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Advances in gelatin bioinks to optimize bioprinted cell functions

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Abstract

Gelatin is a widely utilized bioprinting biomaterial due to its cell-adhesive and enzymatically-cleavable properties, which improve cell adhesion and growth. Gelatin is often covalently crosslinked to stabilize bioprinted structures, yet the covalently-crosslinked matrix is unable to recapitulate the dynamic microenvironment of the natural extracellular matrix (ECM), thereby limiting the functions of bioprinted cells. To some extent, a double network bioink can provide a more ECM-mimetic, bioprinted niche for cell growth. More recently, gelatin matrices are being designed using reversible crosslinking methods that can emulate the dynamic mechanical properties of the ECM. In this review, we analyze the progress in developing gelatin bioink formulations for 3D cell culture, and critically analyze the bioprinting and crosslinking techniques, with a focus on strategies to optimize the functions of bioprinted cells. We discuss new crosslinking chemistries that recapitulate the viscoelastic, stress-relaxing microenvironment of the ECM, and enable advanced cell functions, yet are less explored in engineering the gelatin bioink. Finally, we present our perspective on the areas of future research and argue that the next generation of gelatin bioinks should be designed by considering cell-matrix interactions, and bioprinted constructs should be validated against currently established 3D cell culture standards to achieve improved therapeutic outcomes.

Graphical Abstract

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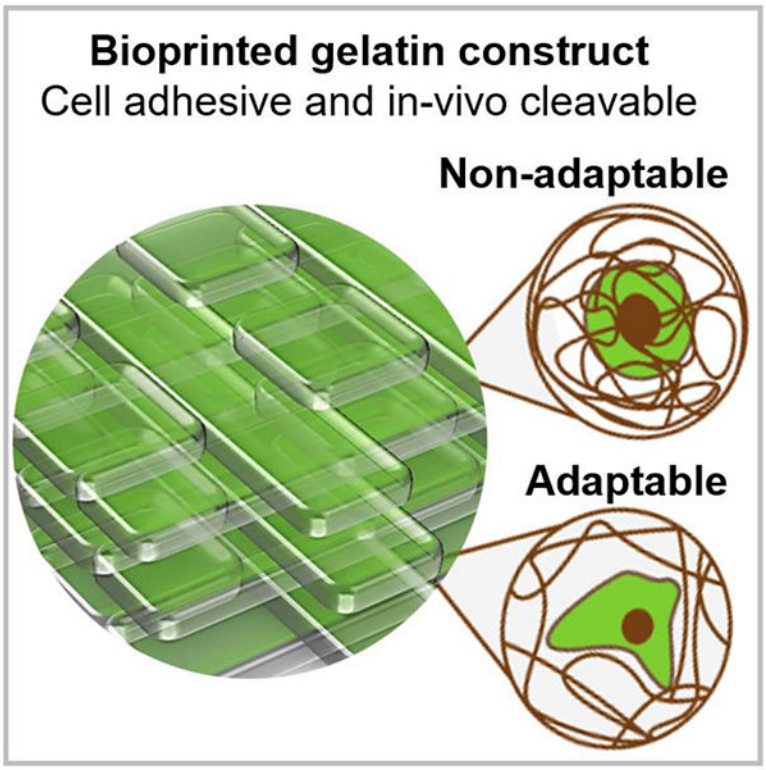

Gelatin bioinks are widely utilized; yet the cell functions in the bioprinted gelatin are often limited. In this review, we summarize the progress in gelatin bioinks for 3D bioprinting, and critically analyze the compositions and crosslinking techniques, with a focus on optimizing the bioprinted cell functions. We discuss new crosslinking chemistries that recapitulate the stress-relaxing microenvironment of the tissue and improve cell functions, yet are less explored in gelatin bioinks.

Keywords

Gelatin bioinks; 3D bioprinting; extracellular matrix; covalent crosslinking; viscoelasticity; stress relaxation

1. Introduction

The current treatment options to replace damaged tissues and organs rely on donor tissue/organ transplantation. The number of available transplantable donor organs is not sufficient to meet the increasing demand due to increased life expectancy [1]. To address the organ shortage, tissue engineering has emerged as an alternative strategy, which promises to provide lab-grown engineered tissues for transplantation [2–4]. Various technologies have been explored for manufacturing bioartificial organs [5, 6]. Two key features that are difficult to replicate in engineered tissues using conventional approaches are the organization of multiple cell types and the development of a vascular system, which are critical for the physiological function of the tissue. To tackle these challenges, 3D bioprinting has emerged

as a viable technology that provides precise control over the spatial distribution of cells and other biologics in a volumetric space with high reproducibility [7, 8]. This results in formation of patterns that are prescribed using computer-aided design (CAD) tools [9]. The 3D bioprinting technology has shown unprecedented advantages in situations where spatial control is essential, thus allowing to bridge the divergence between native and engineered tissue constructs.

Bioink - a mixture of prepolymer and cells - provides a structure for bioprinted constructs and allows cells to survive, proliferate, and grow. One of the challenges in bioink development is maintaining cytocompatibility while fulfilling the physical and mechanical requirements for 3D bioprinting, such as extrudability and shear thinning [10, 11]. For example, in extrusion-based bioprinting (EBB), bioinks play a vital role in dispersing cells prior to bioprinting, maintaining structural integrity during bioprinting, and supporting the spreading and functionality of encapsulated cells post-bioprinting [12, 13]. An ideal bioink should be highly compatible to accommodate living cells, provide high resolution during bioprinting, and possess properties such as high mechanical integrity, stability, non-immunogenicity, shear-thinning behavior, extrudability, and cell adhesion [14]. Bioprinting techniques primarily use hydrogels as bioinks, because, hydrogels mimic ECM due to their hydrophilic polymer network and water content [15]. Hydrogels can retain their structure because of crosslinking via chemical or physical bonds while controlling water absorption [16]. The amount of water that the polymeric network of hydrogels can absorb can be several times greater than their dry weight due to the presence of hydrophilic functional groups such as NH_2, -COOH, -OH, -$CONH_2$, -CONH, and -SO_3H [17]. This high-water intake allows for the encapsulation of cells without inflicting damage. Moreover, this highly hydrated network allows for the exchange of nutrients and gases, making them an attractive option for developing bioinks.

Gelatin, a collagen-derived protein, undergoes physical gelation below room temperature, is shear thinning, contains cell-adhesive ligands, and can be enzymatically cleaved by cells. Due to these characteristics, gelatin bioinks have attracted tremendous attention to develop tissues [18–22]. To date, various gelatin-based bioinks have been formulated to identify bioinks supporting specific cell types [23–26]. Gelatin-based bioinks have been employed in various bioprinting techniques (extrusion, droplet, and light/laser-based) and with different types of encapsulated cells (i.e., primary cells, stem cells, and cancer cells). Gelatin bioink is most stabilized using acrylate-based photopolymerization to crosslink gelatin chains. In recent years, other crosslinking chemistries, such as click chemistry, have been demonstrated to crosslink gelatin [27–29]. Another common theme is to mix natural biomaterials with gelatin to form double-network bioprinted constructs with improved printability and cell functions. However, improving the complex functions of encapsulated cells such as self-organization, differentiation, and migration, remains a challenge. This review outlines recent advances in the development of gelatin bioink formulations for 3D bioprinting with a focus on efforts to improve bioink properties and cell functions. We review progress in double-network gelatin hydrogels and chemical crosslinking methods for gelatin which have the potential to make the cellular microenvironment more biomimetic, thereby improving cell-matrix interactions and cell functions. Finally, we highlight the challenges associated

with the development of gelatin-based hydrogels, discuss overlooked aspects, and provide an outlook to advance this field.

2. Gelatin as a bioink for 3D bioprinting

Gelatin is a low-cost biodegradable protein with molecular weight ranging from 15000 – to 400,000 daltons [30, 31]. It is obtained via thermal denaturation or controlled hydrolysis of collagen, a key fibrous protein in the human ECM (Figure 1a) [32–35]. Gelatin possesses a similar molecular composition as that of collagen, but has a less-ordered macromolecular structure and retains important binding moieties for cell adhesion such as the tripeptide Arg-Gly-Asp (RGD) sequence. In addition, it shows less antigenicity compared to collagen and contains peptide sequences that can be cleaved by the matrix metalloproteinases (MMPs) enzymes [36–38]. Due to the MMP-sensitive nature of gelatin, it can be degraded by the MMP enzymes secreted by encapsulated cells, facilitating remodeling of the microenvironment. Moreover, gelatin displays viscoelastic properties which promote cell migration, differentiation, and proliferation [39, 40]. Due to these characteristics, gelatin is one of the most widely used natural biomaterials in bioprinting (Figure 1b).

Amino acid analysis indicate that gelatin is mostly comprised of glycine (34.7%), proline (15%), alanine (13%), glutamic acid (8.6%), aspartic acid (5.2%), arginine (5.2%), and lysine (3.1%) residues (Figure 1c) [41]. Due to the presence of carboxylic functional groups on aspartic acid, glutamic acid, and amine functional group on lysine, gelatin is highly amenable to chemical modifications to produce chemically crosslinked bioinks. The carboxylic and amine groups present in gelatin are mostly used to introduce crosslinking moieties on gelatin chains. However, the availability of these reactive functional groups can vary depending on the type of gelatin (Figure 1d) [42–45]. Therefore, quantification of these functional groups is important because it influences the rate of polymerization, mechanical stability, and resulting stiffness of the bioink [46, 47].

Gelatin is a thermo-sensitive biomaterial that undergoes sol-gel transition. When cooled to below room temperature, gelatin can form partial triple helical structures, which upon heating, reverts to solution due to disruption of hydrogen bonds [48, 49]. Proline and hydroxyproline residues play a key role in the physical and rheological properties of gelatin [50, 51]. These properties of gelatin can be leveraged to improve the strength of gelatin-based bioinks by using sequential crosslinking strategies (physical gelation followed by covalent crosslinking) [52].For example, as a gelatin bioink solution is cooled from 37 to 4°C, the viscosity will gradually increase due to the formation of triple helices [53]. Thus, flow behavior and extrudability of the gelatin solution can be optimized by tuning the temperature [18–21, 49, 54–56]. As the gelatin liquifies at 37°C, it can also be used as a sacrificial ink for the creation of channels or porous structures within bioprinted constructs for enhancing nutrient delivery and cell growth [57, 58]. Collectively, these characteristics widen the applications for gelatin in the development of bioinks for 3D bioprinting. Table 1 below highlights the advantages, limitations, and properties of gelatin as a bioink material compared to other widely used natural bioinks.

2.1 Gelatin as a support bath for bioprinting

The appealing viscoelastic properties of gelatin have not only widened its use as a bioink but also as a support bath to allow gel-in-gel bioprinting of soft and low-viscosity bioinks while providing an environment that maintains cell viability [99]. The idea of using gelatin as a support bath for the deposition of bioinks in complex 3D constructs was first introduced by Hinton and colleagues in 2015 [100]. Due to the shear thinning property of gelatin, the support bath acts as a rigid body at low shear stresses and flows like a viscous fluid at high shear stresses. Therefore, gelatin makes an ideal material for support baths because a syringe needle can be easily inserted and traversed extruding a bioink. As the needle moves forward, the gelatin solidifies in its wake and holds the extruded material in place. Moreover, the thermo-responsive nature of gelatin makes it easy to wash away the bath and retrieve the bioprinted structure. Several researchers have already made use of these gelatin support baths to bioprint constructs [101, 102]. Kupfer et al. used gelatin microspheres to support the bioprinting of chambered cardiac mimics using low-viscosity gelatin methacrylate bioink [103]. Bao et al. used sodium bicarbonate laden-gelatin slurries as a phase separation inducing matrix (PSIM) to embed a cell-laden chitosan bioink [104]. While gelatin provided support to the bioprinted structure, the reaction between sodium carbonate and the chitosan bioink resulted in micropore formation, which created a hierarchical porous structure within the printed construct. Gelatin was washed away later by incubating the bioprinted scaffold at 37°C. Likewise, Montalbano et al. used gelatin to bioprint bone-like scaffolds using collagen and mesoporous bioactive glass [105]. Since collagen gels at 37°C (sol-gel transition) and gelatin melts at 37°C (gel-sol transition), gelation of the scaffold and removal of the support bath both occur when incubated at 37°C, simultaneously accomplishing two tasks with one stimulus.

3. Improving gelatin bioink properties and cell encapsulation using photo-crosslinking

The use of unmodified gelatin for bioinks results in tissue constructs that suffer from poor stability and structural fidelity. The unmodified gelatin decreases in weight by 50% after 10h of incubation, and complete dissolution within 24h [39]. Therefore, the use of gelatin as a primary bioink requires additional crosslinking to maintain the cellularized structures post-bioprinting. To address this problem, gelatin may be chemically modified to achieve the crosslinking of its chains, thereby improving stability. However, chemical crosslinking, achieved through stable covalent bonds, may limit the ability of encapsulated cells to remodel the microenvironment which is required for their continued growth. This section discusses the developments in photochemically-crosslinked gelatin bioinks and highlights the challenges associated with improving cell functions.

3.1. Photopolymerization of gelatin using methacryloyl crosslinking

One of the most commonly used strategies to stabilize bioprinted gelatin constructs is by methacrylation of the lysine residues; these polymerize to form covalently-crosslinked gelatin methacryloyl (GelMA) in the presence of a photoinitiator and UV-light [43, 106–113]. The GelMA retains cell adhesive properties because the RGD motifs are not

chemically modified during methacrylation [114–116]. GelMA properties can be tuned by changing its concentration, degree of modification, photoinitiator concentration, and exposure time to UV-light [43, 117–120]. Hoorick et. al. functionalized both the primary amines and carboxylic acid of gelatin [42], resulting in the formation of both methacrylate and methacrylamide functionalities in gelatin. This combination of modifications allowed gelatin to have faster crosslinking kinetics compared to other derivates. In addition to this, the resulting superior mechanical integrity and lower swelling ratios improves shape fidelity and higher resolution of bioprinted constructs [121].

In general, a higher concentration of GelMA is easier to print [122]. Yet, higher concentrations of GelMA reduces the proliferation and spreading of encapsulated cells due to the confinement in a dense polymer network, resulting in reduced pore size and diffusion of nutrients [123–127]. Increasing the concentration of GelMA also requires large shear force to eject the bioink, which further reduces cell viability [128]. To overcome this drawback, pore forming GelMA-based bioink formulations have been developed recently [129]. Such formulations rely on aqueous two-phase emulsion, which contain two immiscible aqueous phases of GelMA/cell mixture with encapsulating droplets of polyethylene oxide (PEO). The different volumetric ratios of PEO/GelMA can be used to control pore size, leading to the formation of porous bioprinted tissue constructs [130].

Several research groups have developed strategies to improve the bioprinting of low concentration GelMA bioinks to improve cell functions. For example, GelMA bioinks can physically crosslink when cooled to 4°C prior to bioprinting [131]. These physically crosslinked gels are shear-thinning, thereby allowing direct extrusion of GelMA at a low concentration. Alternatively, unmodified gelatin can be mixed with GelMA to temporarily increase the viscosity of the bioink [127]. The gelatin is then washed away from the bioprinted construct, leaving behind a GelMA polymer network that is conducive for faster cell growth. In another strategy, Liu et al. demonstrated the use of an alginate sheath as a template to provide support for the bioprinting of a GelMA bioink, followed by UV-light exposure to initiate crosslinking [132]. Alginate can also be used to provide temporary structural support to maintain the shape of low concentration GelMA bioinks during printing, which can later be removed to leave behind the desired shape [133].

Even though studies have shown successful outcomes in the EBB of tissue constructs, GelMA bioinks often require an alteration to viscosity, or the addition of other bioprintable materials to overcome the poor printability of low-viscosity GelMA bioinks [134–136]. Other bioprinting methods (e.g., light and droplet -based bioprinting) that are less restricted by bioink viscosity have emerged as strong alternatives for high resolution bioprinting of GelMA bioinks [137–142]. Using volumetric bioprinting, Gehlen et al. successfully bioprinted constructs with 2.5 w/v% and 5 w/v % GelMA bioinks within 30 seconds that both exhibited high cell viability (90%) [143]. Optical tuning of GelMA bioinks using iodixanol has recently been found to be a key factor for improving the resolution in volumetric bioprinting [144]. Using a GelMA/iodixanol combination, complex organoid-laden perfusable liver tissue constructs were fabricated using volumetric bioprinting (Figure 2a). The liver organoids maintained their self-organization in bioprinted constructs and expressed key markers for liver cells (HNF4a, MDR1, CK19). The metabolic activity in

volumetric bioprinted organoids was higher compared to organoids grown using the casting method or EBB, yet lower than the organoids grown in Matrigel control, indicating room for further improvement in cellular functions (Figures 2b–c). Chen et. al. leveraged acoustic bioprinting process, where viscosity does not significantly affect the bioprintability [145] to bioprint low concentration GelMA bioinks (5% w/v) at high cell densities ($> 1.6 \times 10^7$ cells per mL) while maintaining cell viability [146] (Figure 2d). Because the approach is nozzle-free, clogging is not an issue as with EBB. Organization of multiple cell types in various patterns was demonstrated using acoustic bioprinting, indicating the potential of this approach for low concentration inks. Table 2 further highlights some other examples of emerging light and droplet based bioprinting techniques that have utilized GelMA to fabricate high fidelity constructs.

In bioprinting applications, in order to preserve spatial resolution and stabilize the 3D constructs, additional crosslinking is applied post-fabrication [147–151]. However, methacryloyl chemistry is not an ideal strategy to preserve spatial resolution [46, 152]. As chain growth polymerization takes place, crosslinking is slow because of dissolved oxygen acting as a radical scavenger [153]. Increasing the UV intensity can overcome this oxygen inhibition, but it may also result in potential stress and damage to cells [154–156]. Notwithstanding these challenges, GelMA is considered a cost-effective, bio-active, and bio-compatible bioink material.

3.2. Thiol-ene click chemistry in gelatin bioinks

An alternative crosslinking mechanism to stabilize gelatin is based on the dimerization of thiols with reactive carbon-carbon double bond (“enes”), known as thiol-ene click chemistry. This chemistry follows a step-growth radical mechanism requiring low radical initiator concentration, with high conversion of functional groups and low polymerization shrinkage and stress [162, 163]. Thiol-ene chemistry has been used to develop thiol–ene clickable bioinks for various biofabrication applications [164–166].

Among the “ene” functionalities utilized for thiol-ene crosslinking, norbornene is the most common for being resistant to pH induced thiol-Michael addition and competitive homo-polymerization [29, 167]. Thiol-norbornene gelatin is formed by reacting norbornene modified gelatin with thiol containing macromolecules in the presence of free radicals [162, 168–170]. A light-mediated addition of free radicals occurs in the presence of a photo-initiator (Figure 3a). Free radicals interact with thiol groups to form thiyl radicals, which subsequently react with double bonds of norbornene. Due to the presence of highly strained double bonds in norbornene, chain transfer kinetics are faster [171]. This, in combination with the rapid hydrogen abstraction rate, results in brisk photo-crosslinking reactions [162, 165]. As a result, these hydrogels can be processed at low photo-intensities, which has been shown to significantly increase the viability and proliferation of adipose-derived stem cells [172]. Thiol-ene gelatin crosslinking is also faster compared to methacrylate polymerization, and yields better stability of the 3D printed objects, possibly due to the low susceptibility to oxygen inhibition, which significantly slows the methacrylate polymerization [28, 162, 172, 173]. When thiol-ene chemistry is used, cell viability of human umbilical vein endothelial cells (HUVECs) is higher at Day 7 relative to cells cultured in a GelMA bioink (Figure 3b)

[174], potentially due to a decrease in oxidative stress in cells [175, 176]. Gockler et al. demonstrated that the curing time of thiol-ene crosslinking can be significantly decreased by choosing the right thiol crosslinker [177]. For example, the curing time of gelatin norbornene was drastically lower when thiolated gelatin (GelS) was used as crosslinker versus DTT (dithiothreitol) (Figure 3c). Control over functionalization was demonstrated by synthesizing hydrogel of different crosslinking densities, which resulted in different mass swelling ratios. Importantly, the toxicity of the degradation products was lower for GelNB / GelS gels compared to GelMA (Figure 3d). In summary, studies to date, although limited, indicate that thiol-ene photo-crosslinking is superior compared to methacrylate polymerization in terms of printability and cell functions.

3.3. Visible light crosslinking of gelatin

Due to concerns related to the use of UV-light and its unintended harmful effects on cells, visible-light crosslinkable gelatin bioinks have been developed. Furfuryl-gelatin is one such example in which gelatin is derivatized by incorporating furfuryl groups, which can be crosslinked via visible-light irradiation. To date, two modification strategies to introduce furfuryl groups on gelatin have been reported in literature. The first approach involves the primary amines of gelatin to be reacted with furfuryl isocyanate (FI), yielding gelatin – FI with ~99% coupling between furan and gelatin's amino groups [179]. The second approach involves the use of carbodiimide crosslinking to link carboxylic acid groups in gelatin with the primary amine of furfuryl amine (FA) to yield gelatin – FA, followed by crosslinking using visible light [180]. Son et al. reported a novel bioink based on furfuryl - gelatin prepared by incorporating furfuryl groups on gelatin followed by mixing with rose bengal [181] or riboflavin [182] and visible-light exposure to initiate crosslinking. However, a viscosity enhancer such as hyaluronic acid was required to provide the necessary viscosity and shear-thinning properties to the bioink. Although these derivatives have the potential to develop different gelatin bioinks, more work still needs to be conducted to determine new modification strategies to yield furfuryl side groups on gelatin. Furthermore, the effect different strategies had on the resulting properties and crosslinking kinetics for gelatin needs to be studied. For example, compared to gelatin – FI, gelatin – FA exhibits faster crosslinking kinetics with higher flexibility and elasticity [180].

4. Improving bioink properties and 3D cell functions using gelatin - based double network hydrogels

ECM contains multiple proteins and glycosaminoglycans, which play a key role in modulating cell functions. Bioinks comprising of a single biomaterial may not fully meet all the functional and mechanical requirements of cells to form tissue constructs. Therefore, to further achieve biomimicry, other biomaterials such as alginate, hyaluronic acid, and chitosan, are added to develop double-network bioinks. The response of double-network hydrogels to external stimuli, such as pH, temperature, electric, and magnetic fields, can be utilized to recapitulate the dynamic characteristics of the ECM required for cell-matrix interactions and cell spreading [183].The following section highlights recent developments in gelatin-based double network bioink formulations for the 3D printing of constructs.

4.1. Gelatin - Alginate

Alginate is a naturally occurring polysaccharide isolated from brown algae and comprises of block co-polymers composed of 1, 4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues [184, 185]. It has low cell adhesion, yet is extensively used in combination with gelatin due to its biocompatibility, fast ionic crosslinking (e.g., using Ca^{2+}), and easily tunable mechanical properties via physico-chemical modifications [186–188]. For example, a gelatin – alginate bioink has recently been used to fabricate an in-vitro model of the human heart using cardiac spheroids created from human cardiac myocytes, fibroblasts, and endothelial cells (ECs) [189]. The bioink recapitulated the stiffness of human myocardium which allowed cardiac spheroids to maintain their structure and viability for up to 30 days after bioprinting. Similarly, Othman et al. utilized the same multicomponent hydrogel to bioprint HeLA spheroids in a hexagonal shaped scaffold which exhibited in-vivo like mechanical properties with a high cell viability of ~95% [190]. Kang et al. bioprinted a multilayered scaffold using gelatin-alginate bioink for hair follicle (HF) regeneration [191]. The hierarchical and grid structure of bioprinted scaffolds resulted in appropriate orientation and growth of HFs in-vivo.

Alginate is often oxidized to produce alginate di-aldehyde (ADA) which allows chemical crosslinking with gelatin [192–195]. As exogenous aldehydes can damage cells through oxidative stress, the presence of gelatin in ADA – Gel hydrogels can reduce this stress by binding to the aldehyde groups present in ADA [196]. Alginate and gelatin ratios, and the bioprinting parameters, can influence the properties of these double-network hydrogels [197–199]. While most of these studies have focused on increasing alginate amount to improve mechanical properties in gelatin, Mahmoud et al. highlighted that excessive amounts of alginate can lead to decreased bioactivity in the resulting EBB double-network bioink [200]. As alginate provides mechanical and structural stability to support EBB for low-viscosity gelatin hydrogels, it is not necessarily required once the construct is stabilized post-bioprinting. Alginate can be removed from the bioprinted constructs using monovalent ion exchange, or by using Ca^{2+} chelator, which breaks the eggbox like configuration between G blocks in the alginate polymer chain (Figure 4a) [133]. Using these approaches a >50% drop in alginate intensity can be achieved over a period of 3 days (Figure 4b). It is important to note that if oxidized alginate is employed, this approach cannot be used to remove alginate from the matrix because of the chemical coupling of alginate with gelatin chains.

In above-mentioned studies, gelatin – alginate-based double-network bioinks had static, irreversible networks which restricted the movement of cells. To circumvent this issue, Ren et al. introduced reversible bonds within covalently crosslinked GelMA-alginate: they grafted β-cyclodextrin (βCD) on alginate, and mixed N-adamantyl acrylamide (Ad-AAm) as a guest molecule in GelMA-alginate-βCD [201]. Frequency sweep measurements confirmed the presence of covalent and non-covalent interactions as all hybrid hydrogels (AAG1, AAG2, AAG3, AAG5) showed frequency responsiveness with increasing βCD % (from lowest in AAG1 to highest in AAG5) while GelMA (referred to as AAG7) showed constant modulus (Figure 4c (i)). As the amount of GelMA increases (40 mg in AAG4, 80mg in AAG5 and 120mg in AAG6), and as a result the number of covalent crosslinks,

the hybrid hydrogels exhibited a reduced response to frequency and increase to storage modulus (Figure 4c (ii)). When the concentration was decreased to 25% of the original, the hybrid hydrogel exhibited increased frequency responsiveness and decreased storage modulus (Figure 4c (iii)). With a similar objective in mind, Zhu et al. leveraged reversible covalent chemistry to develop a multifunctional bioink based on oxidized alginate and gelatin [202]. The use of amine-functionalized copper (Cu)-doped mesoporous bioactive glass nanoparticles introduced cell adhesive ligands, which in combination with a reversible dynamic microenvironment, resulted in accelerated cell spreading with a viability of over 90%. Furthermore, due to cell mechanosensing in the dynamic matrix and ion stimulation from the incorporated nanoparticles, osteogenic differentiation and angiogenesis of embedded mouse bone marrow-derived stroma cells was also promoted. Collectively, these studies indicate the improved potential of physio-chemical modifications to bioink properties when alginate-gelatin based dual-component bioink is used for 3D bioprinting.

4.2. Gelatin – Fibrinogen

Fibrinogen is a soluble macromolecule that has been used in combination with gelatin to improve mechanical properties, rheological properties, and cellular interactions due to its differential effects on cellular functions [203]. Moreover, it possesses binding sites for a plethora of proteins such as fibronectin, von Willebrand factor, albumin, thrombospondin, fibroblast growth factor-2, and interleukin. It also supports endothelial and cardiomyocyte cell attachment through RGD-specific and non-specific binding sites [204]. De Malo et al. reported a bioink composed of gelatin, GelMA, and fibrinogen, which provided a suitable environment for osteocytes as indicated by their high cell viability (~84%) after 1 week of incubation [205]. Recently, Li et al. also utilized a similar combination to develop a fast stress-relaxing bioink for volumetric muscle loss using gel-in-gel printing strategy [206]. A combination of visible-light crosslinkable furfuryl gelatin and fibrinogen bioink has been shown to improve the proliferation of human cardiomyocytes and fibroblasts [207]. This bioink was further utilized for coculturing and coupling of cardiac fibroblasts with cardiomyocytes [207]. Freeman et al. used a gelatin – fibrinogen blend for the bioprinting of tissue-engineered vascular grafts [208]. Gelatin was heat-treated, and its concentration was varied simultaneously to control the viscosity of the composite bioink which enabled it to hold its shape against gravity during bioprinting. However, the addition of cells liquified the bioink thereby reducing its bioprintability and impairing the gelation of heat-treated gelatin which disrupted the overall microstructure. Furthermore, increasing the amount of heat-treated gelatin (5 to 10 wt %) for a shorter heat-treatment time (1h) decreased the cell viability from ~88% to ~69%.

While several researchers have tuned alginate amount in gelatin – fibrinogen bioinks to improve printability [209–213], Somasekhar et al. improved the bioprintability by varying the gelatin amount in gelatin-fibrinogen-alginate bioink containing HUVECs [214]. Gelatin was kept in its un-crosslinked form so that it could leach out of the matrix post-bioprinting to create porous structures, which allowed the spreading of cells, while fibrinogenwas retained in the matrix and improved cell attachment. Higher gelatin ratios (1:9 instead of 2:3 alginate: gelatin) for both 5% and 10% w/v bioink concentrations resulted in higher cell

viability during culture. This was attributed to cellular remodeling and porous nature of the scaffold resulting from the removal of un-crosslinked gelatin [215].

4.3. Gelatin – Chitosan

Chitosan, a natural polysaccharide, is being explored for bioprinting applications due to its biocompatibility and biodegradability [216]. Chitosan contains protonated amino groups making it a positively charged polyelectrolyte. This results in electrostatic interactions with charged carboxylic acid groups in gelatin, resulting in a stable polyelectrolytic complex [217, 218]. Therefore, it is often combined with gelatin to form hybrid hydrogel bioinks that exhibit good biocompatibility, high shape fidelity, and bioprintability at room temperature [219]. Yet, the polyelectrolytic complex may not be stable for a long-term 3D cell culture which is often required in tissue engineering. Thus, several groups have engineered gelatin-chitosan bioink formulations consisting of GelMA and methacrylated chitosan [220, 221]. This strategy results in a more stable double-network formation due to the chemical coupling of gelatin and chitosan chains during photo-curing, which increases the crosslinking degree and storage modulus. Bone marrow derived stem cells (BMSCs) remained viable, proliferated, and were distributed homogenously in the gelatin-chitosan bioink. Furthermore, cells formed aggregates and expressed chondrogenesis markers, indicating a primitive sign for cell – cell interactions [222].

Recently, granular hydrogels have emerged as an attractive candidate for 3D bioprinting due to their dynamic, self-healing properties [223]. Wang et al. combined GelMA with granular hydrogel composed of hydroxypropyl chitosan microspheres to form a compound bioink containing adipose derived stem cells (ASCs) [224]. The bioink was then 3D bioprinted to make cylindrical rings. The granular hydrogel showed superior storage modulus, self-healing ability, and significantly higher proliferation of ASCs. Due to the advantage of dynamic structures, Chen et al. used slow stable crosslinking of gelatin/ 4-arm poly(ethylene glycol) succinimidyl glutarate (PEG-SG) and fast dynamic crosslinking of aldehyde hyaluronic acid (AHA)/N-carboxymethyl chitosan (CMC) to develop a structurally-stable hydrogel bioink with high permeability [225]. Compared with Gel – Alg constructs containing same cells (fibroblasts, C2C12 myoblast, Ne-4C neural stem cells), AHA/CMC gel showed higher proliferation and viability of ~94%.

4.4. Gelatin – Hyaluronic Acid (HA)

Hyaluronic acid (HA), a glycosaminoglycan, is a key component of the ECM and is involved in cell matrix interactions through CD44 HA receptors on the cell membrane [226–231]. HA has been used for bioprinting applications but its use has been limited due to its non-cell-adhesive nature and poor mechanical properties [232]. To circumvent this issue, gelatin - hyaluronic acid combinations have been reported as bioinks for 3D printing [233, 234]. Hossain Rakin et al. used a methacrylated hyaluronic acid (MeHA) - GelMA combination to develop a hybrid bioink [235]. MeHA, with a high degree of substitution, enabled rapid photocrosslinking while GelMA was added to improve cell adhesion. Similarly, Jiang et al. used the same two prepolymers to bioprint multilayered scaffolds with spatially differentiated adipose-derived mesenchymal stem cells (ADMSCs), which improved tendon to bone interface regeneration [236]. Skardal et al. reported

bioprinting tubular tissue constructs using a combination of methacrylated ethanolamide gelatin (GE-MA) and methacrylated hyaluronic acid (HA-MA) [237]. The hydrogel was biocompatible and supported cell attachment and proliferation for HepG2 C3A, Int-407, and NIH 3T3 cells in-vitro. Moreover, cells were able to gradually remodel the environment and secret ECM as they matured into a viable tissue. Enzymatic crosslinking can also be used to develop hyaluronic acid – gelatin composite hydrogels, where covalent crosslinking between HA and gelatin occur via oxidative coupling of phenolic moieties in both conjugates [234]. Due to the synergistic biological effect of gelatin and HA, this hybrid hydrogel results in excellent cellular activity and viability (81%) of human dermal fibroblasts.

HA is an essential component of the tumor ECM, where HA is known to regulate proliferation of cancer cells, metastasis, and tumor growth [238]. The expression of CD44 receptor is also upregulated in cancer cells. Thus, gelatin-HA based bioinks are particularly suitable to create a niche for cancer research and to model cancer growth in-vitro. Future research studies may focus on this frontier to fully utilize the synergistic combination of gelatin and HA in composite bioinks.

4.5. Gelatin – Silk Fibroin (SF)

Silk fibroin (SF), a protein containing repeating patterns of Gly-Ser-Gly-Ala-Gly-Ala residues has been used to develop a plethora of bioinks due to its natural degradability, mechanical strength, and rheological properties [61, 239–243]. Gelatin and silk interact via entanglement and physical crosslinking, leading to the formation of stable bioprinted constructs without the need for a crosslinker. The bioink also supported the growth and proliferation of encapsulated chondrocytes [244]. In another study, to further improve the functions of encapsulated cells, an enzymatically-crosslinked gelatin and SF were used to prepare microporous 3D environment for the differentiation of stem cells in bioprinted constructs [245]. Importantly, upon implantation in the rabbit cartilage defect model, the bioprinted cells regenerated cartilage tissue, indicating translational potential for gelatin-silk formulations [245]. In contrast, Yang et al. used visible-light crosslinking for methacrylated gelatin and methacrylated-SF bioinks [246], where encapsulated BMSCs and HUVECs displayed over 98% viability. The incorporation of hydroxyapatite in a gelatin-SF composite bioink has been shown to mimic collagen fibers in the natural articular cartilage [247]. Increasing the silk content reduced the degradation rate due to an increased number of hydrogen bonds being formed within the molecules. The increased hydrogen bonding led to a rearrangement of peptide chains, transforming the random coiled structure into crystallized regions, thereby enhancing the intermolecular interaction and reducing the degradation rate. This also reduced the swelling property of the hydrogel due to a smaller number of polar OH groups available to accommodate water. Bioink with 10% SF exhibited the best bioprinting performance and mechanical properties with a tensile elastic modulus of ~11 MPa and a compressive elastic modulus of ~1 MPa. Together, the limited number of studies thus far indicates that both the bioprintability and cellular functions can be improved by incorporating silk in the gelatin bioink.

5. Improving cell function in gelatin bioinks using reversible crosslinking

Current strategies to crosslink and stabilize gelatin or gelatin-based bioinks are mostly based on covalent crosslinking such as methacrylate polymerization and thiol-ene coupling. These strategies are based on permanent covalent bonds, which restrict the spreading and growth of encapsulated cells due to cellular confinement (Figure 5a) [248, 249]. Moreover, to improve the mechanical properties in the bioprinted construct, a high density of covalent crosslinks is employed to ensure print fidelity and stability. However, these crosslinks are irreversible and high crosslinking density further restricts cellular activities (spreading, migration, differentiation), as well as the diffusion of nutrients/bioactive molecules through the scaffold [250, 251]. Encapsulated cells rely on the remodeling of the surrounding microenvironment, either through enzymatic cleaving or physical rearrangement of the matrix, to perform their functions [252–254]. Recent studies indicate that matrix viscoelasticity strongly influences cell functions [255]. Viscoelasticity refers to the ability of the matrix to resist deformation under applied stress (elastic nature), and simultaneously dissipate energy in a time-dependent manner (viscous nature) [256]. Viscoelasticity is often measured in terms of the stress relaxation rate when the hydrogel is subjected to a constant compressive load. Natural tissues, such as adipose tissue, brain, or liver, are viscoelastic and demonstrate stress relaxing behavior over a timescale from tens to hundreds of seconds (Figure 5b) [257–262]. Thus, viscoelasticity is an important characteristic to emulate in engineered bioinks.

Crosslinks that are reversible, such as hydrogen bonding, imine bonding, or hydrazone bonding, improve the viscoelasticity of the matrix. These reversible crosslinks can be either covalent crosslinks or supramolecular physical interactions (Figure 5c). In either case, the crosslinked network junctions can undergo frequent cycles of association and dissociation, which provides a way to dissipate energy and give rise to stress relaxation behavior. Cells encapsulated in a viscoelastic matrix can physically remodel the matrix by transiently breaking the crosslinks using contractile forces, which is difficult to perform in a matrix stabilized by rigid, covalent crosslinks such as methacrylate bonds. Several researchers have reported the effect that matrix viscoelasticity has on cellular activity [263–268]. Recently, gelatin-based matrices have been developed using reversible crosslinks that show stress-relaxing behavior [269–272]. The following section highlights these reversible physical and covalent crosslinking strategies that are, or can be, applied either alone or in combination with irreversible covalent crosslinking chemistries to develop gelatin-based bioinks (Figure 5d).

5.1. Improving bioprinted cell functions using physical crosslinking

Physical crosslinking results in reversible intermolecular interactions such as hydrophobic interactions, hydrogen bonds, host-guest interactions, polymerized entanglements, and ionic/electrostatic interactions [274–276]. These interactions are ubiquitous in nature; hydrogen bonding keeps enzymes and DNA in equilibrium with their bound and unbound states while electrostatic and hydrophobic interactions assist in the localization of biomolecules [277, 278]. The physical crosslinking strategy is more biocompatible due to the absence of any chemical crosslinkers [279]. Physical crosslinking can provide tissue-like viscoelasticity to hydrogels [273, 280]. Electrostatic/ionic interactions have been extensively applied

to develop gelatin-based bioinks, where crosslinking occurs between two molecules of opposing electric charges [214]. Duan et al. 3D printed heterogenous aortic valve conduits with a gelatin and alginate hydrogel, which was ionically crosslinked with $CaCl_2$ [281]. The encapsulated aortic root sinus smooth muscle cells (SMCs) and aortic valve leaflet interstitial cells (VICs) showed over 80% viability. Moreover, SMCs expressed elevated alpha-smooth muscle actin, while VICs expressed elevated vimentin, indicating the maintenance of cellular phenotypes.

Host-guest interactions is another strategy to obtain affinity-based crosslinking of the gelatin chains [59, 282]. Host molecules such as cucurbiturils and cyclodextrins generally possess large cavity volume to encapsulate the guest [283]. Usually, the external property of the host molecules causes interactions to occur with the surrounding solvent, while the guest inclusion is facilitated by the internal features of their cavities via hydrogen-bonding interactions, electrostatic interaction, hydrophobic interactions, specific molecular shape, or size matching (Figure 6a). Dai et. al. reported a novel host-guest modulated gelatin hydrogel that combined dopamine modified GelMA (MeHG DN), dopamine modified gelatin (HG DN), and acrylate β-cyclodextrin [284]. Acrylate β-cyclodextrin was used as a host molecule to be crosslinked by host-guest interactions with dopamine side groups. These complexes acted as reversible bonds to provide the final hydrogel with excellent resilience and toughness. MeHG DN hydrogels displayed 3.4 times higher compressive modulus compared to pure GelMA and HG DN hydrogels (Figure 6b (i)). This was attributed to host-guest crosslinking which absorbed energy and resisted fracture, while methacrylate crosslinks hindered fracture propagation, thus increasing the threshold stress of the hydrogel (Figure 6b (ii)). Both HG DN and MEHG DN hydrogels demonstrated temperature-dependent viscosity and shear-thinning behavior, making them suitable for EBB (Figure 6b (iii–iv). Wang et al. used cyclodextrin-adamantine guest-host interaction to mimic the stiffness of the soft tissues on GelMA [285].

Another strategy of physical gelation is to use hydrogen bonding. The combination of hydrogel bonding and covalent bonding has been shown to improve organoid formation in viscoelastic hydrogels [286–288]. Xu et al. showed that the quadruple hydrogen bonding in gelatin introduces self-healing capability [289]. However, it is yet to be seen if bioprinting can be achieved using such formulations. Presumably, a combination of covalent bonding (to increase stability) and quadruple hydrogen bonding (to increase viscoelasticity) can provide bioinks with increased cellular proliferation and functions.

5.2. Improving bioprinted cell functions using reversible covalent crosslinking

One of the challenges associated with physically crosslinked matrices is the lack of long-term stability which limits the potential duration of 3D culture. For example, host-guest crosslinking matrices are only stable for up to a few days in cell culture. To overcome this issue, reversible covalent crosslinks are being developed to provide both improved stability and viscoelasticity. Reversible covalent crosslinking results in the formation of reversable covalent bonds under mild and controllable conditions [15, 290]. The dynamic properties of these bonds are acquired from a shift in the chemical equilibrium of reversible reactions; at equilibrium, a definite number of chemical crosslinking groups are present in the crosslinked

state, which maintain the 3D structure. The dynamic nature of the crosslinking increases the viscoelasticity of the hydrogel, thereby recapitulating the features of the ECM [291]. Some of the common reversible crosslinking methods that can be potentially applied to form gelatin-based bioinks to improve cell functions are listed in Table 3 and are discussed below.

5.2.1. Gelatin crosslinking using imine bonding—Gelatin naturally contains amine functional groups on lysine residues. These amine groups can be reacted with aldehyde groups, which is known as imine crosslinking and results in a crosslinked gelatin hydrogel [193, 292]. The rate of imine crosslinking can be controlled by tuning pH [293, 294]. Imine bonds are highly reversible and as a result produce a viscoelastic hydrogel. Alginate and dextran are examples of crosslinkers that can be oxidized to generate aldehyde functionalities which can react with gelatin [193, 295, 296]. Musilova et al. achieved imine-crosslinked bioinks by combining oxidized dextran with gelatin from three different sources [297]. These bioinks displayed strong shear-thinning behavior with excellent viscoelastic properties, thereby enabling EBB. No cytotoxicity was observed in any of the gelatin-based bioinks, and fibroblasts cells were distributed homogenously without any disruption of the cell structure. To enhance crosslinking and provide a better environment for cellular growth, Somasekharan et al. covalently crosslinked oxidized alginate with amine groups on gelatin and platelet rich plasma [298]. This imine-crosslinked, bioprinted construct demonstrated a highly interconnected porous structure that is required to promote cell growth and migration with over 80% cell viability.

It is important to note that imine bonds have the fastest bond exchange dynamics, and thus, result in the highest viscoelasticity of the matrix (fast stress-relaxation rates). However, imine crosslinking is also less stable due to the fast hydrolysis rate. Thus, imine crosslinked matrices are typically limited to short-term cell culture (<7 days) [299, 300]. This can be improved either by forming a double-crosslinked network such as those discussed in Section 4, or by introducing additional covalent crosslinks such as thiol-ene or oxime crosslinking [168, 301, 302]. Therefore, imine crosslinking is a potential strategy to tune the properties of gelatin-based bioinks.

5.2.3. Gelatin crosslinking using Diels-Alder click reaction—Diels Alder is a thermo-reversible [4+2] cycloaddition reaction between a dienophile (e.g., maleimide) and diene (e.g. furan) [309, 310]. Reversibility of the reaction occurs at low temperatures through retro DA reactions. The reaction does not require light, initiators, or other catalysts, and produces high yield without generating any side products. To achieve Diels-Alder crosslinking, gelatin can be modified with furans and crosslinked using either a maleimide crosslinker such as PEG-bis-maleimide, or by mixing with maleimide-modified biopolymers [311, 312]. The Diels-Alder crosslinked gelatin hydrogel showed a storage modulus similar to that of liver and breast gland tissue and possessed pH responsive properties. Although the hydrogel showed promising properties for biomedical applications, it has not been utilized for cell encapsulation or bioprinting of 3D constructs. Magli et al. functionalized both gelatin and chitosan by methyl furan to develop a bioink [313]. Star-PEG maleimide was utilized as dienophile to enable crosslinking by Diels – Alder cycloaddition and embedded

glioblastoma cells not only remained viable post-bioprinting, but continued to proliferate in the hydrogel.

Importantly, the Diels – Alder reaction proceeds slowly at physiological pH levels which could result in the sedimentation of encapsulated cells [314, 315]. To enable rapid crosslinking via Diels – Alder chemistry, Madl et al. reported an alternative Diels-Alder reaction pair (fulvene–maleimide) to develop hydrogels under physiological conditions [316]. Fulvene–maleimide gels crosslinked ~10 times faster and exhibit cell adhesive ligands to support the culture of human MSCs due to the use of a RGD-based crosslinker coupled with elastin- like protein. Furan can also be substituted with methyl furan, a more electron rich diene which allows cell encapsulation via fast gelation at neutral pH [317, 318]. These recent developments are promising and make Diels-Alder reactions more suitable for cell encapsulation and bioprinting.

5.2.4. Gelatin crosslinking using hydrazone bonding—Hydrazone crosslinking belongs to pseudo click reactions (moderate orthogonality) which result from the reaction between hydrazide and aldehyde or ketone groups. The reaction has high yields and lacks harmful side products [319]. Formation of hydrazone is generally carried out at a pH of 4.5 and 5.0. Higher or lower pH results in decreased yield and slower kinetics [320].Taking advantage of the dynamic nature, Wang et al. developed a bioink using GelMA and hyaluronic acid, which were crosslinked using a combination of dynamic hydrazone bonding and photocrosslinking [305]. The dynamic hyaluronic acid network provided shear-thinning and self-healing properties, while post-extrusion photocrosslinking of GelMA reinforced the double-network hydrogel, enhancing the overall mechanical properties of the scaffold. BMSCs remained viable and proliferated, migrated, and aggregated to form an interconnected multicellular network, highlighting conducive cell-matrix interactions possibly caused by the presence of a hydrazone bonding that allowed matrix remodeling. Similar strategies have been used in other studies as well where hydrazone and thiol-ene crosslinking were combined in the same gelatin matrix to gain insights into how cells respond to sequential crosslinking reactions, which enable temporal modulation of the matrix [306, 307]. This temporal change in the physicochemical matrix properties can be used to study various cell – cell and cell – matrix interactions in bioprinted gelatin. Studies thus far indicate that the physiochemical microenvironment of 3D bioprinted cells can be tuned using hydrazone crosslinked gelatin.

5.2.5. Gelatin crosslinking using disulfide linkages—Disulfide linkages can also be used to crosslink a gelatin matrix [321, 322]. These crosslinks are also reversible. For example, the presence of excess thiols or the use of oxidative triggers (e.g., hydrogen peroxide), increase the thiol-disulfide exchange. The pKa value also affects the thiol–disulfide exchange rate, which can be tailored using electron-withdrawing groups [323]. These disulfide groups are reductively cleavable and allow fast gelatin kinetics, thus enabling cell encapsulation [324–326]. Due to the reversible nature of this chemistry, thiolated gelatin hydrogels may be a suitable candidate for bioink development [327, 328]. Due to the lack of cysteine amino acids in gelatin that contain thiols, the thiol groups need to be introduced to enable disulfide crosslinking. This can be accomplished by reacting

primary amines with n-acetylhomocysteinethiolactone or Traut's reagent to yield gel-SH [329]. Other possible thiolation strategies for gelatin includes the reaction of carboxylic acid with cysteamine [330], or by reacting carboxylic acids with 3,3-dithiobis (propionic hydrazide) followed by the cleaving of disulfides using dithiothreitol (DTT), which results in thiolated gelatin [331]. Shu et al. reported disulfide crosslinked HA-gelatin hydrogel films that improved cellular growth. Although this crosslinking strategy has not yet been widely adopted for gelatin-based hydrogels, it has been used to prepare other biomaterial hydrogels for cell encapsulation [322, 332, 333]. Notably, although the disulfide linkages are reversible, its effect on the viscoelastic properties of hydrogels are less explored. Moreover, the use of oxidative triggers such as hydrogen peroxide can potentially induce cellular damage, which should be studied, as well as the presence of free thiols and their effect on cells [327].

5.2.6. Gelatin crosslinking using boronate ester bonding—Boronate ester bonds are reversible covalent bonds formed via the combination of cis-1,2 or cis-1,3 diols and boronic acid to form a cyclic ester. The reaction occurs under physiological conditions without any catalyst [334]. The properties of the dynamic covalent network formed by boronate ester crosslinks are regulated by temperature, pH, and the specific molecular structure of the boronic acid-diol binding pair [335]. Due to the fast reaction kinetics of boronate ester bonds, dynamic hydrogels can form quickly, and show rapid self-healing ability [336, 337]. Xie et al. reported a composite self-healing hydrogel by utilizing phenyl boronic acid (PBA) modified gelatin and polyvinyl alcohol for wound healing applications [271]. Step strain-sweep measurements confirmed the reversibility of the crosslinks (Figure 7a). Moreover, the hydrogels demonstrated self-healing behavior (Figure 7b). Gelatin functionalized with different amounts of PBA showed different degradation rates (Figure 7c). Similarly, Nguyen et al. crosslinked gelatin and PVA using dual crosslinking (boronate ester and thiol-ene crosslinking), and demonstrated that the addition of PVA to gelatin increased viscoelasticity due to the formation of reversible boronate-ester linkages between PVA and gelatin [270] (Figure 7d). The viscoelastic nature of the matrix resulted in growth and cytokine secretion of embedded MLO-A5 pre-osteocytes. In another study, human MSCs were shown to spread faster in boronate-ester crosslinked viscoelastic gelatin-PVA matrix, thereby emphasizing the importance of reversible crosslinking chemistry to improve 3D culture conditions for encapsulated cells (Figure 7e) [306]. Recently, Hu et al. also utilized this crosslinking chemistry to develop shear-thinning, viscoelastic gels using gelatin for treating brain lesions [338]. Although, researchers have started using this strategy to develop dynamically crosslinked gelatin hydrogels, it has not yet been explored for the development of gelatin-based bioinks with tunable viscoelasticity.

6. Challenges and future outlook

Formation of a functional tissue requires a balance between cell-based and cell-ECM interactions [339]. The composition and mechanical properties of the ECM drastically influence cellular growth and other functions [340, 341].The current gelatin bioinks should be designed by considering the cellular needs to improve cell functions. Designing gelatin-based bioinks capable of supporting cell attachment, proliferation, and spreading with

long term functionality post-bioprinting still remains challenging [342]. Notwithstanding the tremendous progress that has been made so far, cell-matrix interactions and cellular functions can be further improved in bioprinted gelatin constructs by choosing the right composition and crosslinking strategy that mimic the native ECM dynamics [15, 343]. To date, researchers have focused on improving the bioprintability and stability of gelatin-based bioinks using chemical crosslinking strategies that result in static bonds, which inhibit cellular functions [344]. As these covalent crosslinks are permanent, encapsulated cell functions are hampered due to the confining microenvironment [15]. Cell-mediated enzymatic [345, 346] or hydrolytic degradation [347, 348] is required to allow cell migration and spreading. To some extent, gelatin allows for matrix remodeling due to being enzymatically cleavable, however, it should be combined with dynamic crosslinking to achieve optimum performance.

Currently, most of gelatin-based bioinks, especially GelMA, utilizes static covalent chemistries to enable stable constructs. The reversible covalent crosslinking chemistries for developing gelatin-based 3D constructs are underexplored [338, 349]. Scalable constructs for mammals, and eventually humans, not only require high mechanical properties and structural integrity, but should also provide an ECM mimicking environment to improve cell functions. The ECM is a highly dynamic network that exhibits time and frequency dependent properties in response to deformation and loading [273]. To incorporate this non-enzymatic mode of remodeling, above-mentioned reversible crosslinking strategies need to be applied to gelatin-based bioinks to have a sufficient degree of relaxation and viscoelasticity. The literature also reports that increasing the viscoelasticity increases spreading and proliferation of encapsulated cells. Chaudhuri et al. reported that encapsulated human MSCs exhibited enhanced spreading, osteogenic differentiation, and proliferation with faster stress relaxation [258]. Lou et al. reported human MSCs growth in viscoelastic gels crosslinked via dynamic hydrazone bonds, which modified focal adhesions and spreading [265]. This suggests that viscoelasticity is a critical factor that must be considered when developing new gelatin-based bioinks. The reversible covalent crosslinks also impart shear-thinning and self-healing behavior which may improve the extrudability of the bioinks while reducing the amount of pressure required.

How much viscoelasticity is required in a matrix to obtain optimum cell functions following bioprinting? This question requires careful consideration of the viscoelasticity (stress relaxation rate) of the native tissue, and a rational crosslinking strategy emulating that in the bioink. For example, we recently reported that a hydrogel with a stress relaxation rate matching that of liver tissue can significantly improve 3D cellular growth and liver organoid formation [350]. However, it is yet to be seen if bioprinted cells in a similar composition of the hydrogel can form organoids in bioprinted constructs. The time scales of cell action and hydrogel rearrangement needs to be matched. This requires further in-depth study of the dynamics for 3D cell behavior and kinetics of hydrogel rearrangement. Lastly, the growth of cells and organoids in gelatin bioprinted constructs should be benchmarked against the commonly used reconstituted basement membrane matrices such as Matrigel, which are viscoelastic, to determine the performance.

Crosslinking strategies involve the chemical modification of gelatin., and The effect of such modifications on the bioactivity of gelatin needs careful consideration. Modification of amine groups on gelatin can decrease the degradability of gelatin by proteases such as MMPs [351–353]. Therefore, in addition to the evaluation of mechanical and rheological properties, bioprintability, and cell viability for gelatin-based bioinks, the influence of chemical modifications on protease degradability, cell adhesion, and cell signaling should also be evaluated. The in-vivo microenvironment contains numerous proteases capable of degrading the gelatin matrix. However, in-vitro studies often employ only a single type of protease to study the biodegradation of gelatin-based matrices, which does not recapitulate the complex in-vivo microenvironment. Thus far, most of the studies on gelatin-based 3D constructs has been done in-vitro with limited in-vivo studies [304, 354]. More in-vivo studies should be performed as the behavior of these bioprinted constructs in complex in-vivo environments is largely unknown.

From a translational point of view, it is important that the bioinks are sterile and lack pyrogenicity. As gelatin has a biological origin, it often has a high level of endotoxins [355]. Unfortunately, the endotoxin levels in gelatin scaffolds, particularly for in-vivo transplantation, are rarely considered or characterized. Endotoxins, drastically affect the performance of biomaterials and are known to promote an excessive pro-inflammatory environment. Thus, it is critical to develop endotoxin removal strategies for gelatin-based constructs, specifically for immunomodulation studies and in-vivo applications. The choice of sterilization technique for gelatin is also known to alter its bioprintability, biodegradation, and mechanical properties [356]. Therefore, the effect of FDA-approved sterilization techniques should be considered when designing new gelatin-based bioinks. This is important, as this could alter the mechanical properties of the constructs, which need to be defined before conducting any clinical study.

Multiple studies have utilized the approach of blending an auxiliary material or increasing the prepolymer concentration of gelatin to improve printability. Although this has proven effective in improving shape fidelity, it compromises the cell viability [357]. This is one of the key hurdles that has prevented further development of gelatin-based bioinks. Furthermore, even though gelatin provides cell supporting motifs to a bioink, its ability to shield the cells from shear stress during the extrusion is low [358]. Given that extrusion-based bioprinting is the most widely used bioprinting modality, more effort is needed to find formulations that protect cells during extrusion.

ECM is ubiquitous in the human body, but its quantity varies from organ to organ. For example, a healthy liver is mostly populated by cells while the ECM comprises up to only 10% of its total volume. In contrast, ECM in the skin constitutes up to 70% of total volume [359, 360]. Thus, gelatin-based bioinks require substantially different cell densities depending upon the application. High cell densities can greatly affect the printability and shape fidelity of the resulting bioprinted constructs [361]. For example, a very high cell density can result in nozzle clogging in extrusion-based bioprinting due to increased bioink viscosity, and can also affect droplet formation in case of droplet-based bioprinting which affects the printing resolution [128, 362]. Furthermore, as currently most of gelatin-based bioinks are stabilized by UV crosslinking, the effect of cell density on

crosslinking efficiency and viscoelasticity are underexplored. For example, Diamantides et al. reported the alterations in rheological properties of collagen bioinks with high cell densities [361]. A high cell density (up to 100×10^6 cells/ml) increased the viscoelasticity but reduced the storage modulus and slowed down the gelation kinetics of the resulting gel. Recently, Martorana et al. demonstrated the effect of different cell lines on rheological properties of gellan gum-based bioinks [363]. Human colon tumor cells showed an increase in viscoelastic modulus compared to acellular constructs. Therefore, future studies are likely to improve the design of gelatin constructs by focusing on the impact of different cell types and their densities on the viscoelastic and crosslinking properties of gelatin-based bioinks.

Currently available gelatin-based bioprinted constructs have dense networks with nanoscale pores and lack bio-functional heterogeneity found in native tissues. In recent years, the use of hydrogel microparticles (microgels) to form granular hydrogel scaffolds have gained considerable interest as these scaffolds provide innate porosity (void spaces between microparticles), shear-thinning, and self-healing properties [223, 364–366]. Microgels are microscale hydrogel particles that when assembled and packed together form granular hydrogels with inherent porosity favoring cellular infiltration and growth both in-vitro and in-vivo [367]. A key structural aspect of microgels is that the macro-scale porosity and pore geometry can be tuned using microgels of different shapes (e.g., polygonal fragments, high aspect ratio rods, etc.) and sizes or by varying the packing density [368, 369]. The size of these particles in combination with the shear-thinning nature provides an advantage of easy extrusion/injection through small orifices resulting in minimal damage to cells. Considering the advantages of microgels, researchers have started to develop gelatin-based bioinks to bioprint porous scaffolds with improved porosity [370]. Song et al. recently developed a gelatin-based composite bioink using gelatin microgels within gelatin solution [371]. Shao et al. also utilized gelatin microgels to bioprint GelMA constructs with mesoscale pore networks [57]. Embedded osteoblasts and human umbilical vein endothelial cells in large-scale (>1cm) bioprinted constructs showed greater spreading in comparison to constructs without mesoporous network highlighting the importance of porous nature for enhanced cell growth. Thus, the microgel approach provides an improved method for designing gelatin-based bioinks for 3D bioprinting. Given the unique properties, the use of microgel in bioprinting of gelatin is likely to expand significantly in the future.

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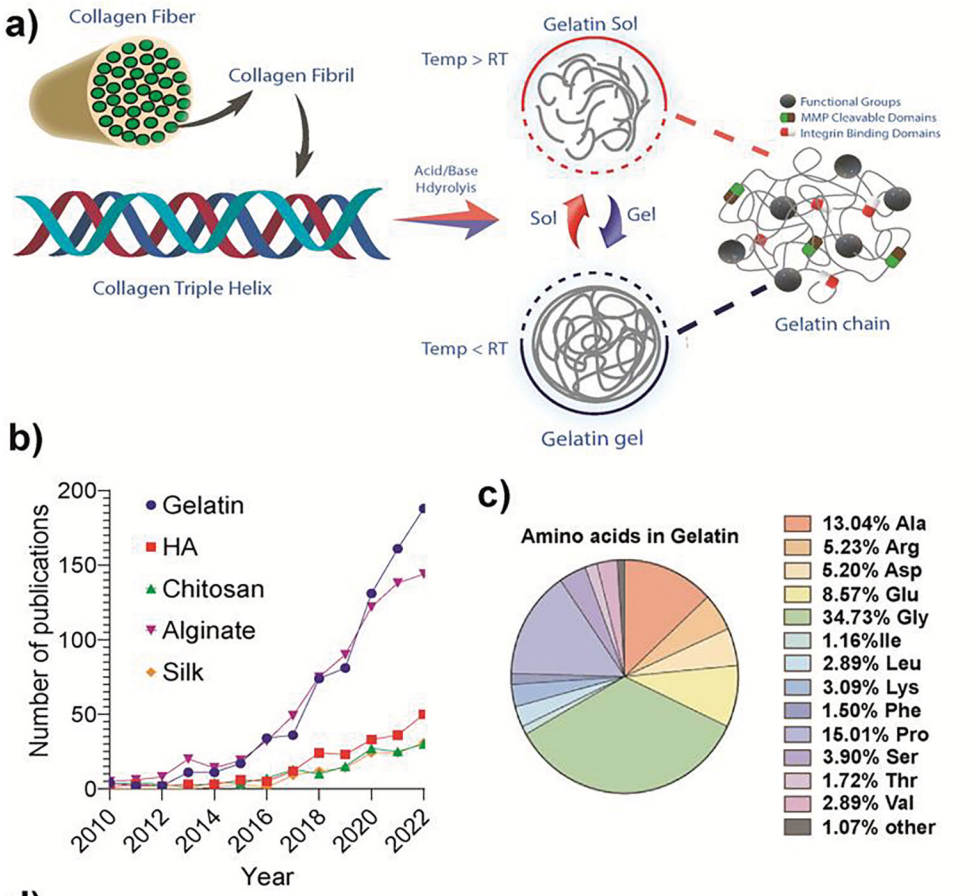


d)

Gelatin Type	Source	COOH (mmol/g)	Amines (mmol/g)
Gelatin Type B	Bovine Hides	1.0980	0.385
Gelatin Type A	Porcine skin	0.846	0.292

Figure 1.

a) Schematic description of the collagen structure and hydrolysis to gelatin, **b)** The application of gelatin in bioprinting in terms of the number of publications as per Scopus database [search string: ABS (gelatin/hyalur/chitosan/alginate/silk* AND bioprint* OR biofabr*)]. **c)** The amino acids present in gelatin. Adapted with permission [41]. Copyright 2022, Wiley-VCH Verlag GmbH & Co. **d)** The amount of carboxylic acid and amine functional groups in gelatin type A and type B [42, 43, 45, 59]

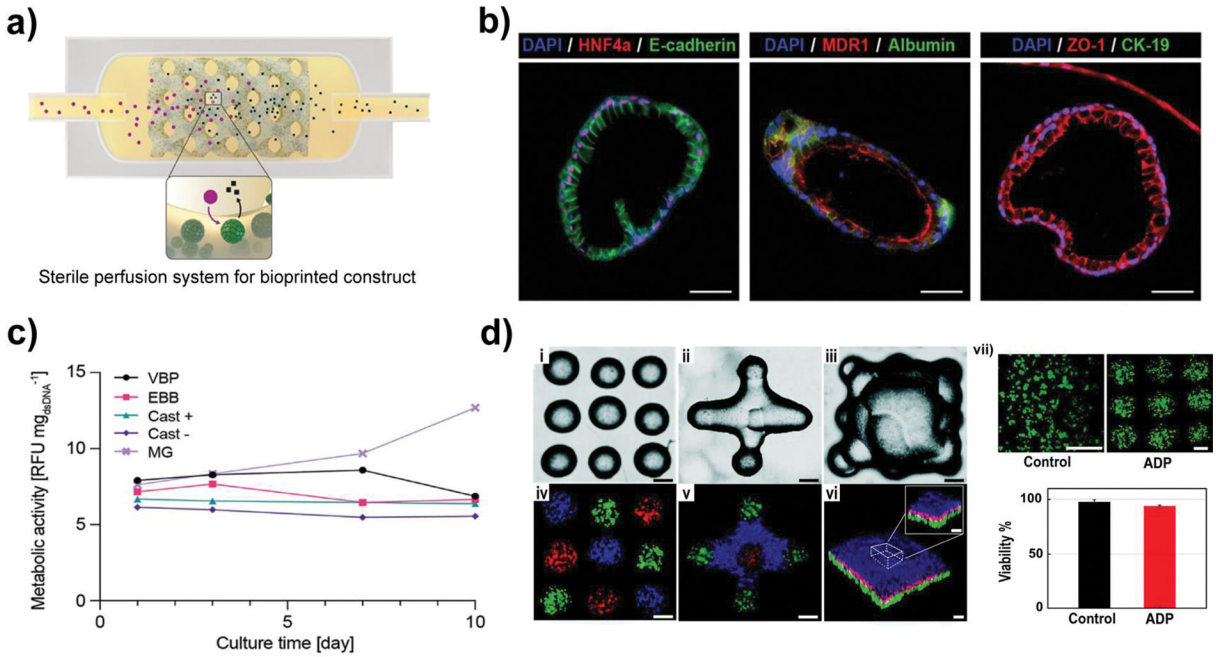


Figure 2.

a) Schematic showing the 3D bioprinting of organoid laden and perfusable GelMA construct using volumetric bioprinting. **b)** Liver organoids maintained self-organization and expressed key liver markers. **c)** Metabolic activity of liver organoids compared to extrusion bioprinting, casting with and without iodixanol and in Matrigel. Adapted with permission [144]. Copyright 2022, Wiley-VCH Verlag GmBH & Co, **d)** Optical images and confocal images of various patterned cellular structures using acoustic bioprinting (i-vi) (all scale bars: 500 μm), Viability of acoustic bioprinted cells vs. control group (vii). Adapted with permission [146], Copyright 2021, Royal Society of Chemistry.

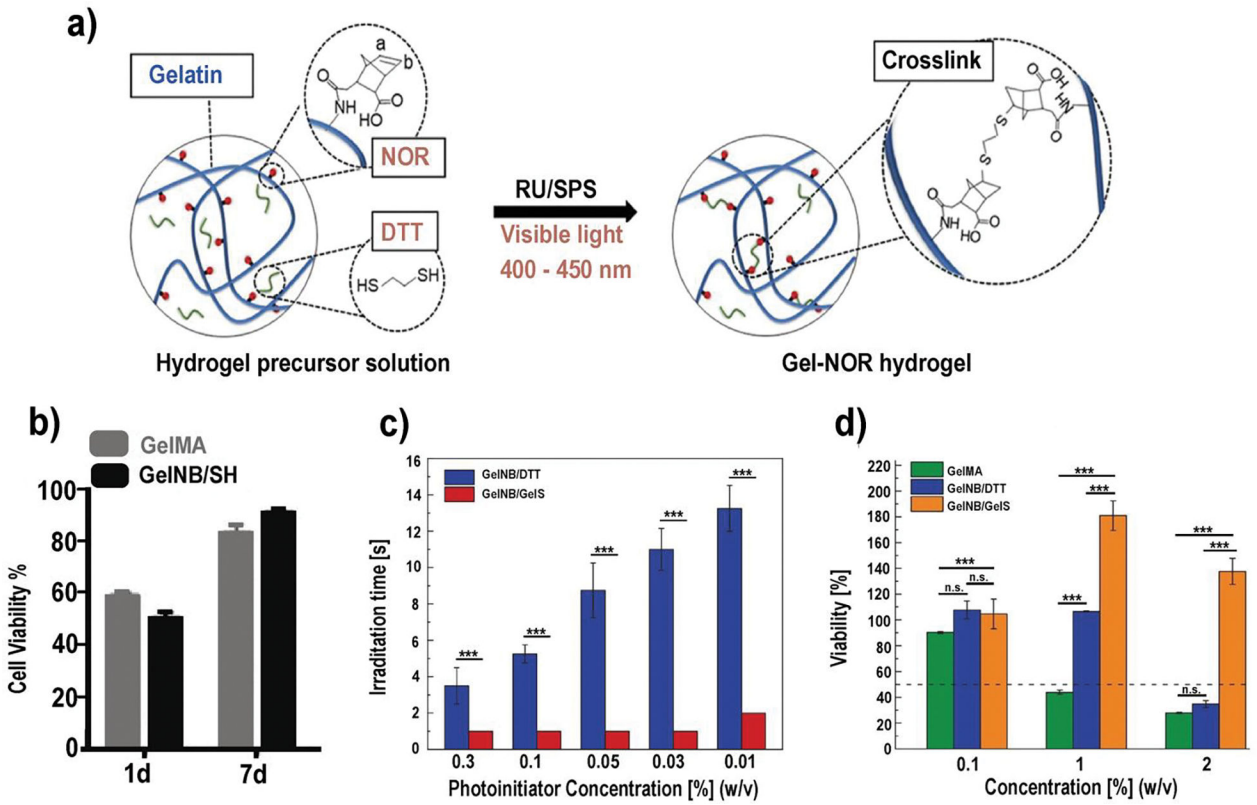


Figure 3:

a) Schematic diagram for thiol-ene crosslinking of gelatin – norbornene. Adapted with permission [178], Copyright 2021, Wiley-VCH GmbH. **b)** Comparison of cell viability of HUVECs in GelMA vs GelNB/SH. Adapted with permission [174], Copyright 2021, American Chemical Society. **c)** Low irradiation time required for the curing of GelNB with GelS crosslinker compared to DTT crosslinker. **d)** The effect of the degradation products of GelMA, GelNB/DTT and GelNB/GelS on the viability of hepatocarcinoma (HepG2) cells. Adapted with permission [177], Copyright 2021, Wiley-VCH GmbH, Weinheim.

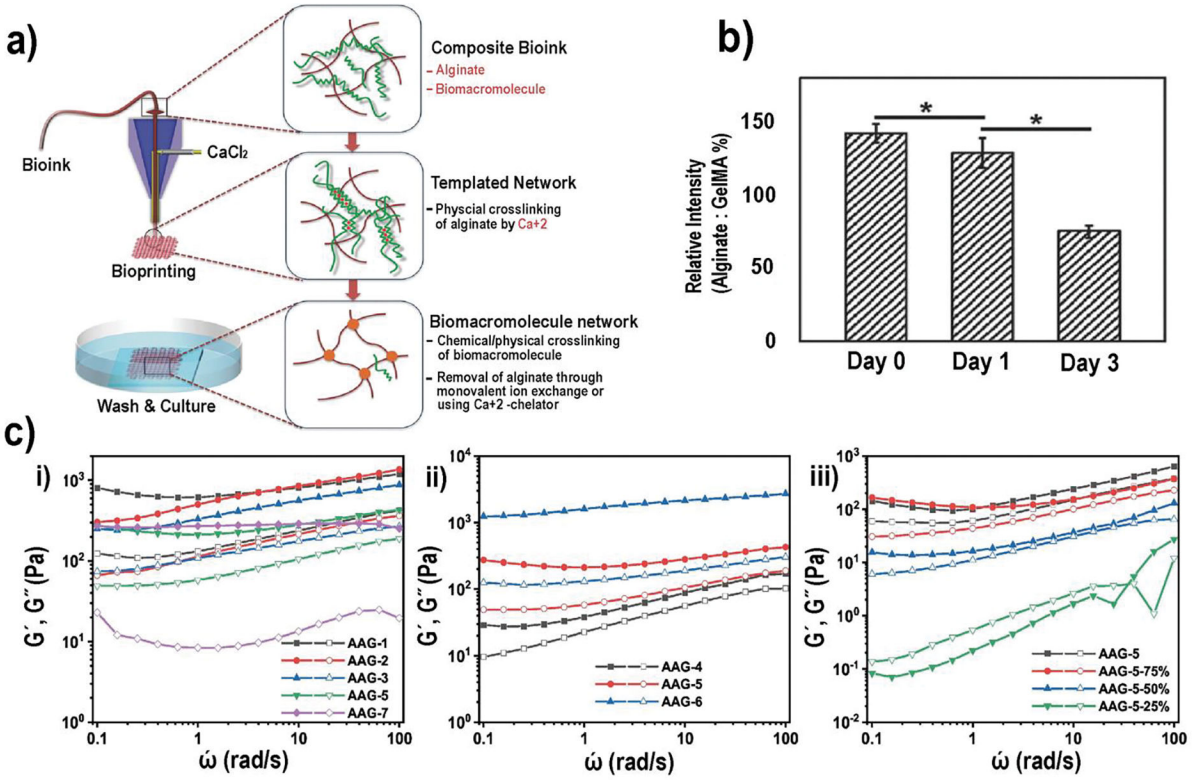


Figure 4.

a) Schematic representing preparation of GelMA construct using alginate as a sacrificial support material. **b)** Reduction in the intensity of alginate within GelMA when alginate is gradually dissolved. Adapted with permission [133], Copyright 2018 WILEY-VCH Verlag GmbH & Co, **c)** Frequency sweep of hybrid hydrogels, i) increasing frequency responsiveness in hybrid hydrogels with increasing βCD % (from lowest in AAG1 to highest in AAG5, AAG7 represents GelMA only), ii) increased storage modulus and decreased frequency responsiveness as a result of increased GelMA amounts (from lowest in AAG4 to highest in AAG6), iii) Reduced polymer density increases frequency responsiveness but reduces storage modulus (AAG-5–75%, AAG-5–50%, and AAG-5–50% represents hybrid hydrogel prepared from 75%, 50%, and 25% substances of AAG-5, respectively. Solid symbols (G′) & hollow symbols (G′′)), Adapted with permission [201], Copyright 2022, Elsevier.

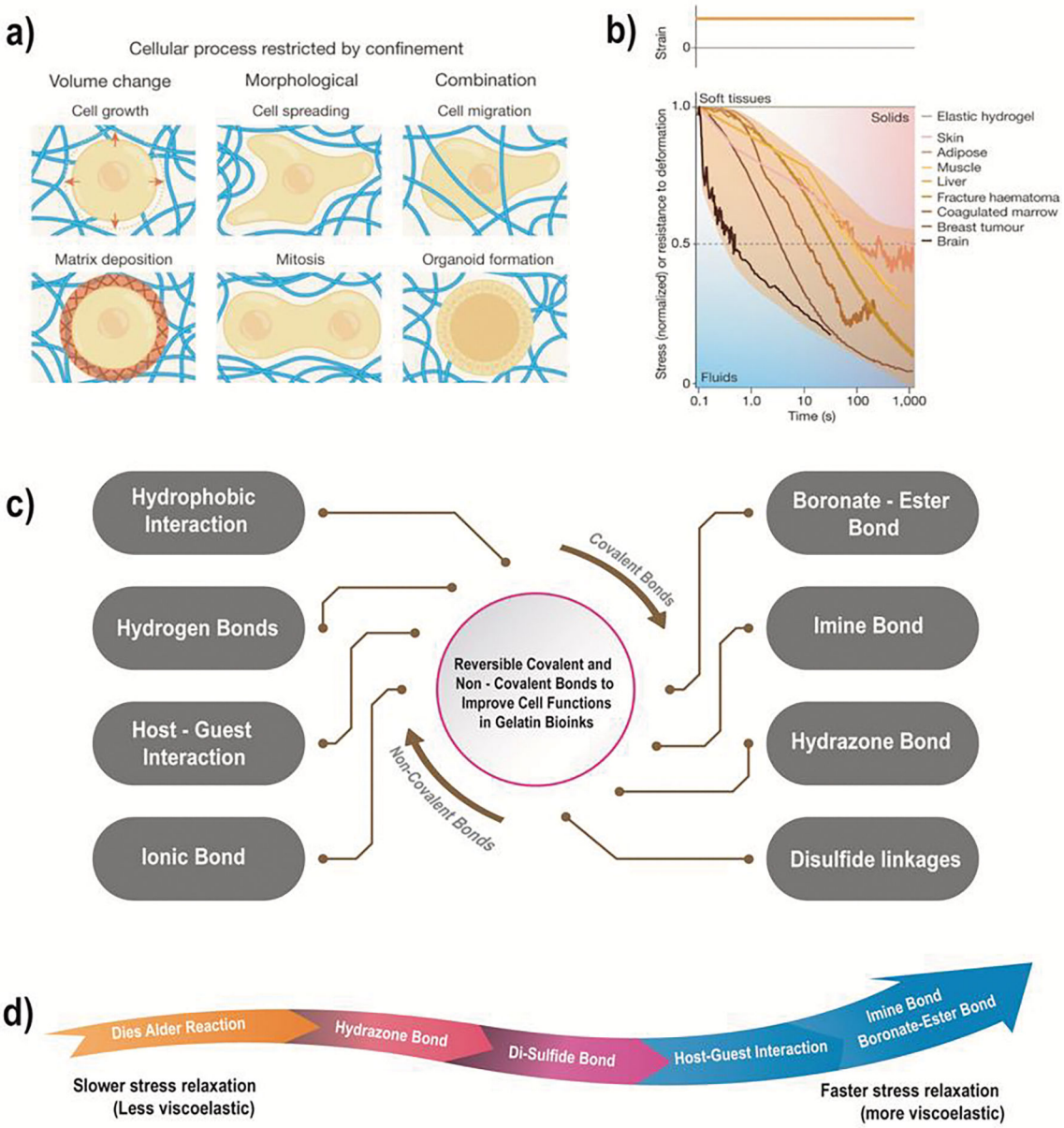


Figure 5.

a) Static covalent networks confine cells, restricting their cellular functions. **b)** Stress relaxation rates of different natural tissues. Adapted with permission [273], Copyrights 2020, Springer Nature. **c)** schematics representing the reversible covalent and non-covalent bonds that can be introduced in gelatin-bioinks to improve viscoelasticity. **d)** Stress relaxation rates of different dynamic bonds (Increasing from left to right).

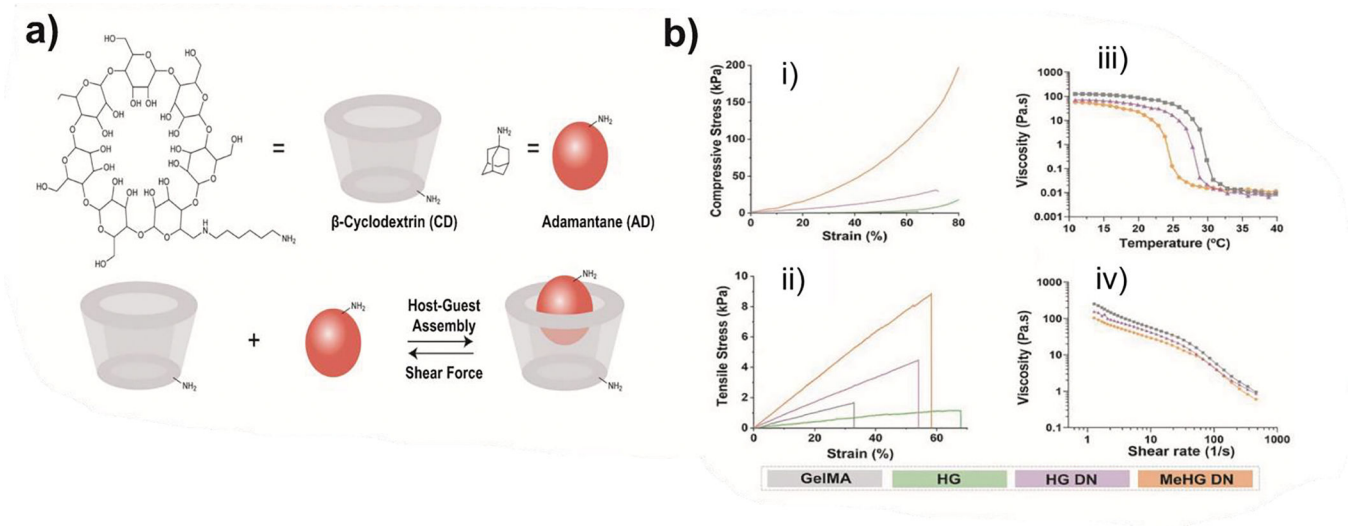


Figure 6.

Guest-host crosslinking: **a)** A schematic representation of host-guest interactions between β-cyclodextrin (host moiety) and adamantane (guest molecule). Adapted with permission [59], Copyright 2020, Wiley Periodicals. **b)** Mechanical characterization of GelMA, HG DN and MEHG DN hydrogels. i) Compressive stress – strain curve, ii) change in viscosity as function of temperature, iii) tensile stress-strain curve, iv) shear thinning behavior of hydrogels (viscosity vs shear rate), Adapted with permission [284], Copyrights 2022, Wiley-VCH GmbH.

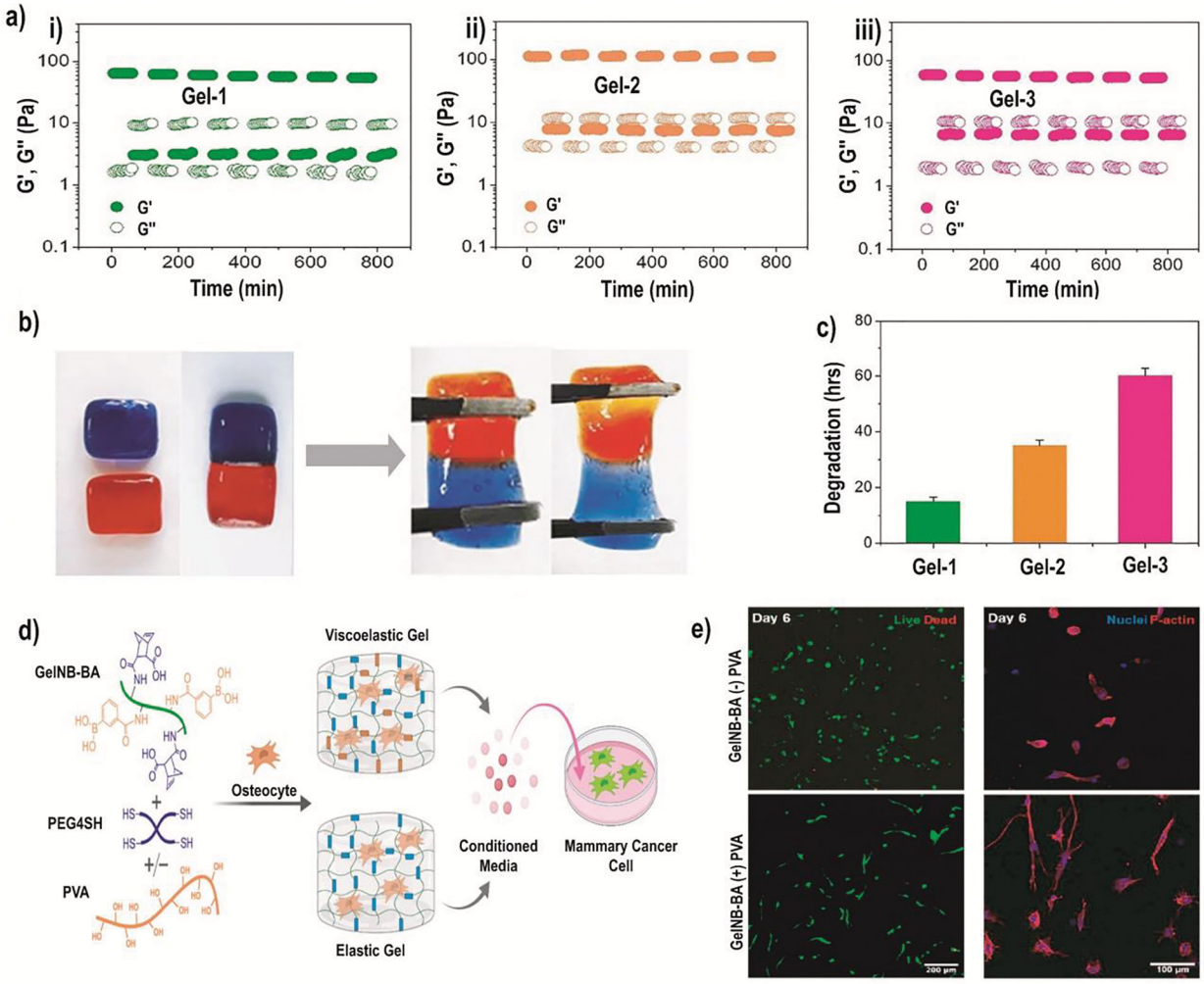


Figure 7.

(a i – iii) Step strain-sweeps of composite hydrogels (phenyl boronic acid (PBA) modified gelatin and polyvinyl alcohol) with different amounts of PBA (PBA amounts in Gel 3 > Gel 2 > Gel 1). **b)** Self-healing behavior of composite hydrogel system, **c)** In-vitro degradation rate of dynamic gels with respect of increasing PBA amount, Adapted with permission [271], Copyright 2022, Elsevier. **d)** A schematic representation of reversible boronate-ester-diol bonding between GelNB-BA and PEG4SH. Adapted with permission [270], Copyright 2021, American Chemical Society. **e)** Live/Dead and F-actin staining of human MSCs encapsulated in GelNB-BA / PEG4SH hydrogels in the absence or presence of PVA, which improved viscoelasticity due to boronate-ester bonding of PVA and gelatin. Adapted with permission [306], Copyright 2021, American Chemical Society.

Table 1:

Comparison of gelatin with other natural polymers as a bioink material for 3D bioprinting

Bioink Material	Material Class	Common bioink crosslinking strategies	Cell binding motifs	Enzymatic degrad-ability in-vivo	Advantages	Drawbacks
Gelatin	Protein	Covalent, enzymatic [60, 61], and physical	✓	✓ [62]	• Low cost • Thermoresponsive behavior • Low antigenicity and high biocompatibility [63]	• Poor mechanical integrity • Poor thermal stability • Less control over MW
Silk Fibroin (SF)	Protein	Enzymatic [64] or physical	✗ [61, 65]	✓ [66, 67]	• MW can be controlled by degumming [68–70] • Excellent mechanical properties [71] • Genetically modifiable into variety of morphologies	• Generates immunogenic response [72, 73] • High cost
Chitosan	Poly-saccharide	Ionic [74]	✗	✓ [75]	• Mucoadhesive [76] • Antimicrobial [77] • MW can be controlled by different production methods [78–80]	• Loses mucoadhesive capacity in physiological environment [81] • Insoluble at physiological pH [82] • Enzymatic production of chitosan is highly costly
Alginate	Poly-saccharide	Ionic [83–85]	✗	✗ [86]	• Low toxicity • Range of MWs (60,000 – 700,000Da) are commercially available [87] • Cheap and renewable source of biopolymer [88]	• Low cell attachment due to lack of cell binding motifs • Alginates obtained from cold water species have poor viscosity [89]. • Precipitates at low pH [90]
Hyaluronan (HA)	Poly-saccharide	Covalent [91] or physical	✗	✓ [92]	• Excellent biocompatibility and biodegradability • Promotes angiogenesis and cell proliferation [93] • MW of HA can be controlled using different production methods [94–96]	• Biosynthesis of HA is costly • Bacterial production of HA involves risk of bacterial endotoxins [97] • Undergoes fast hydrolysis by hyaluronidases [98]

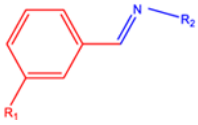
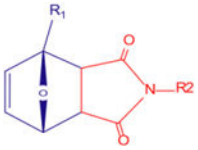
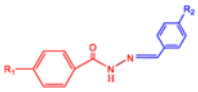
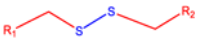
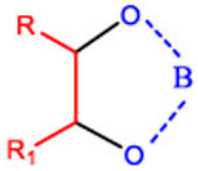
Table 2:

Application of GelMA hydrogels in bioprinting high fidelity constructs using emerging light and droplet based bioprinting technologies.

Bioink Name	Bioprinting Method	Aim / Application	Bioprinting Resolution	Source
GelMA	Digital light processing based stereolithography (DLP – SLA)	To study the effect of various GelMA synthesis parameters on the printability of final bioink and properties of printed constructs.	50, 100, 250 μm	[142]
GelMA	SLA	To bioprint precise geometry of the human corneal stroma for tissue regeneration.	400–500 μm	[141]
GelMA	SLA	To design a low-volume novel vat for retrofitting an existing additive manufacturing equipment, evaluate the effect of the machine and material setup parameters for printability.	100 μm	[157]
Eosin Y (EY) - GelMA	Dynamic Optical Projection SLA	To develop a new bioink solution that provides both visible light crosslinking and cell adhesion.	NA	[158]
GelMA + iron-oxide nano particles.	Two-photon Polymerization	To develop a magnetically controllable GelMA-based biodegradable microrobot f for stem cell delivery.	NA	[159]
GelMA	Two-photon Polymerization	To study the biological properties, machinability of GelMA hydrogel and the biocompatibility of 3D scaffolds fabricated by two-photon polymerization.	250 nm	[160]
GelMA	Volumetric Bioprinting	To provide a new method to fabricate hetero-cellular bone-like tissues by leveraging the advantages of ultrafast tomographic Volumetric Bioprinting technique and 3D hMSC/HUVEC co-culture.	34 μm	[143]
GelMA	Acoustic Bioprinting	To develop an acoustic droplet printing (ADP) method to fabricate 3D native-like tissues for recapitulating cellular function.	Droplet size = 200 μm, height = 100 μm	[146]
Saponified-Heat Treated GelMA	Therma Inkjet Bioprinting	To improve GelMA printability in a thermal inkjet print-head.	NA	[161]

Table 3:

Common reversible covalent bonds with different stability and stress relaxation rates used to provide viscoelastic nature in gelatin.

Reversible Covalent Bonds	Chemical Structure	Stability in Culture (weeks)	Stress Relaxation Rate ($\tau_{1/2}$) (s)	Applications in gelatin
Imine Bond		1	< 10	[297, 298, 303]
Diels – Alders Reaction		4	< 1000	Not yet used for gelatin bioinks
Host- Guest Interaction		3	< 30	[272, 304]
Hydrazone Bond		1	> 1000	[305–307]
Di-sulfide Bond		2	> 100	Not yet used for gelatin bioinks
Boronate – Ester Bond		1	< 10	[270, 271, 306, 308]