting is a biotechnology that uses biomacromolecules and biomedical engineering to create 3D structures that regulate cell functions, tissue regeneration



and disease treatment. It has applications in drug screening models, medical engineering, dentistry, surgery, and human-created structures like meniscus, vascular junctions, bone, tumor models, nerve and skin tissues. By harnessing the power of 3D bioprinting, researchers and medical professionals can advance healthcare and improve patient outcomes, making it a promising technology with significant implications in medical and biological fields.<sup>35</sup> However, there are no established standards or criteria for bioprinting or bioinks, which makes them challenging for researchers to determine which type of bioprinting or bioink they require for given tissue engineering applications.

Even while there are now a lot more commercially available bioprinting systems and bioinks than ever before, certain research organizations will probably still need to use unique setups and custom formulations of bioinks for specific tissues with different shapes and compositions.<sup>36</sup> Extrusion-based 3D bioprinting is one of the most frequently employed 3D bioprinting methods in tissue engineering applications, which deposits layers of tissue-specific cell-filled bioink on top of one another, indicating the importance of each layer of the bioprinted scaffolds. Natural polysaccharides have consistently attracted considerable attention in tissue engineering and extrusion-based bioprinting,<sup>37,38</sup> and their hydrogels have gained broader applications in tissue engineering in recent decades. Notably, polysaccharides have diverse applications, for example, regeneration of encompassing bone and cartilage, muscle and cardiac tissue, neural tissue, skin, and other tissues. However, for the fabrication of large, multilayered complex structures through the techniques of polysaccharide-based hydrogel printing, the self-standing gel strength and post-printing shape fidelity are the issues of major concerns for the researchers working with such biomaterials in tissue engineering.<sup>39,40</sup>

This review discusses the key properties of polysaccharides, their application in extrusion-based bioprinting technologies, and their role in tissue engineering. It delves into recent developments in polysaccharide-based hydrogels for creating multilayered scaffolds suitable for complex tissue structures. The review underscores the adaptability of these natural polymers in developing 3D bioprintable hydrogels and outlines their advantages and challenges in bioprinting. It also emphasizes the importance of bioink composition for effective 3D bioprinting and highlights extrusion-based bioprinting as a favorable method due to its versatility and reduced cell damage.

## 2. Characteristics of bioinks

Bioinks come in two categories: those that utilize scaffolds and those that do not. Scaffolds are fibrous or 3D structural biomaterials that permit not only transformation into tissues, but also biological liquids and gases to pass through. This is accomplished by making it simpler for cells to interact, deposit ECM and maintain viability with no or mild toxicity

and inflammation.<sup>41</sup> The bioink should be printable and capable of maintaining cells without any cell damage, and also preserve the physio-mechanical and biological characteristics of the targeted tissue after bioprinting. The bioinks can be altered depending on the printing systems used to print on the target tissue for all the qualities to be achieved. Combining two or more nano-biomaterials is frequently necessary to improve the mechanical and biological properties, particularly for extrusion-based bioprinting.<sup>42</sup> Multi-biomaterial bioinks are superior in bioprinting to those formed from a single biomaterial. Most single polymer-based bioinks lack the bioprintability, outstanding mechanical qualities, and functionality required to create tissues that mirror biological ones. The selection of polysaccharides is more constrained in 3D bioprinting because cells and post-printing fidelity are involved. It is important to consider mechanical qualities, biocompatibility, porosity, pH neutrality, hydrophilicity, and biodegradability while currently selecting the biomaterials for bioink.<sup>43,44</sup> The majority of bioprinting techniques use additive manufacturing concepts by depositing tissue-specific cells and biomaterials collectively known as “bioinks” spatially and temporally in a regulated and sequential manner. Even though numerous bioinks have been created, no single bioprinter/bioink combination has proven effective in all tissue engineering applications to date, which is why the sector is still quite experimental. The following are some of the important characteristics that a bioink must have.

### 2.1 Cell source

When living cells are incorporated into a hydrogel, it becomes a measurable bioink. Since a necessary, key element of bioink is cells, the cell types and sources should be considered important factors. Since the chosen cell must continue to operate, reproduce successfully, imitate its physiological state, and regenerate the target tissues throughout the bioproduction process,<sup>45</sup> the right cell type, source, and density should be chosen carefully to create bioink and tissue engineering scaffolds. One of the most fundamental prerequisites for bioink's compatibility is that it must be robust and secure to support the loaded cells' survival and differentiation.<sup>46</sup>

### 2.2 Hydrogel and its synthesis

A hydrogel, a 3D network of polymer, transforms into a bioink by adding cells into a hydrogel in 3D bioprinting and tissue engineering. It is synthesized by using diverse polymers such as HA, chitosan, gelatin, and chondroitin sulfate through the methods of chemical or physical crosslinking. The gelation process with cell incorporation should be easy to be handled, sterilizable and nontoxic because it can affect not only the cell viability but also the bioprinting processing and post-printing fidelity of the scaffolds.<sup>47</sup> Depending on how the composition and properties of the hydrogel and bioink are prepared, various

alternatives are available for the gelation procedure such as photo-crosslinking, ionic, hydrophobic, thermal, enzymatic, and stereo-complexing methods.

### 2.3 Biocompatibility

For the development of tissue engineering constructs by 3D bioprinting, cells in bioink should maintain their capacity to differentiate and proliferate *in vitro* without any damage. Biocompatibility and biodegradation were the primary concerns for the hydrogels and 3D printing.<sup>48</sup> Cutting edge researchers have expanded the concept of biocompatibility from the need for minimal cytotoxic effects in cell and biomaterial levels to a favorable biological relationship with the host, especially in tissue engineering and regenerative medicine area. Because they can simulate the natural cellular environment, the bioinks from natural biomaterials offer a nontoxic biomanufacturing approach, but they have to overcome the issues of poor mechanical qualities and immune response issues.<sup>49</sup>

### 2.4 Bioprintability

Bioprintability refers to a biomaterial's ability to be printed in a controlled manner and shapes within a certain time frame. The idea behind 3D bioprinting is the layer-by-layer, controlled production of three-dimensional living objects using computer-aided design.<sup>50</sup> Several techniques, such as extrusion printing,<sup>51</sup> inkjet printing,<sup>52</sup> laser-assisted printing,<sup>53</sup> and stereolithography,<sup>54</sup> are used to bioprint cell-laden biomaterials, or bioinks. At present, 3D bioprinting technology has garnered significant interest and is being extensively researched for a variety of biomedical uses.<sup>55</sup> Rheology is essential to the extrusion-based 3D printing of hydrogels because it influences the behavior of the material during the printing process through its flow and deformation characteristics. Viscosity, shear thinning behavior, and viscoelasticity are examples of rheological qualities that are important in influencing the extrudability, shape retention, and general quality of printed structures.<sup>56</sup> Organ and tissue bioprinting relies on bioprinting of a gel with cells; however, the demanding bioprinting environment limits the bioink material options. Since the bioink's nature can substantially impact the bioprintability, the scaffold properties such as size accuracy and structural construction are all important considerations in 3D bioprinting and tissue engineering. Bioprinting technology varies based on its purposes; for example, micro-extrusion bioprinting requires adequate rheological properties and shear-thinning behaviors of bioinks, and preserves the preliminary shape fidelity of the post-bioprinting scaffolds.<sup>57</sup>

### 2.5 Mechanical properties

Bioprinted hydrogels should have convincing mechanical qualities to withstand diverse biopressures throughout the bioprinting process and to sustain the scaffold structure after bioprinting for successful practical use as well as

differentiation during its biodegradation into specific tissue regeneration. At the same time, the bioink should guarantee the scaffold's mechanical qualities that can adapt to the target tissue's native environment. Due to their low mechanical properties, natural polysaccharide hydrogels do not provide good sacrificial nourishment during bioprinting and implant in the patient's body. Hence, synthetic polymers or nano-biomaterials are often used for higher mechanical qualities, keeping the bioink structurally and mechanically stable like genuine tissue, especially under mechanical stress like cartilage, cardiovascular tissues or bone.<sup>58</sup>

## 3. Natural polysaccharides in bioinks

Polysaccharides and their derivatives are becoming more and more popular among the variety of biopolymers that can create hydrogels for 3D bioprinting applications, especially for the engineering and development of bioink formulations. Their primary characteristics—which include their vast diversity of chemical structures, ease of derivatization and functionalization, suitable mechanical and rheological qualities, and inherent biocompatibility and biodegradability—are what make them so appealing.<sup>59</sup> When it comes to bioprinting of the intricate and multilayered tissues, natural polysaccharides are essential building elements. Natural sources of polysaccharides as depicted in (Fig. 1) have the extraordinary capacity to produce hydrogels, which offer an environment that is favorable for cell encapsulation and growth as well as tissue regeneration. These polysaccharides function as the structural backbone of bioinks, enabling precise multilayer deposition during bioprinting procedures.<sup>29</sup> This integration gives the printed structures both mechanical stability and tissue engineering potential, by simulating the intricacy of original tissues in physical and biological aspects, presenting a possible path toward the creation of complex and useful biological constructs. The appropriate utilization of natural polysaccharides in bioprinting has enormous promise to transform regenerative medicine and advance the creation of realistic, multilayered tissues as researchers explore the frontier of tissue engineering.<sup>60</sup>

### 3.1 Alginate or sodium salt of alginic acid (ALG)

One of the most common products made from brown algae is an alginate, ALG, with anionic, edible and hydrophilic properties. Due to its extremely low cost and biocompatibility, ALG is widely employed in biomedical applications.<sup>61</sup> While ALG's low bioactivity also prevents the growth and proliferation of encapsulated cells, its stability is obtained by oxidation processing.<sup>62,63</sup>  $\text{Ca}^{2+}$  and other divalent and trivalent cations are commonly used in the ionic gelation process to create ALG hydrogels in a stable state.<sup>64</sup> The drug release rate and the stability of the gels are modulated by control of the increase in ionic cross-linking.<sup>65</sup> ALG hydrogels' gel formation, mucoadhesive ability, biodegradability and nontoxicity, among other qualities, have

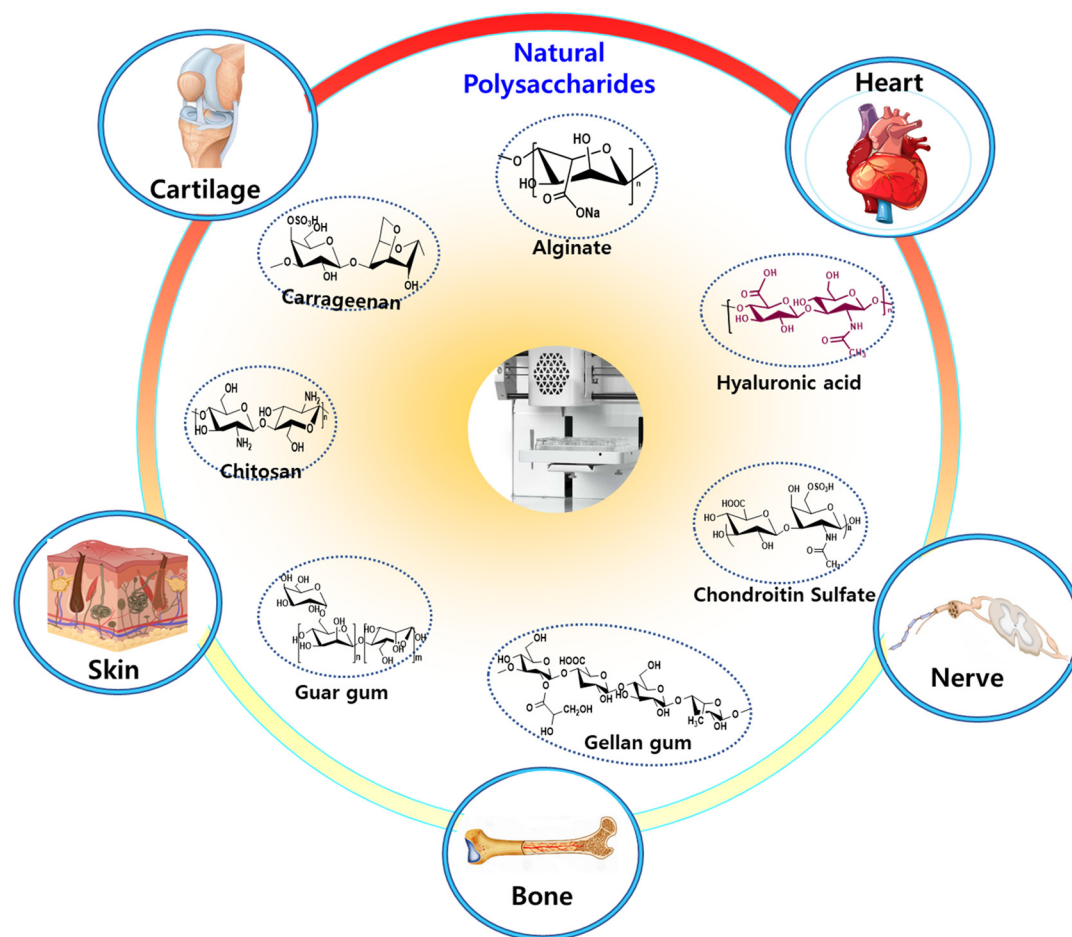


Fig. 1 Examples of natural polysaccharides used for 3D bioprinting tissue engineering.

encouraged the use of these biomaterials in multilayer 3D bioprinting, tissue engineering and drug delivery applications including wound healing, bioinks for 3D printing, *in vitro* models, or the delivery of anticancer medications.<sup>66</sup>

In order to create hydrogels with potential uses in various multilayer 3D bioprinting and tissue engineering techniques, ALG has also been mixed with other polymers, in order to create skin tissue analogues using extrusion bioprinting; Somasekharan *et al.* reported a bioink based on alginate mixed with gelatin and diethylaminoethyl cellulose (DCEL). Because the gelatin backbone naturally contains RGD peptide sequences, the inclusion of gelatin boosted cell adherence, while the fibrous character of DCEL gave matrix stability and improved mechanical qualities. When compared to skin tissue, the bioprinted scaffolds had appropriate mechanical properties, such as elasticity, with an elongation of break of  $91.7 \pm 9.36\%$  and a Young's modulus of  $125 \pm 22$  kPa. For 21 days, the co-culturing of fibroblasts and keratinocytes was seen, along with the progression of cell differentiation within the scaffolds. Histological examination at this stage revealed the development of comparable dermal and epidermal components.<sup>67</sup>

Dogan *et al.* went one step further in a recent study, creating bioprinted scaffolds using an alginate–collagen I

bioink loaded with mesodermal progenitor cells (hiMPCs) derived from human-induced pluripotent stem cells that were cultured with the addition of vascular endothelial growth factor (VEGF) to induce blood vessel formation. Following a 21 day incubation period, the bioprinted scaffolds showed signs of both small and big vessel creation. To evaluate their functionalities, the scaffolds were subsequently inserted into a chicken embryo's chorioallantoic membrane (CAM). The CAM model's appropriate blood perfusion was made possible by the printed vessels.<sup>68</sup>

### 3.2 Chitosan

Chitosan is a linear polysaccharide, made up of randomly distributed-(1 → 4)-linked D-glucosamine (deacetylated unit molecules from chitin).<sup>69</sup> Chitosan's motivating properties are its low renewability, lack of toxicity, lower cost, biocompatibility, bio-adhesiveness, anti-bacterial activity, and biodegradability. Chitosan degrades more slowly than animal-derived polymers like collagen and fibrin<sup>70,71</sup> and has several functional groups, including amine, amino, and hydroxyl groups, which can interact with both cationic and anionic molecules. This is particularly significant in the case of proteins. With the addition of various groups along the

polymer chain,<sup>72</sup> the functionalization of the polysaccharide was possible, opening up a variety of applications for it, including hydrogel, bioink, 3D bioprinting, tissue engineering<sup>73</sup> and drug administration.<sup>74</sup>

Chitosan hydrogels have been used for diverse purposes such as the repair of bone and cartilage, and other tissues. As examples, polyelectrolyte hydrogels can be produced in a simple way by combining chitosan with polysaccharides or proteins with opposed charges.<sup>75</sup> In recent years, further applications for chitosan hydrogels have included heart regeneration and wound healing.<sup>76,77</sup> Tonda-Turo *et al.* created a bioink based on chitosan that combines photocrosslinking and thermally induced gelation in a dual crosslinking mechanism. To do this,  $\beta$ -glycerol phosphate salt and chitosan-methacrylate were combined to provide thermosensitive behavior, having an elastic modulus of about 6 kPa. After 48 hours, cell proliferation was observed from the extrusion bioprinting of these hydrogels loaded with NIH 3T3 cells, resulting in 3D constructs with good cell dispersion even after 24 hours.<sup>78</sup> The simultaneous inclusion of additional ingredients or nanostructures in the bioinks is another popular tactic used to boost the effectiveness of chitosan-based bioinks. For example, the development of a bioink based on chitosan and poly( $\gamma$ -glutamic acid)  $\gamma$ PGA is described in the work of Pisani *et al.* The amine groups in chitosan and the carboxylic acid groups in  $\gamma$ PGA interacted ionically to produce the hydrogel. Human dermal fibroblasts were utilized by the authors to 3D bioprint grid-shaped constructs using this hydrogel. Cell viabilities of about 70% were seen 24 hours after the bioprinting procedure and maintained for 14 days in culture.<sup>79</sup>

### 3.3 Hyaluronic acid (HA)

HA is available in all joints, neural, connective, and epithelial tissues in animals. The extracellular membrane of mammalian tissue (muscle), present in some organs including the central nervous system, contains HA with a repeating unit of *N*-acetyl-D-glucosamine and D-glucuronic acid in its chemical structure.<sup>80</sup> The capacity of HA to maintain moisture levels makes it a perfect substance for promoting wound healing. Depending its molecular weight and water solubility, HA has diverse properties of bioprinting, mechanical strength and stability.<sup>81,82</sup>

By adding gelling agents, or chemical modifications, HA hydrogels can be created.<sup>83</sup> Combining HA with gelling agents, such as poloxamer,<sup>84</sup> ALG,<sup>2,85</sup> collagen,<sup>86</sup> methylcellulose (MC),<sup>87</sup> or gelatin,<sup>6</sup> is one of the frequently used techniques for producing physically cross-linked HA hydrogels. Cross-linking substances like divinyl sulfone or glutaraldehyde can be added to HA polymers to create crosslinked HA hydrogels.<sup>88</sup> However, the usage of cross-linking agents may be constrained since they might be hazardous or produce toxic byproducts to tissue engineering applications. For the creation of HA hydrogels, more

biocompatible chemical modification techniques including enzymatic cross-linking, click chemistry, or physical crosslinking have been investigated<sup>89</sup> such as oligonucleotides. For these new options to work, HA must be functionalized with additional chemical groups, which can be added to the carboxylate, hydroxyl, or amide groups.<sup>90</sup>

Methacrylate groups can be chemically crosslinked with HA to generate HA hydrogels, a method that is frequently employed with various biopolymers. These platforms have been used to release PLs and GF. Further, even MSCs have been cultivated in these matrices to stimulate their differentiation for uses in periodontal regeneration,<sup>91</sup> cartilage regeneration,<sup>92</sup> bone regeneration, or skin regeneration.<sup>93</sup> A HA-based bioink made of HA, hydroxyethyl acrylate, and gelatin-methacryloyl was reported by Noh *et al.* They demonstrated that the HA-based bioink did not adversely affect the viability of embedded bone cells when used to manufacture multilayered construction. The hydrogel consisting of three components was biocompatible, and the gel printing procedure was good.<sup>81</sup>

### 3.4 Cellulose

Due to its rigid structure, cellulose, one of the most prevalent polymers in plant cell walls, contributes to its construction. It consists of a repeating unit of (1  $\rightarrow$  4) connected-D-glucopyranosyl. As a renewable polysaccharide, cellulose offers intriguing properties due to its adaptability and sustainability.<sup>94,95</sup> Natural cellulose possesses specific chain length, crystallinity, and intra-intermolecular hydrogen bonding, among other physicochemical properties. The great thickness of free OH groups in cellulose allows for hydrogen bonding, but they also contribute to the insoluble nature of cellulose.<sup>96,97</sup>

Given that cellulose is not soluble in water, it makes sense to alter its structure to develop soluble derivatives with a high affinity for water, which can then be used to make hydrogels. Methylcellulose (MC), one of them, has been used in the creation of several hydrogels for their multilayered bioprinting.<sup>98</sup> For instance, Boonlai *et al.* combined CNCs, methylcellulose, and  $\kappa$ -carrageenan, and then used ionic crosslinking (with aqueous KCl) to make an appropriate hydrogel for 3D extrusion-based bioprinting of live constructions for applications such as bone tissue creation and regeneration. Better shear-thinning behavior resulted from adding 2 and 4 wt% of CNCs to the hydrogels because nanocellulose enhanced the bioink's rheological characteristics. Furthermore, while increasing the CNC content from 2 to 4 wt%, the compressive stress at 30% strain increased in the CNC-reinforced hydrogels from  $20.03 \pm 0.02$  to  $23.28 \pm 0.01$  kPa. The vitality of L929-loaded printed constructs was greater than 90% five days after bioprinting, demonstrating that the bioprinting procedure and the prepared biomaterials did not damage these cells.<sup>99</sup>



### 3.5 Dextran

Dextran polymer is a naturally occurring, non-toxic, hydrophilic homopolysaccharide containing  $\alpha$ -(1  $\rightarrow$  6)-linked-D-glucopyranose. Dextran comprises solely hydroxyl(-OH) groups, which do not contribute to cellular adhesion,<sup>100,101</sup> but its hydroxyl groups are simple to functionalize, making hydrogel preparation possible. DEX hydrogels can be created using physical or chemical cross-linking, including photo-crosslinking,<sup>102</sup> Michael addition,<sup>103</sup> Schiff-base reaction,<sup>104</sup> and enzymatic cross-linking. Physically cross-linked hydrogels typically have lower mechanical strengths than chemically cross-linked ones, but because they may be made at softer circumstances, there is less risk of harm to the included biomacromolecules.

Actually, utilizing a promising core/shell extrusion-based 3D bioprinting technology, only one study to date has described the bioprinting of a vascularized construct for wound care employing oxidized dextran.<sup>69,105</sup> This was accomplished by using a hydrogel consisting of periodate-oxidized dextran and peptide-functionalized succinylated chitosan (C) as the shell and GelMA as the core. In this experiment, two cell types were used: BMSC in the shell and HUVECs in the core. With HUVEC-specific markers visible 21 days after bioprinting, the 3D bioprinted peptide-CD/GelMA constructs offered a suitable milieu for cell growth and development, indicating the presence of endothelial cells within the tube-like structures. Although this work is the only one on the usage of dextran-based bioinks to date, it provides a way to employ this polysaccharide to create vascularized structures, which addresses one of the primary obstacles in 3D bioprinting live tissues.<sup>106</sup>

### 3.6 Gellan gum

An anionic polysaccharide with a linear chemical structure is GEG. As a result of the fermentation process, it is secreted by the bacteria *P. elodea*. L-rhamnose, two D-glucose subunit chains, and repeated chain units of D-glucuronic acid are all present in gellan gum. It has effective mechanical and gelling capabilities and exhibits cytocompatibility, biocompatibility, and biodegradation.<sup>107,108</sup> Gellan gum is used in tissue engineering to bio print skeletal cartilage, tissue, and structures that resemble the human brain.<sup>109</sup> In a different direction, gellan gum and pure starch were mixed to create 3D-printed scaffolds with varied printing gaps for Schwann cell seeding. The printed constructs were shown to be stable, to have sufficient swelling ratios, and not to be harmful to the L929 fibroblast cell line. Because these methods work well for 3D bioprinting, the usage of gellan gum is expected to be increased in the formulation of cell-laden bioinks in the future.<sup>110</sup>

### 3.7 Agarose

A repeating chain like arrangement of D-galactose and 3,6-anhydro-L-galactose units makes up the agarose polymer. Along with agarpectin, agarose is a naturally occurring

linear polysaccharide composed of D-galactose and 3,6-anhydro-L-galactopyranose units.<sup>111</sup>

Because of its thermo-responsive reversible gelling, agarose polysaccharide, generated from red seaweed, is frequently used in the tissue engineering sector.<sup>112,113</sup> Instead of using bioink components, alternative uses for agarose hydrogels in bioprinting procedures have also been investigated.<sup>114</sup> Agarose hydrogels are easily molded and used to cast sub-millimetric geometries at low concentrations (less than 1% (w/v)).<sup>114</sup> Utilizing this characteristic, Aydin *et al.* created a bioink using alginate and agarose that was created using a microwave-assisted technique. The agarose acted as a self-eroding sacrificial component to cast tubular structures within the alginate that was packed with cells, creating living printed constructs with a vascularized network. In particular, this sacrificial bioink made it possible to bioprint a 2 cm tubular structure in 2 minutes, preserving its shape and enabling very high cell survival (up to 95%) in a 3 day culture of MSCs generated from human adipose tissue (AT MSCs).<sup>115</sup>

### 3.8 Konjac gum

A water-soluble hydrophilic polymer derived from konjac tubers is called konjac glucomannan (KJG). China, Japan, and Southeast Asia have traditionally used KJG as a food source and traditional medicine. The  $\alpha$ -(1  $\rightarrow$  4)-pyranoside bond between D-glucose and D-mannose polymerizes the main structural chain of KJG, with a decreased number of acetyl groups at C-6 locations in the side chain unit.<sup>116</sup> KJG is a desirable biopolymer because of its notable properties, which include biodegradability, biocompatibility, the ability to form films, and the capacity to gel.<sup>117</sup> Alves *et al.* presented a KJG-xanthan gum hydrogel for wound dressing application. The dressing was then assessed by physicochemical and biological experiments.<sup>116</sup> The use of a KJG hydrogel in the treatment of acute wounds was investigated by Yang *et al.* after its synthesis by adjusting the KJG concentration during the preparation process. The hydrogel was demonstrated to have antibacterial activity, biocompatibility, and water-holding capacity.<sup>118</sup>

### 3.9 Guar gum(GG)

GG is a plant-based polysaccharide derived from *Cyamopsis tetragonolobus* and has a high molecular weight. It is made up of  $\alpha$ -(1  $\rightarrow$  6), connected galactose side chain units and  $\beta$ -(1  $\rightarrow$  4)-D-mannan linear chains.<sup>108</sup> An affordable hydrophilic polyhydroxy polysaccharide is guar gum. Due to its strong biocompatibility, biodegradability, and outstanding rheological qualities, it is frequently utilized in multilayered and complex tissue engineering application.<sup>119,120</sup> Indurkar *et al.* presented a novel, cost-effective bioink made of gelatin and varying guar gum concentrations. This novel biopolymer combination was created to provide bloom strengths,  $G'$  and  $G''$ , that range widely, and then  $\tan \delta$  was computed using  $G'$  and  $G''$ , and then the bloom test was conducted. Following the study of filament creation, these physical parameters



were associated with the printability of the bioink. The guar gum–gelatin bioinks with bloom strength between 480 and 750 and  $\tan \delta$  between 0.15 and 0.2 were reported to be printable because they could form filaments during extrusion.<sup>120</sup>

### 3.10 Xanthan gum (XTG)

XTG is an anionic polysaccharide generated from *Xanthomonas* bacteria that produces a viscous solution (high molecular weight) and has a low shearing rate. The side chains of XTG are composed of a trisaccharide made up of D-mannose beta ( $\beta$ )-(1  $\rightarrow$  4), D-glucuronic acid beta( $\beta$ )-(1  $\rightarrow$  2), and D-mannose, which are linked to substitute residues of glucose groups by alpha( $\alpha$ )-(1  $\rightarrow$  3)-linkage in the backbone. XTG is a branched polysaccharide.<sup>121</sup> The remarkable qualities of XTG, which include exceptional physicochemical properties, stability over a wide pH range and temperature range, high viscosity at low concentration, high degree of pseudoplastic behavior, biodegradability, and non-toxicity, are suitable for multilayered and complex tissue engineering application.<sup>122</sup>

Since xanthan gum has outstanding rheological properties, it is often used to enhance the mechanical and rheological properties of bioinks.<sup>123,124</sup> To be more precise, Lim *et al.* used xanthan gum's ability to thin shear and mixed it with CMC as well as with alginate, GelMA, and hMSCs to create a suitable bioink for extrusion-based bioprinting.<sup>125</sup>

## 4. Polysaccharide-based hydrogels in complex/multilayered tissue engineering applications

Polysaccharide-based hydrogels are increasingly used in tissue engineering for regenerating various tissue types, including soft, hard, connective, and functional tissues. These hydrogels are formulated into bioinks that encapsulate cells and bioactive molecules, offering properties such as biocompatibility, appropriate rheology, and elasticity, crucial for bioprinting of complex or multilayered scaffolds like those needed for cartilage, bone, skin and nerves. However, single-polymer hydrogels often fall short of meeting the complex demands of multilayered tissues. To address this, combinations of multiple bioactive polymers, nanobiomaterials, and crosslinking techniques are used to enhance the mechanical, biological, and post-printing properties of the constructs, ensuring effective regeneration of multilayered complex tissues. Further control of the interactions between layers is a crucial requirement to achieve multi-layer post-printing stability and integration.

Table 1 summarizes polysaccharide-based hydrogels used for 3D bioprinting of multilayered complex structures. To successfully regenerate specific functional tissues, cellular level bioengineering is essential during and after 3D bioprinting. The details of each tissue reconstruction using

polysaccharide-based bioinks are discussed in the subsections below.

### 4.1 Cartilage

Collagen fibers, matrix, and chondrocytes make up cartilage tissue.<sup>141</sup> Cartilage is devoid of lymphatic and blood vessels. From the perichondrium's blood arteries, nutrients enter the intercellular matrix and feed the osteocytes.<sup>142</sup> This histological trait significantly restricts the injured cartilage's ability to regenerate itself. Thus, physicians and researchers have been baffled by the damage healing challenge.<sup>143</sup> Combining cartilage scaffolds with 3D bioprinting technology offers a potent strategy to address and revitalize cartilage imperfections. These scaffolds are central in fostering essential cellular processes such as adhesion, differentiation, proliferation, and migration of the cells in the 3D bioprinted scaffolds. Moreover, they actively promote the regeneration of new tissue after implantation.<sup>144,145</sup>

3D printing of bone and cartilage has utilized ALG, a biocompatible and organic polymer, for bioink synthesis. Although progress has been made in 3D bioprinting of ALG for orthopedic applications, issues such as poor bioink component distributions and mechanical properties, lack of long-term stability, the absence of functional moieties for cell adhesion and proliferation, *etc.* remained as problems to be solved.<sup>146</sup> Hence, it is mixed with other proteins, polypeptides, and bioactive molecules to strengthen its structure and improve its biological activities. The uniform biomixing of multiple components present in today's advanced bioink is crucial for the controlled and homogeneous tissue regeneration. In a recent study, Bhattacharyya *et al.* demonstrated a screw extruder head with an ionic gel (ALG), alpha-tricalcium phosphate ( $\alpha$ -TCP) micro/nanoparticles, and osteoblast cells. 10 layers were 3D bioprinted using the bioink samples with a screw extruder which showed better bioprintability, with better mechanical and biological properties than the conventional mixing ones. The live cell distribution in the printed constructs was significantly superior to conventional mixing even with continuous feeding and extrusion-based bioink printing. The extrusion head ensured the control of uniform micro/nanomaterials and cell distribution throughout the directly mixed printable bioink with minimal cell damage as shown in Fig. 2(a and b).<sup>147</sup>

Govindharaj *et al.* developed a bioink hydrogel combining dECM with ALG and *Phallusia nigra* supports. When 3D bioprinted using extrusion with human mesenchymal stem cells (hMSCs), the hydrogel exhibited high cell viability, substantially increasing hMSC proliferation and promoting chondrogenesis for cartilage repair applications.<sup>149</sup> Chae *et al.* developed a 3D meniscus graft using mesenchymal stem cells and ECM. The implants showed good biocompatibility and strong mechanical properties in a rabbit meniscectomy model. They also stimulated the development

**Table 1** Polysaccharide-based extrusion 3D bioprinting of multilayered complex structures for tissue engineering applications

| S. No | Polysaccharide      | Preparation of bioink                               | Cell type                        | TE application | Features/limitations  | Ref. |
|-------|---------------------|---|----------------------------------|----------------|---|------|
| 1     | HAMA-CSMA-gel       | UV-crosslink  | SMSCs                            | Cartilage      | 1. Easy extrusion and stable 15-layer scaffolds w/ fidelity<br>2. Significant elastic and compressive strengths; robust after cross-linking<br>3. <i>In vitro</i> proliferation and differentiation of SMSCs<br>4. <i>In vivo</i> cartilage regeneration and functional recovery                                  | 126  |
| 2     | HA-ALG              | Ionic Ca <sup>+2</sup>                              | ADSCs                            | Cartilage      | 1. Double cross-linking w/ biotinylated HA and streptavidin for printability and structural integrity<br>2. Strengthened w/ Ca <sup>2+</sup> for mechanical stability & durability<br>3. High precision in printing both square & round shape<br>4. Chondrogenic differentiation for cartilage tissue engineering | 127  |
| 3     | GEG/lignin          | Magnesium (Mg <sup>+2</sup> )                       | hMSCs                            | Cartilage      | 1. Elation properties & lignin's shear-thinning behavior<br>2. High stability mimicking the cartilage environment<br>3. Chondrogenic differentiation  | 128  |
| 4     | GEG/gel             | Ionic Ca <sup>+2</sup>                              | MSCs                             | Cartilage      | 1. Rheological properties, shear recovery, and shape fidelity<br>2. The 30-layer scaffold w/ mechanical strength<br>3. Initial cell viability in scaffolds is over 80%  | 129  |
| 5     | ALG/gel             | Ionic Ca <sup>+2</sup>                              | MSCs                             | Bone           | 1. Stiffness controlled by alginate concentrations<br>2. Matrix mineralization and cellular organization<br>3. Osteogenic development effectively at high cell densities<br>4. Softer scaffolds excel in matrix deposition and cellular activity  | 130  |
| 6     | GEG                 | UV-crosslink  | MC3T3-E1                         | Bone           | 1. Good shear-thinning properties<br>2. UV cross-linking post-printing significantly w/ mechanical stability<br>3. High shape accuracy and structural integrity in complex structures   | 131  |
| 7     | ALG                 | UV-crosslink  | HASCs                            | Bone           | 1. Osteogenic potential of bioink by bone-derived ECM<br>2. Modified dECM enhances the mechanical properties of the bioink<br>3. Printing of complex, cell-laden structures w/ precise multi-layered, micro-sized struts<br>4. Osteogenic development w/ high <i>in situ</i> cell viability (>90%)                | 132  |
| 8     | GEG MA/GelMA        | Ionic Ca <sup>+2</sup> and UV-crosslink             | BMSCs                            | Bone           | 1. Structural stability of scaffolds w/ double cross-linking<br>2. Post-printing shape integrity<br>3. Diverse printing across a broad temperature range<br>4. Angiogenic and osteogenic properties   | 133  |
| 9     | XTG/ALG             | Ionic Ca <sup>+2</sup> (electrostatics interaction) | CCD-986                          | Skin           | 1. Viscoelastic properties, printability & structural stability w/ catechol-functionalization<br>2. Compressive and tensile strengths of the gel<br>3. Catechol functionalization w/ the biocompatibility   | 134  |
| 10    | ALG/gelatin         | Ionic Ca <sup>+2</sup>                              | PBMCs                            | Skin           | 1. Gel stability and offers strong antibacterial properties w/ gallium<br>2. Bioink's reproducibility and scalability<br>3. High-resolution & free printing of refined structures   | 135  |
| 11    | Pectin methacrylate | Ionic Ca <sup>+2</sup>                              | Human neonatal dermal fibroblast | Skin           | 1. Tunable mechanical properties w/ UV cross-linking & ionic gelation<br>2. Shear-thinning behavior in extrusion printing<br>3. Mechanical strength for dermal tissue support   | 136  |
| 12    | ALG/DCEL/gel        | Ionic Ca <sup>+2</sup>                              | hFBs                             | Skin           | 1. Enhanced mechanical integrity and stacking<br>2. Printing various shapes durable for manual handling<br>3. Supports growth and function of skin cells  | 67   |
| 13    | ALG/gel             | Ionic Ca <sup>+2</sup>                              | Schwann cells                    | Nerve          | 1. Post-printing stability<br>2. High viability rate of 91.87% in the scaffold<br>3. Enhanced release of neurotrophic factors (NGF, BDNF, GDNF <i>etc.</i> )<br>4. Positive immunofluorescence after 4 weeks  | 137  |
| 14    | HA acid/ALG         | Ionic Ca <sup>+2</sup>                              | Schwann cells                    | Nerve          | 1. Multilayer scaffolds w/ varied geometries<br>2. Balance stabilization speed & flowability for effective bioprinting  | 138  |

Table 1 (continued)

| S. No | Polysaccharide  | Preparation of bioink  | Cell type     | TE application | Features/limitations   | Ref. |
|-------|-----------------|------------------------|---------------|----------------|--|------|
| 15    | ALG/BNC/GelMA   | Ionic $\text{Ca}^{+2}$ | Schwann cells | Nerve          | 3. High Schwann cell viability and functional activity<br>1. BNC addition improves mechanical & rheological properties<br>2. Post-printing shape fidelity & line thickness<br>3. Oriented growth and high viability of Schwann cells   | 139  |
| 16    | HA acid/ALG/gel | Ionic $\text{Ca}^{+2}$ | Schwann cells | Nerve          | 1. Curable <i>via</i> iron(III) chelation or photocuring, adjusting mechanical properties & degradation rates<br>2. Intricate tissue architecture printing w/ post-printing stability<br>3. High cell viability in Schwann cells, neuronal cells, and glioma cell-laden constructs | 140  |
| 17    | HA/gelatin      | Genipin                | MC3T3         |                | 1. Gelatin concentration affects hydrogel's viscosity and shear-thinning, improving extrusion<br>2. Printed complex, multi-layer (50 and 100 layers) structures<br>3. Robust cell growth & high cell viability   | 6    |
| 18    | ALG/HA          | Ionic $\text{Ca}^{+2}$ | MC3T3         |                | 1. Good printability for complex structures w/ stable viscosity<br>2. 100 layers printing w/ post-printing integrity under stress by $\text{Ca}^{2+}$<br>3. Cell survival and controlled release of medicinal substances   | 2    |

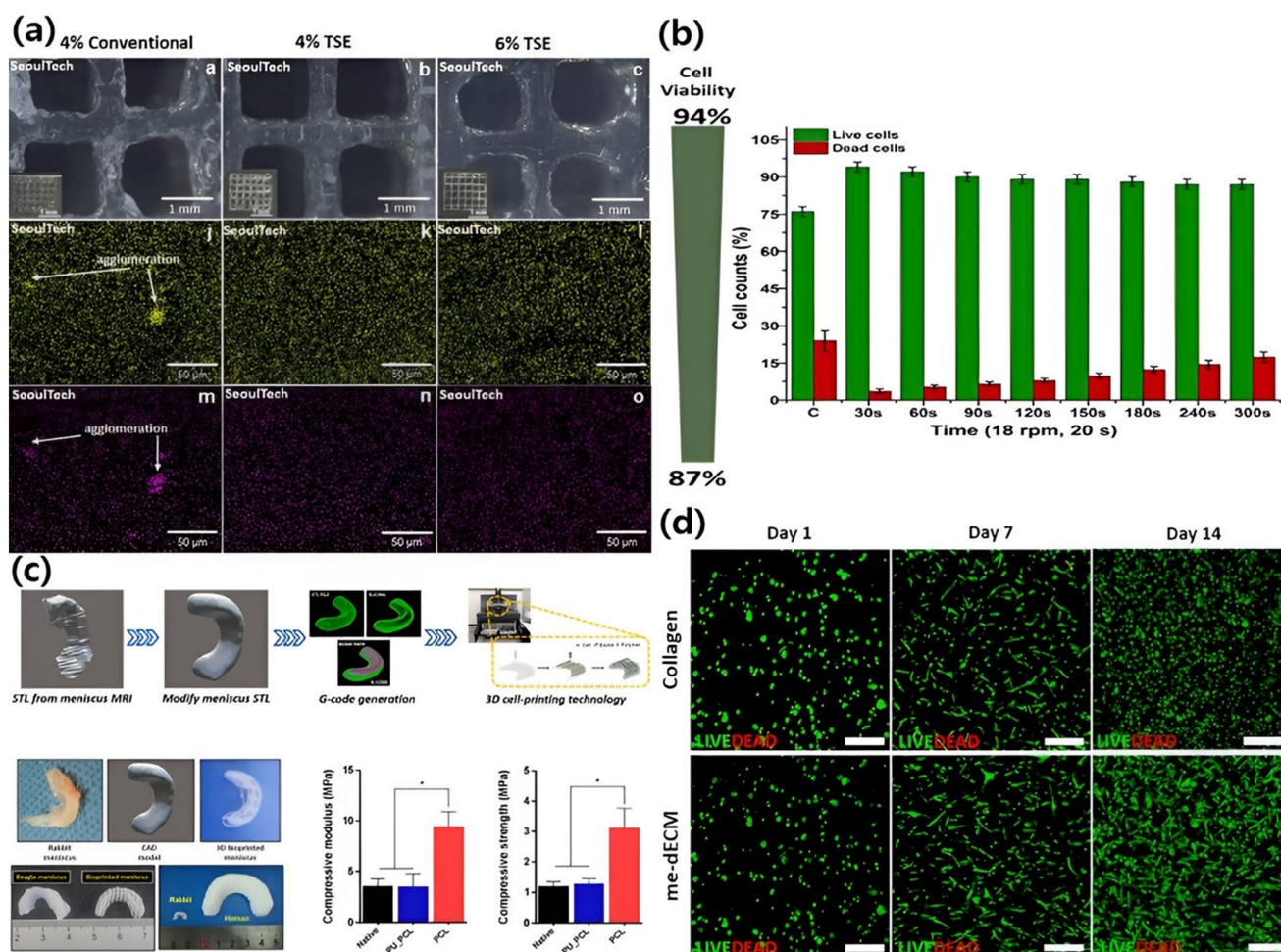


Fig. 2 (a) 3D biprinted (top view) micro/nanocomposite ALG gels showing improved dispersion of  $\alpha$ -TCP particles in the bioprinting pen process; (b) cell viability of ALG gels (permission from Elsevier<sup>147</sup>); (c) bioink from the meniscus was used to 3D print living, functional meniscus grafts; (d) live/dead staining (permission from Elsevier<sup>148</sup>).



of neo-fibro cartilage *in vivo* due to a meniscus-specific environment. This approach, combining cells, biomaterials, and bioactive proteins, holds promise for promoting regenerative healing of complex cartilage tissues (as depicted in Fig. 2(c) and (d)).<sup>148</sup> Sadeghianmaryan *et al.* enhanced 3D bioprinting using a chitosan-based bioink with optimized crosslinking methods. The biocompatibility of the chitosan-printed bioink was validated through ATDC5 cell testing. This bioink holds promise for applications in cartilage regeneration and joint repair due to the articular cartilage's role as a shock absorber and lubricant in the joints.<sup>150</sup>

To increase the strength of the chitosan hydrogel, He *et al.* first treated the chitosan with calcium and ethylene diamine tetraacetic acid (EDTA), and then 3D printed a scaffold. The bioink has been altered to exhibit enhanced mechanical characteristics and to encourage the growth of chondrocytes and the expression of genes related to cartilage.<sup>151</sup> One derivative of chitosan with outstanding injectability and biocompatibility is hydroxybutyl-chitosan (HBC). Liu *et al.* synthesized the HBC-nanofiber (NF) hydrogel by combining HBC hydrogel with poly(lactic acid-co-glycolic acid) copolymer nanofiber. The use of nanofibers considerably improved the HBC-NF hydrogel's capacity to encourage cartilage development. The HBC-NF hydrogel was then injected into a 3D printed PCL skeleton that had internal microchannels to produce a mechanical concentration scaffold resembling a real cartilage.<sup>152</sup> Antich *et al.* printed 3D scaffolds for articular cartilage by combining HA and alginate. The novel biological ink that encourages the regeneration of articular cartilage and has appropriate mechanical qualities is made of HA and alginate.<sup>153</sup> Angelis *et al.* printed 3D CH/HAA scaffolds using chitosan (CH) and hydroxyapatite (HAA) as raw materials. During a protracted culturing period, the printed scaffold's adequate porosity offers ideal conditions for chondrocyte colonization and synthesis. A unique idea for articular cartilage tissue engineering is provided by the scaffold cultured in platelet lysate (PL), which can better retain the phenotype of chondrocytes and enhance gene expression of the most relevant ECM components, utilizing the fact that HAA encourages cartilage regeneration.<sup>154</sup>

Olate-Moya *et al.* mixed alginate, gelatin, and chondroitin sulfate (CS). To enhance the shape fidelity and resolution of the 3D printing scaffold, graphene oxide was further added to the composite hydrogel as a nanofiller material. A  $30 \times 30 \times 1 \text{ mm}^3$  (length  $\times$  width  $\times$  height) scaffold was designed. An interior pattern resembling a mesh was established, with a thread spacing of 1.5 mm. Human adipose tissue mesenchymal stem cells were used to demonstrate that the novel nanocomposite hydrogel had a higher rate of cell proliferation than pure sodium alginate. Additionally, the scaffold made with the innovative hydrogel nanocomposite shows superior cartilage induction.<sup>155</sup>

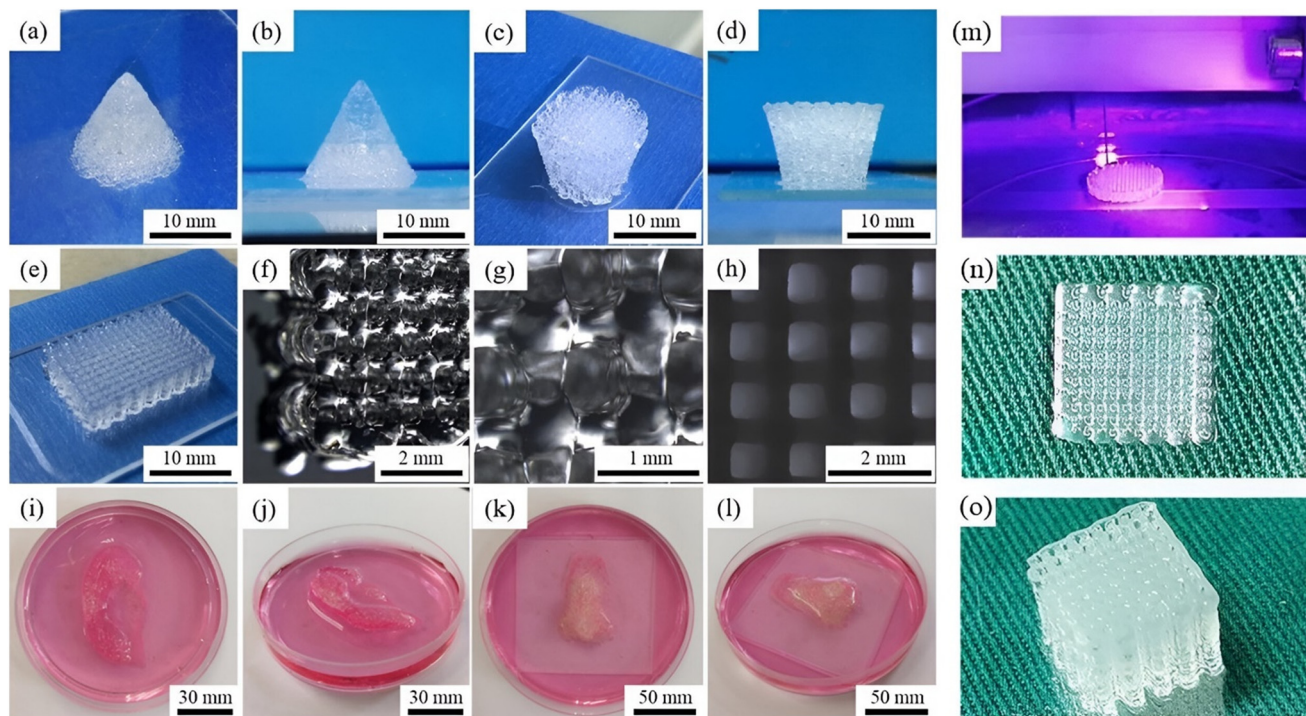
## 4.2 Bone

Bone is a sophisticated composition comprising hierarchical tissue, mineralized collagen fibers, and a network of blood vessels. Remarkably, bones can autonomously mend minor cracks and fractures due to their strong regenerative capacity. However, when a bone injury exceeds the size of two centimeters, its natural healing mechanisms become insufficient. Restoring both form and function necessitates tailored grafts with tissue regeneration capacity. Conventional hydrogels lack precise control for internal structure and growth factor distribution.

Innovative 3D bioprinting methods have been proposed to get over these constraints.<sup>156,157</sup> In a recent advancement, Curti *et al.* created a scaffold-based 3D bioprint bioink from fish gelatin(FG), cuttlebone (CB), and ALG. It was accomplished by bonding the bioink after it had been produced. Conical nozzles with an inner diameter of 0.25 mm (G25) were employed; eight layers with a deposition direction ranging from 0 to 90°, and a layer thickness equal to 75% of the nozzle diameter. The composite FG-CB-ALG bio-ink hydrogel encapsulated to pre-osteoclast cells MC3T3-E1 and most bone ECMs were replicated using marine materials. The *in vitro* biocompatibility assay revealed that the composites could support MC3T3-E1 murine pre-osteoblast growth and proliferation and that the hydrogel may boost the bone regeneration potential of the 3D bioprint scaffold.<sup>158</sup> Seunghyun *et al.* introduced PDANPs for their notable affinity to bind with calcium ions, ALG, and temporally oxidized cellulose nanofibrils, all vital components within the bioink designed for bone tissue construction. Once integrated into each bioink, osteoblasts were extruded using 3D printing technology, and hydrogels were printed as a grid structure of 6 layers, followed by a curing period lasting up to 7 days. Notably, most cells in each sample remained viable. The printed cells' viability rate (% of living cells) surpassed 80% for all three models on both days 1 and 7.<sup>159</sup>

For the objective of bone tissue engineering, a unique MeGC-based cell-laden 3D bioink was created. For creating biocompatible and structurally stable cell structures, the bioink solutions were tuned. MeGC bioink was created by photocuring the precursor solution for 70 seconds. The produced five-layer 3D bioprinting constructions showed increased cell proliferation, distinct bone differentiation, and good shape fidelity.<sup>160</sup>

Wu *et al.* introduced a dual-network hydrogel approach, by combining the shear-thinning and recovery properties of GEG with the rapid photo crosslinking capabilities of poly(ethylene glycol)-diacrylate (PEGDA). The bioprinted multilayered scaffold provided an open and interconnected network (Fig. 3(a to l)), facilitating efficient oxygen and nutrient exchange. Within these hydrogels, several human-scale structures were printed with good stability and fidelity. Furthermore, researchers incorporated MC3T3-E1 cells and murine BMSCs. Throughout the 21 day cell culture, both cell



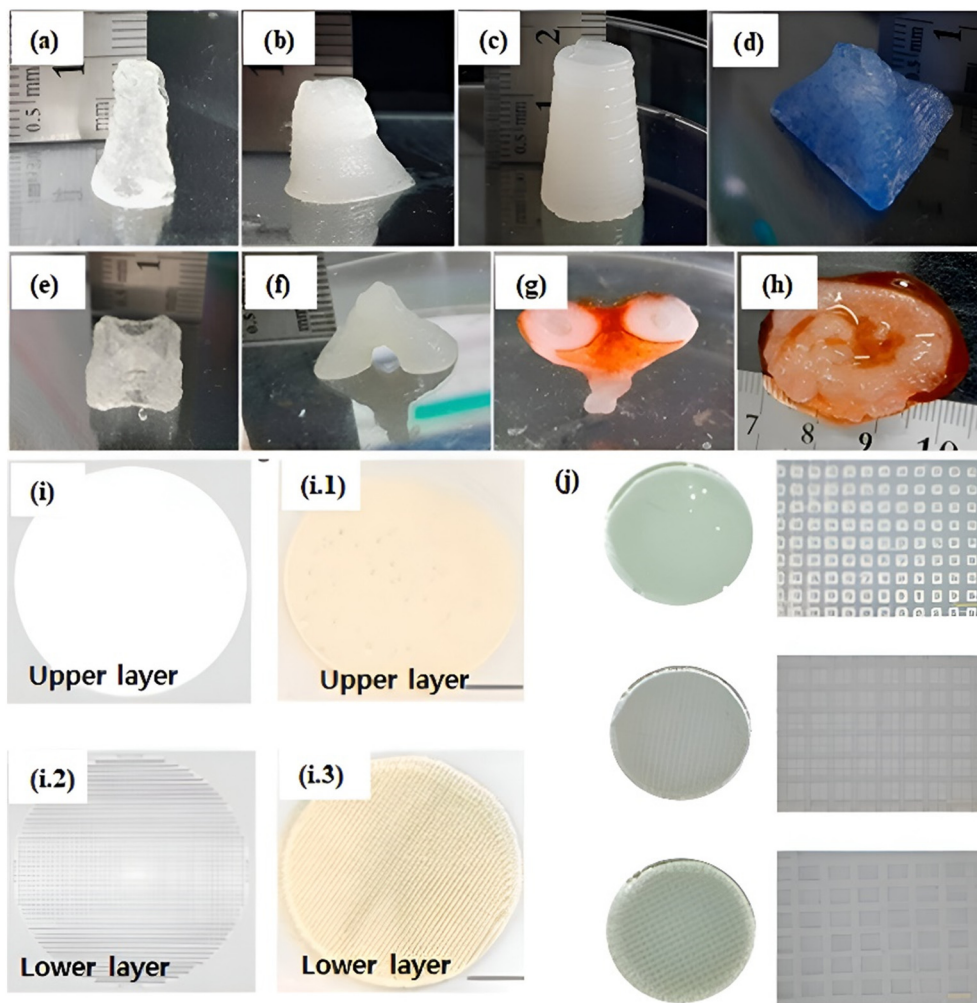
**Fig. 3** Images of several 3D printed scaffolds by Ink G1.5P10. Aerial view (a) and left view (b) images of a sharp cone structure. Aerial view (c) and left view (d) images of a reverse square prism structure. (e) The aerial view image of a cuboid structure. Its partial enlarged images obtained by a stereoscope shown as (f)–(h) (after being treated with liquid nitrogen). Top view (i) and aerial view (j) images of a 3D printed gel human ear. Top view (k) and aerial view (l) images of a 3D printed gel human nose. (permission from Elsevier).<sup>131</sup> (m)–(o) Printing process of the scaffold, top view and side view of the scaffold with 60 layers (permission from Elsevier<sup>133</sup>).

types demonstrated both stability and viability, with cell survival exceeding 87%.<sup>131</sup> The deferoxamine-loaded GelMA/GGMA-based hybrid bio-ink was improved by Zhihui *et al.* using ionic and light cross linking techniques to create a 3D-printed scaffold of 60 layers as shown in (Fig. 3(m)–(o)). The hydrogels made of GGMA/GelMA had the best printing and tensile qualities. The regulated release of pharmaceuticals from GGMA/GelMA hydrogels could be used to simulate *in vivo* bone reserve, HUVEC tube improvement, and cell migration. A rat cranial defect model study demonstrated that angiogenesis and bone repair might occur *in vivo* by activating the hypoxia-inducible factor 1 (one)-alpha signaling pathway. To repair the bond defect, hybrid hydrogel 3D bioprinting can assist with osteogenesis and angiogenesis.<sup>133</sup>

### 4.3 Skin

The body's largest organ, the skin, serves as a protective barrier against the external environment. Bioprinted skin must possess key attributes to facilitate effective nutrient/waste exchange and promote robust skin regeneration. These include biocompatibility, mechanical properties aligned with natural skin tissue, and a meticulously interconnected network of pores.<sup>161,162</sup> Lately, there has been a growing trend in developing 3D multicellular HA-based constructs.<sup>6</sup> In addition to HA, researchers have explored various other biocompatible materials like collagen, fibrin, decellularized

dermis and epidermis, ALG,<sup>147</sup> chitosan,<sup>7,147</sup> gelatin, and cellulose,<sup>95</sup> aiming to facilitate skin wound healing. Liu *et al.* introduced an innovative approach by combining ALG/gelatin to produce a bioink with enhanced printability of multilayered scaffold structures. Through an in-depth rheological analysis, they identified the optimal blend comprising 2% ALG and 15% gelatin. Introducing 2% CaCl<sub>2</sub> triggered a remarkable ionic gelation process driven by the ALG component, which substantially stabilizes the fabricated structures. This hybrid bioink encapsulated hAECs and was employed for bioprinting a skin-like, bi-layered membrane construct. The outcomes of the *in vitro* study demonstrated a notable enhancement in the epithelial cell phenotype of the hAECs.<sup>163</sup> In a study by Zhao *et al.*, the approach involved the incorporation of poly(*p*-phenylene vinyl acetate) (PPV) and HA into ALG/gelatin ink blends, resulting in skin patches imbued with distinctive biological properties. The ALG/HA/Gel-A5G81/PPV skin patches proved instrumental in effectively healing the wound and fostering the development of new skin tissue. Importantly, these patches exhibited *in vivo* biodegradability, signifying their potential as a promising avenue for advanced wound healing approaches.<sup>164</sup> Due to the uniform distribution of chitosan and kaolinite nanoclay utilizing the screw-based pen-type extruder, Bhattacharyya *et al.* in this study demonstrated the structural stability of the multicomponent ALG-based hydrogel. Extrudable multilayered big and sophisticated



**Fig. 4** (a)–(c) 3D printed hollow cylinder with varying nanocomposite concentration, (d) pyramid, (e) star, (f) top view of the bifurcated tube, (g) bottom view of the bifurcated tube, (h) and human ear (permission from Elsevier<sup>7</sup>). (i) & (i.1) The upper dense layer is dense mimicking the epidermis, (i.2) and (i.3) the lower porous layer resembling the dermis. (j) The printed products by DLP-based 3D printing showed complex structures of CAD images of 3D biprinted products.<sup>165</sup>

tissue engineering constructs can be created using this multiple crosslinking method of the self-standing hydrogel with good post-printing shape fidelity. For large scale 3D bioprinting, the “Biowork™” pen with a roller head attached to a 3D bioprinter is utilized. Bioinks made from osteoblast and fibroblast cells in varying concentrations of kaolin-dispersed alginate/chitosan hydrogels were used, and various multilayered 3D biprinted constructs were demonstrated as given in (Fig. 4(a)–(h)). Additionally, it increases the cellular activity of two distinct cell types (osteoblast and fibroblast), which are targeted towards bone and skin tissue engineering.<sup>7</sup> The double crosslinked hydrogel with HA as its foundation showed excellent swelling characteristics, biocompatibility, and extremely controlled breakdown. To treat diabetic foot ulcers, this material is being evaluated as a novel gel wound dressing. Zhou *et al.* printed functional live skin using a newly developed biomimetic GelMA/HA-NB/LAP hydrogel using digital light processing based 3D printing technology (Fig. 4 (i), (i.1)–(i.3) and (j)).<sup>165</sup>

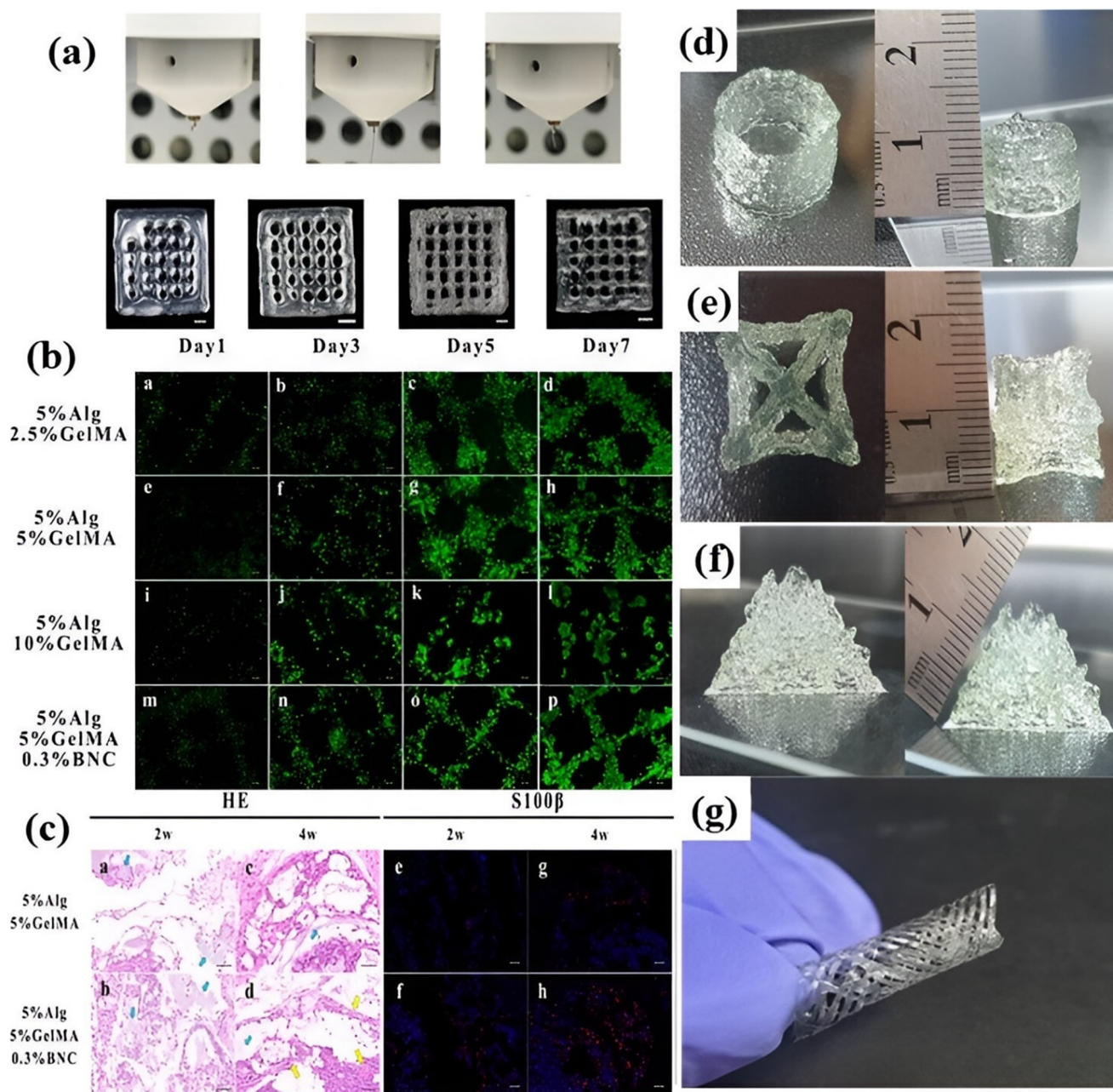
#### 4.4 Nerve

The nervous system is pivotal in transmitting impulses across different body regions, thereby governing their coordinated movement. In addressing central and peripheral nerve injuries, the restoration of nerve function through regeneration is of paramount importance. However, the intricacy lies in the fact that nerve regeneration is particularly challenging for the central nervous system compared to the peripheral nervous system, as the former exhibits limited inherent self-repair capabilities.<sup>166</sup> Efforts directed at brain tissue regeneration through bioprinting entail the creation of 3D models that encompass elevated cell densities and a diverse array of cell types. This orchestrated complexity serves as a vital substrate for mimicking the intricate architecture of brain tissue. Moreover, the success of these models hinges upon the precise administration of external cues, which govern cell behavior and sustain the intricate web of cellular interactions.<sup>167</sup> By harnessing bioprinting, a dynamic



platform is established for fostering the renewal and preservation of the nervous system, addressing the challenges posed by nerve injuries and contributing to the advancement of regenerative therapies.<sup>168</sup> Peripheral nerve damage presents a clinically intricate and challenging scenario, often leading to compromised sensory and motor capabilities due to inherent regenerative potential limitations. Despite various efforts, their efficacy is frequently confined. In the pursuit of addressing peripheral nerve regeneration, nerve-guided catheters have emerged as captivating alternatives. Among

the therapeutic options available, autologous nerve transplantation, while popular, is burdened with shortcomings such as donor scarcity and potential neurological issues in the donor area.<sup>169,170</sup> Presently, the predominant choice for malleable nerve cells in neural tissue engineering revolves around Schwann cells. These versatile cells possess the capacity to express an array of nerve growth factors (NGF), encompassing NGF, BDNF, GDNF, and PDGF, thereby instigating the formation of nerve synapses. Particularly adept at promoting the regeneration of the



**Fig. 5** Application of nerve tissue regeneration: (a) different concentrations of ALG BNC, and GelMA, excessive fluidity of extruded liquid, extruded lines suitable for printing. (b) and (c) Cell viability staining is performed on the hydrogel (permission Elsevier).<sup>139</sup> 3D bioprinted structures: (d) hollow tube structure, (e) star shape, (f) pyramid shape & (g) four-axis printed tubular shape with a bio responsive gelatin-hyaluronic acid hydrogel (permission MDPI<sup>6</sup>).

peripheral nervous system, Schwann cells find extensive application. Consequently, integrating ALG-based hydrogels housing Schwann cells can effectively facilitate the expression of multiple nerve growth factors, contributing to a conducive environment for nerve development.<sup>137</sup> As an illustration, the hydrogel framework, crafted from a blend of low-viscosity ALG, HA, and fibrinogen, notably fosters heightened activity and proliferation of Schwann cells, accompanied by the expression of essential biological proteins. Moreover, this scaffold exerts a regulatory influence on the maturation of the dorsal root ganglion, concurrently promoting the alignment of Schwann cells.<sup>138</sup> The study explored the printability aspects of ALG, GelMA and a hybrid bioink composed of bacterial nano-cellulose (BNC), accompanied by an in-depth analysis of the resulting material's physicochemical attributes reported by Wu *et al.* Subsequently, RSC96 cells were integrated into the bioink and used for bioprinting. The incorporation of ALG and BNC in the composition reveals that post photo-crosslinking and chemical crosslinking, the BNC and GelMA composite exhibited the most delicate lines compared to other groups. This suggests that the addition of BNCs enables the preservation of the scaffold's original shape, highlighting its potential to maintain inherent structural integrity as shown in (Fig. 5a). Furthermore, the biocompatibility of the engineered construct was confirmed through experiments involving unclothed mice. To facilitate nerve regeneration, the combination of BNC with ALG/GelMA was employed in the hydrogel construct. Notably, *in vivo* investigations revealed compelling outcomes, wherein directed growth, proliferation, and gene expression of rat Schwann cells were promoted as shown in (Fig. 5b and c).<sup>139</sup> Haring *et al.* employed a combination of natural and synthetic polymers to successfully bioprint 3D micro-physiological brain tissue, facilitating research on disease models and regenerative potential. The bioprinting process involved using a specially formulated bioink of HA, ALG, and gelatin, which could be crosslinked using  $\text{CaCl}_2$ . This investigation was conducted in comparison to the stiffness of the original brain tissue. Intriguingly, it was discovered that the hydrogel attained a stiffness level comparable to that of native human tissue at higher concentrations of gelatin.<sup>140</sup> Similarly, Khatun *et al.* demonstrated genipin crosslinked with a gelatin–HA bioresponsive hydrogel for high shape fidelity and resolution. A variety of forms, including hollow tubes and star shapes that were each made up of about 50 layers and could reach a height of 1 cm (Fig. 5d and e), were successfully printed. These intricate printed forms have a porous surface morphology that was visible after lyophilization. The gelatin–HA gel-based 3D printed structures, in particular the 1.5 cm tall pyramid-shaped structure (Fig. 5f) with about 100 layers, demonstrated exceptional stability and shape integrity. An intriguing finding was that the printed structures stuck to the substrates very well without the need for further adhesive, indicating how highly sticky the hydrogel is to the surface.<sup>6</sup>

#### 4.5 Muscle

Muscle tissue provides for around 45% of total body weight and accounts for a considerable percentage of the body's composition, with a significant portion interwoven with bones. Peripheral nerves intricately innervate muscles, particularly those within motor organs. In nerve injury, particularly to peripheral nerves, the innervated muscles undergo a process of atrophy, leading to a decline in size and function.<sup>171</sup> Hence, the restoration of muscle tissue involves a combination of strategies encompassing nerve and vascular regeneration techniques. Currently, the principal avenues for promoting muscle regeneration involve administering growth factors and cell transplantation. Notably, ALG is a widely favored biomaterial for 3D printing muscle constructs, particularly in ALG-based bioink with cells.<sup>172</sup> Lee *et al.* demonstrated a bioink composed of collagen, GelMA, and dECM, restoring muscle tissue using 3D bioprinting with a novel photo-crosslinking technique. This crosslinking method was used to create multilayered 3D cell-laden structures, mechanically stable and biologically safe, cell-laden struts. When administered to a volumetric muscle defect injury in mice, the bioprinted hASC-containing constructs showed noticeably improved muscle regeneration over those made with a traditional bioprinting method.<sup>173</sup> The C2C12 myogenic cells, a widely used cell type in tissue engineering for muscle regeneration, were studied in those 3 commercial biomaterials. The results showed robust cell proliferation and successful differentiation across all 3 muscular scaffolds. The cells also demonstrated significant myotubular organization along a linear structure and the ability to coalesce and fuse, generating structurally aligned multinucleated myotubes.<sup>174</sup>

#### 4.6 Other tissues

Tuning the polysaccharide-based hydrogels through a simple modification or crosslinking method enabled researchers to use them in tissue engineering and bioprinting tissues such as retina, liver, heart, *etc.*<sup>175</sup> The kidney's substantial complexity and intricate structure pose a formidable challenge for complete bioprinting in a single endeavor. The formidable nature of this task arises from both the intricacy of the organ's design and its considerable size. The most practical approach involves employing 3D bioprinting to create microscopic components, often called “mini tissue building blocks.” Subsequently, these intricate building blocks can be assembled to construct larger, functional kidney structures.<sup>175</sup> To circumvent the intricacies of organ size and cellular diversity, the utilization of organoids presents a viable avenue for bioprinting endeavors. However, achieving functional viability for the kidney necessitates addressing critical concerns related to the organ's ductal system and vasculature, which present their own complex challenges.<sup>176</sup> Cardiovascular disease (CD) is the foremost global cause of mortality, accounting for 31% of all deaths. This prevalent health issue primarily arises from plaque

accumulation, a condition known as atherosclerosis, which constricts arteries and other vital blood vessels. This broader category of cardiovascular ailments encompasses conditions like myocardial infarction, congestive heart failure, stroke, and valvular heart disease.<sup>177</sup> A composite bioink (ALG/GelMA) is intricately deposited using the coaxial extrusion bioprinting technology, encasing endothelial cells, and creating micro fibrous scaffolds with intricate micro-channels. This novel approach significantly promotes the process of scaffold reendothelialization, encouraging the growth and proliferation of HUVECs. After this, the deendothelialized scaffold serves as a foundation for introducing cardiomyocytes, culminating in developing a deendothelialized myocardial scaffold. This scaffold configuration closely mimics the structural attributes of native myocardial tissue. Remarkably, the scaffold orchestrates synchronized and spontaneous contractions and maintains a consistent rhythm, yielding an impressive heartbeat rate ranging from 55 to 75 beats per minute.<sup>178</sup> LA *et al.* created axially symmetric aortic valve scaffolds with a 12 mm internal diameter using PEGDA and ALG, similar to double nozzle extrusion printing. The hybrid scaffolds had an elastic modulus of 5.3–74.6 kPa and a nearly 100% survival rate for cultured PAVIC over 21 days.<sup>179</sup> The bioink derived from ALG can be used to create 3D scaffolds that enhance the mechanical and biological properties of the hydrogels. These scaffolds, when adapted to the liver's honeycomb architecture, can significantly improve hepatocyte proliferation, replication, and overall liver function preservation.<sup>180</sup> Rania *et al.* used a core-shell bioprinting technique to enhance the complexity of hepatocytes' microenvironment. They used distinct core-shell structures housing different cell types and incorporated Matrigel into ALG/MA to mimic the honeycomb-like arrangement in liver tissue. This led to significant increase in hepatocyte activity and proliferation within the scaffold. The introduction of plasma or fibrin into the core of ALG/MA facilitated the organization of fibroblasts into a network, creating a more intricate and realistic microenvironment.<sup>181</sup> A 3D retina-equivalent construct was 3D printed using GM and triethylamine to methacrylate-HA at different degrees. Cell-binding motifs were added to HA-GM polymers, followed by photoirradiation using a LAP photo initiator. The hydrogels were further enhanced with RGD to improve adhesion properties. The construct, with a base layer of 125  $\mu\text{m}$  and a top layer of 250  $\mu\text{m}$ , showed over 70% vitality and matured into retinal photoreceptors, ganglion cells, and bipolar cells within 14 days, resembling real retina layers.<sup>182,183</sup>

## 5. Future directions

3D bioprinting has made significant progress, but the challenge lies in creating tissue engineering structures with desired bioink properties. Natural polysaccharides continue to be the preferred option for creating bioinks for 3D

bioprinting tissue engineering. Because of their greater bioactivity, lower immune response and biocompatibility, polysaccharides are frequently preferred over proteins and synthetic polymers like PCL, PEG, and Pluronic. These naturally occurring biomaterials are excellent at simulating the ECM and offer vital signals that support cell adhesion, proliferation, differentiation, and tissue regeneration, all of which are necessary for not only tissue regeneration but also proper integration with host tissues and operation of bioengineered tissues. Although they have limitations in terms of processing and cell interactions in 3D bioprinting tissue engineering, a number of interesting methods and chemical alterations have already shown how to overcome these restrictions. 3D bioprinting has made substantial progress using polysaccharide-based bioinks due to their biocompatibility and effective mimicry of the ECM in different tissues. These hydrogels can work as a major bioink component for engineering complex, multilayered structures and are favored for their non-toxicity, natural degradability with minimal side effects, and diverse crosslinking properties. Current research is enhancing these bioinks' properties with modifications of hydrogels to their chemical and physical structures similar to ECM's proteoglycans including cell-adhesive oligopeptides, aiming to closely replicate native tissue environments and improve the functionality of the engineered tissues. The field is moving towards integrating bioactive molecules such as growth factors and nanoparticles, as well as incorporating real-time monitoring technologies to refine the bioprinting process. This integration is expected to drive significant advances in personalized regenerative medicine, leading to more effective tissue and organ replacements. Especially in complex tissue regeneration, fabrication of multilayered tissues such as skin, blood vessel and osteocartilage emerges as an essential tool. Furthermore, development of bioinks and hydrogels is critical in tissue engineering and regenerative medicine with post-printing fidelity, *i.e.* higher interaction between the layers of the tissue engineering constructs. The convergence of these technologies holds promise for accelerating the development of personalized, functional tissue replacements, ushering in a new era of 3D bioprinting of multilayered complex tissue scaffolds, regenerative medicine and tissue engineering.

## 6. Abbreviations

|      |                             |
|------|-----------------------------|
| DECL | Diethylaminoethyl cellulose |
| ALG  | Alginate (sodium salt)      |
| CHN  | Chitosan                    |
| HA   | Hyaluronic acid             |
| CEL  | Cellulose                   |
| DEX  | Dextran                     |
| ECM  | Extracellular matrix        |
| GEG  | Gellan gum                  |
| AGR  | Agarose                     |
| KJG  | Konjac gum                  |



|                   |   |
|-------------------|---|
| GG                | Guar gum  |
| XTG               | Xanthan gum                                     |
| CS                | Chondroitin sulfate                             |
| PRP               | Platelet-rich plasma                            |
| BMSCs             | Bone marrow mesenchymal stromal cells           |
| dECM              | Decellularized extracellular matrix             |
| MFC               | Meniscal fibrocartilage cells                   |
| TGF               | Transforming growth factor beta-1               |
| MSCs              | Mesenchymal stem cells                          |
| MECM              | Meniscal extracellular matrix                   |
| SMSc              | Synovial mesenchymal stem cells                 |
| hMSCs             | Human mesenchymal stem cells                    |
| ATDC5             | Mouse teratocarcinoma cell                      |
| MC3T3-E1          | Clonal murine cell line of immature osteoblasts |
| MA                | Methacrylate                                    |
| HASCs             | Human adipose-derived stem cells                |
| BMP-2             | Bone morphogenetic protein-2                    |
| GGMA              | Gellan gum methacrylate                         |
| HUVEC             | Human umbilical vein endothelial cells          |
| hAECs             | Human amniotic epithelial cells                 |
| NGF               | Nerve growth factor                             |
| BDNF              | Brain-derived neurotrophic factor               |
| GDNF              | Glial cell-derived neurotrophic factor          |
| PDGF              | Platelet-derived growth factor                  |
| BNC               | Bacterial nanocellulose                         |
| RSc               | Rat Schwann Cell 96                             |
| CaCl <sub>2</sub> | Calcium chloride                                |
| CD                | Cardiovascular disease                          |
| PAVIC             | Porcine aortic valve interstitial cells         |
| BTE               | Bone tissue engineering                         |
| CCD-986           | Human skin fibroblast cells                     |
| PBMCs             | Human peripheral blood mononuclear cells        |
| hFBs              | Human fibroblasts                               |
| MeGC              | Methacrylated glycol chitosan                   |
| FG                | Fish gelatin                                    |
| CB                | Cuttle bone                                     |
| CMP               | Cuttlefish melanin nanoparticles                |
| PLs               | Platelet lysates                                |
| GFs               | Growth factor                                   |
| CNC               | Carboxylated-cellulose nanocrystal              |
| PDANP             | Polydopamine nanoparticles                      |

## Conflicts of interest

There are no conflicts to declare.

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