Functionalizing bioinks for 3D bioprinting applications

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3D bioprinting has emerged as the intersection between chemistry, biology and technology. Through its integration of cells, biocompatible materials and robotic-controlled dispensing systems, the process enables the production of structures that are biomimetic and functional, thus revolutionizing the concept of tissue engineering. One of the biggest limitations of 3D bioprinting for tissue engineering is the lack of printable materials (bioinks) with all-inclusive properties desirable for the construction of engineered 'bio-physico-functional' tissues and organs. Thus, bioinks are required to be functionalized or altered to produce the most desirable bioarchetypes. Functionalization methods vary across chemical, mechanical, physical and biological methods, and common methods include blending of materials, coatings, crosslinking and exploiting functional groups. In this short review, a description and critical comparison of reported functionalization methods, focusing on their effects and contributions toward bioinks, have been presented.

Introduction

A major challenge in the field of 3D bioprinting for tissue engineering is the lack of printable materials – known as bioinks [1]. Currently utilized bioinks are based on natural and synthetic polymers [2]. Most natural polymers possess cellular interactivity and biocompatibility [3] but often lack the mechanical properties needed for them to maintain their structural integrity and support the physical stress within the *in vivo* microenvironment. By contrast, synthetic polymers are advantageous owing to their potential for modification, which could be explored to induce bioactivity along with providing more control over their structure and architecture [3]. However, synthetic polymers prove to be challenging as a result of their poor biocompatibility, poor cellular adhesion, toxic degradation products, as well as a loss of mechanical properties during the degradation process [4]. This review focuses on presenting available design strategies, herein known as functionalization methods and techniques, used to alter and optimize synthetic bioinks (Fig. 1). The methods used for functionalization, as well as their effects and successes, will be discussed and evaluated to promote the applicability and sustainability of bioinks. A comparison of the bioinks commonly employed is elaborated on in Table 1.

3D bioprinted scaffolds for tissue engineering

Bioprinting utilizes the concept of computer-generated 3D designs, adjustable parameters and bioadditive manufacturing technologies [5] (Fig. 2) to 'print' precise geometries that will mimic anatomically correct biological structures [6]. Because the purpose of these engineered tissues is to replace and/or repair damaged tissues [7], bioinks used to fabricate scaffolds (printed constructs) must meet certain criteria to be considered suitable for clinical application. The most obvious specification is

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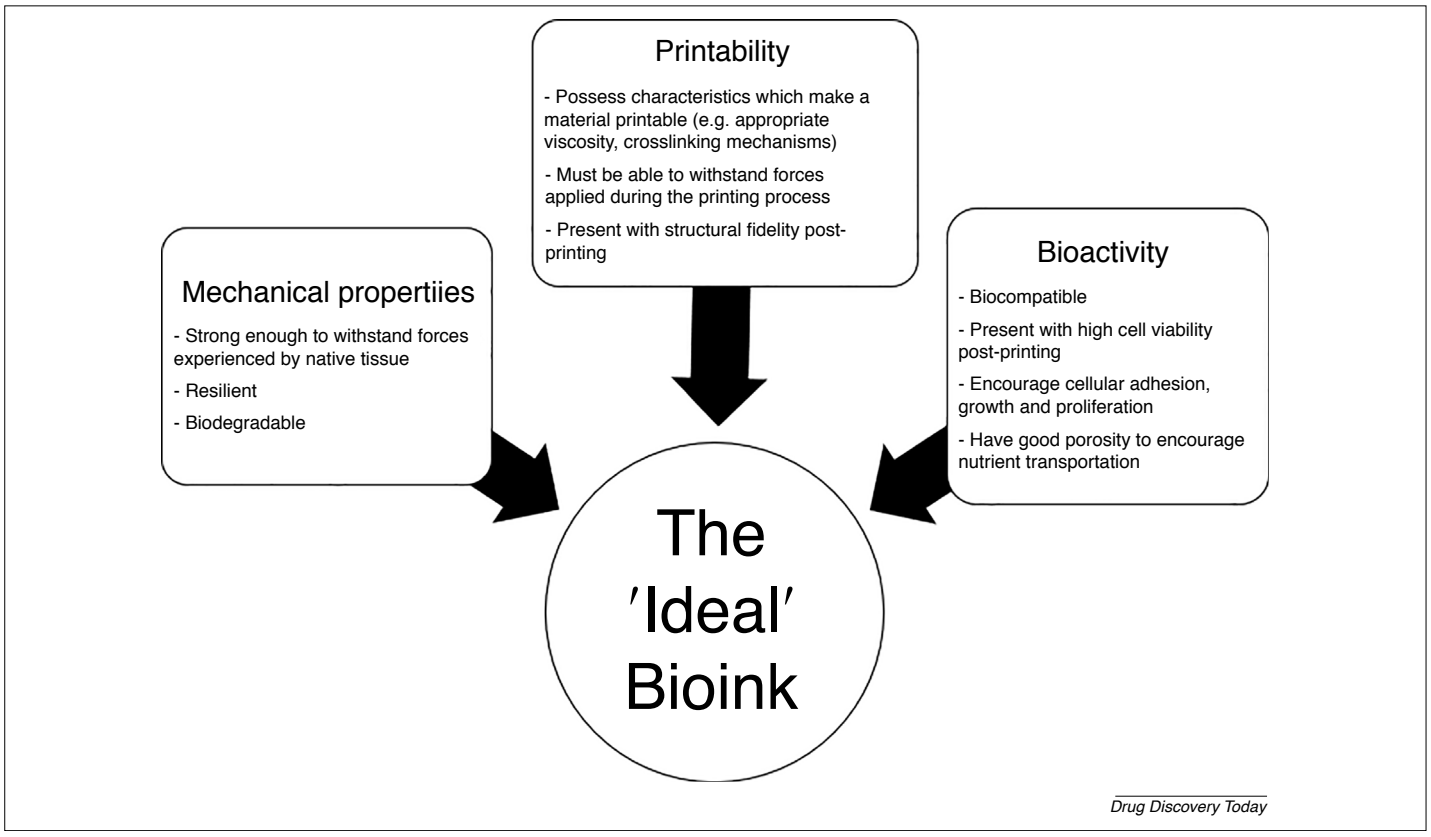

FIGURE 1

A summary of commonly desirable properties in bioinks.

biocompatibility and biodegradability [8], with original materials and degradation products being nontoxic [7,9]. Cells must be able to attach and adhere to the scaffold, which should also be capable of encouraging cellular proliferation and differentiation [10] to promote bioactivity. Scaffold architecture is also vital [8] in terms of porosity, morphology, connectivity and orientation, which can be controlled through the choice of bioink and printing method. These parameters play a paramount part in determining the transfer and movement of nutrient, oxygen and waste through the engineered complex [7,10]. Good mechanical properties are vital to scaffolds, because the engineered tissue must prove stable over time, as well as strong enough to withstand applied forces during implantation and *in vivo* [8,9].

TABLE 1

Comparison of commonly used nonfunctionalized bioinks

Material	Description	Advantages	Refs	Disadvantages	Refs
Alginate	Natural	• Gels rapidly when ionically crosslinked • Biocompatible • Cheap	[11] [69] [69]	• Minimal cell recognition and adhesion • Slow degradation when not crosslinked • Low mechanical strength	[69] [59] [2]
Gelatin	Natural	• Encourages cellular growth • Thermoresponsive sol-gel transition	[2,13] [2,12,28]	• Liquifies at physiological temperatures • Poor mechanical properties	[2] [69,70]
Hyaluronic Acid	Natural	• Biodegradable • Biocompatible	[2,70] [2,69]	• Highly hydrophilic • Not mechanically stable • Slow gelation rate	[51] [51] [69]
Polyethylene glycol (PEG)	Synthetic	• Biocompatible • Hydrophilic • Exhibits shear thinning behavior	[71] [71,72] [11]	• Poor mechanical strength • Poor cell adhesion	[69] [73]
Poly(lactic acid) (PLA)	Synthetic	• Excellent mechanical strength • Biodegradable • Biocompatible	[74] [71,74] [74]	• Poor cell interaction and adhesion • Low hydrophilicity	[74] [74]
Poly(ε-caprolactone) (PCL)	Synthetic	• Biocompatible • Biodegradable • Thermoplastic • Excellent mechanical properties	[75] [75] [24] [19,75]	• Absence of biological properties	[54]

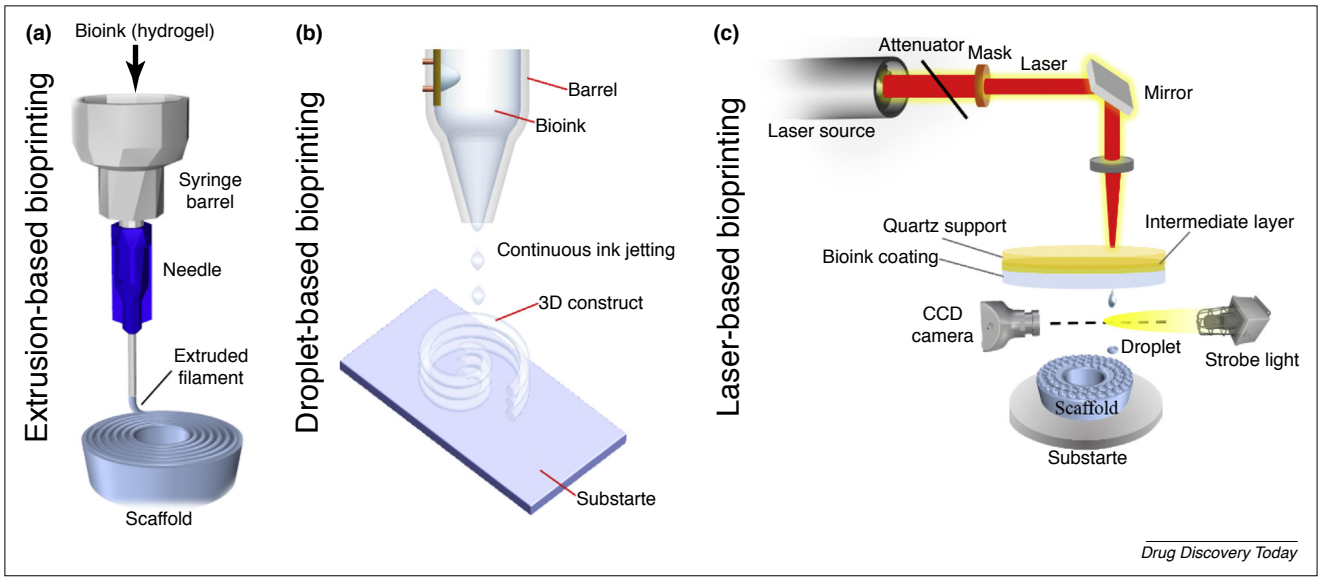

FIGURE 2

Various common bioprinting techniques. **(a)** Extrusion-based bioprinting, **(b)** droplet-based bioprinting, **(c)** laser-based bioprinting. Reproduced, with permission, from Ref. [76].

Functionalization of scaffolds for optimal properties

Because there is currently no 'perfect' bioink, functionalization is a practical way to incorporate desirable properties and overcome limitations (Table 1). However, it is important to note that the characteristics of the material as well as of the produced construct significantly depend on the desired outcome (i.e., the target tissue or organ). Because of the complexity of the human body, there is immense variability among different tissues and organs with regard to their structural and physiological requirements [11] and, as such, functionalizations are dependent on these requirements and must be tailored as necessary. The outcomes of various functionalization methods are discussed below, with a focus on optimizing mechanical properties, printability, biocompatibility and bioactivity (Fig. 3).

Functionalization for improving mechanical integrity

In the quest for attaining the desirable properties in the polymer for 3D printing, chemical functionalization has been explored in numerous ways and one such method is the introduction of methacrylate groups. Functionalization of polymers [such as gela-

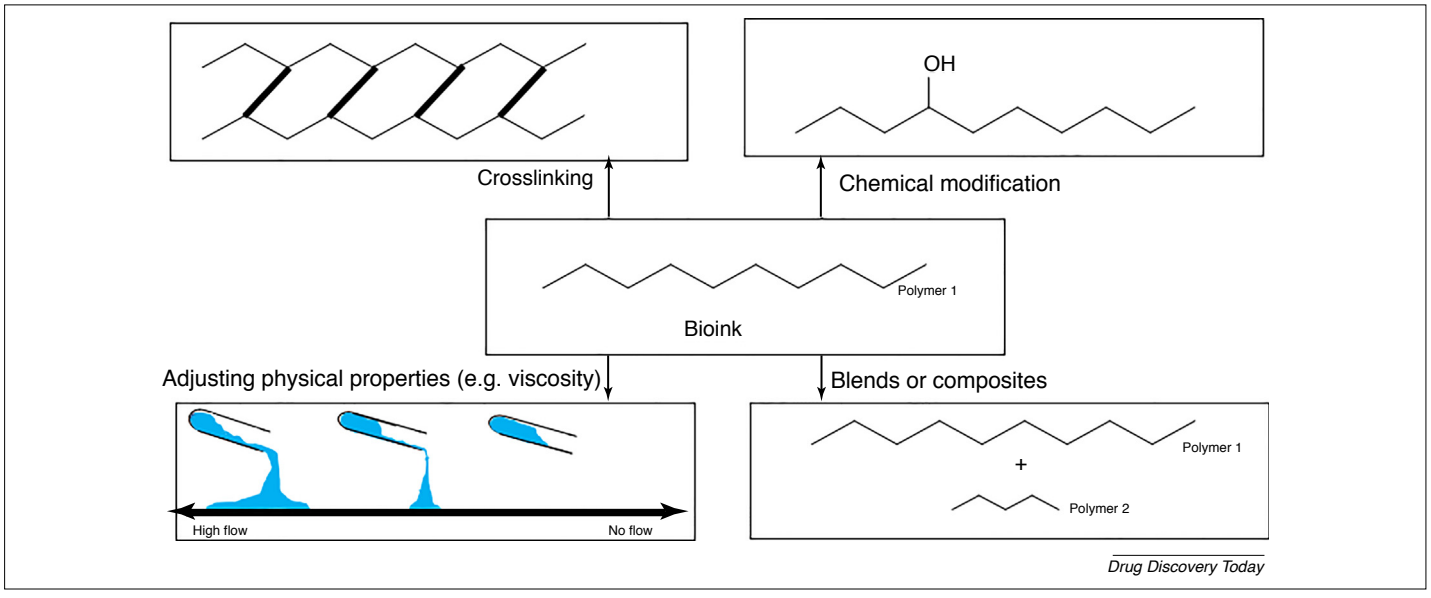

FIGURE 3

Schematic summary of various methods of functionalization.

tin, hyaluronic acid and poly(hydroxymethylglycolide-co-ε-caprolactone)] with unsaturated methacrylate groups [12] results in polymers that are photopolymerizable [13] and form a more mechanically stable construct [14]. An increase in the degree of methacrylation [14] has been proven to increase the stiffness of constructs [15] when measured against various strain levels; however, the compressive modulus is mainly affected by the methacrylated polymer concentration [14,16]. Combining methacrylated polymers with each other – such as covalently binding methacrylated gelatin (gelMA) to methacrylated poly (hydroxymethylglycolide-co-ε-caprolactone) blended with poly-ε-caprolactone – produced constructs with a higher mechanical resistance (7.7 ± 2.4 N) and integrity [17], when compared with a compound of gelMA bound to a nonmethacrylated blend that showed low compressive strength (71 ± 4% for the unmethacrylated blend compared with 43 ± 5% for the methacrylated blend), decreased recovery and a lack of integration of materials. This inferred that the presence of methacrylated polymers positively influences the mechanical properties. Mechanical properties are also shown to be influenced by covalent grafting of a reinforcing thermoplastic network to a hydrogel [18]; or blending of a thermoplastic polymer with another polymer [19] also results in constructs with improved mechanical strength and integrity. The thermosensitive nature of constructs enables immediate solidification of constructs when deposited onto thermoregulated platforms [18].

A study compared various hydrogels (agarose, alginate, gelatin methacrylamide and BioINK – a PEGMA-based hydrogel) and their respective equilibrium moduli to evaluate their potential to be used as articular- and fibro-cartilage. Results showed that the equilibrium moduli of the studied polymers were not of an equal or superior standard to that of native human cartilage but that, with reinforcement with Poly(ε-caprolactone) (PCL; a thermoplastic material) microfibers, the equilibrium modulus improved to within the acceptable range [20]. The mechanical properties can be varied according to the potential tissue by altering the fiber spacing [21], fiber diameter and the molecular weight of the polymer. Another method of employing thermoplastic fibers is to produce constructs via the alternate deposition of thermoplastic fibers and hydrogels, and this process can be tailored by altering characteristics such as fiber spacing, thickness and orientation [21,22].

Hydrogel reinforcing gels have also been studied with favorable results [21]. The use of a hydrogel maintains the improved mechanical properties of the support structure, as well as improving control over degradation kinetics [23]. The use of a hydrogel also contributes to the usage of these inks for bioprinting soft tissues – for which not many reinforcing options exist [21]. Blends of a polymer with different molecular weights can also affect the mechanical strength of constructs. This would be a noteworthy method of functionalization because of the absence of any additives, and therefore the chance of adverse interactions is minimized. A combination of a high molecular weight alginate blended with less low molecular weight alginate (ratio 2:1) produced a mechanically stable construct, similar to that of native tissue [24].

Conditions surrounding crosslinking are important as well. UV exposure, used in photopolymerization, can be used to control the stiffness and swelling of the hydrogels [12], with longer exposure leading to stiffer constructs and slower degradation [25]. Temperature has also proved to play a significant part in the mechanical properties of the gels, with solutions stored and allowed to form thermal gels before exposure to UV appearing to produce significantly stiffer constructs than solutions maintained at higher temperatures [12]. Lower temperatures can also slow the crosslinking process down and lead to a greater ordered crosslinked structure with improved mechanical properties [26]. An increase in crosslinker concentration and crosslinking density [27] has been shown to lead to higher degrees of crosslinking, thus enhancing structural integrity [28] and mechanical properties [20]. Crosslinking agents themselves also play a part in the mechanical properties of scaffolds. Glutaraldehyde was shown to improve the stability and mechanical strength when used as a crosslinker for collagen-based scaffolds and has shown an impressive decrease in degradation behavior with an increase in concentration. However, glutaraldehyde was also proven to decrease the flexibility of scaffolds and result in a drastic increase in the stiffness of scaffolds with increasing concentrations [28]. Functional crosslinkers also provide an interesting avenue for enhancing mechanical properties. Bioactive glass possesses impressive bioactive properties and, when used in combination with alginate, acts as a crosslinker. The alginate self-crosslinks through the Ca^2 provided by the bioactive glass [29] and produces constructs with impressive compressive strength as well as bioactivity.

Interpenetrating networks have been used to enhance mechanical strength by combining the properties of each material [27] and improvements have been made upon the concept through utilizing 'double networks' [30] – specialized interpenetrating networks, composed of materials with opposing mechanical properties, that have been proven to possess high fracture stress and toughness – and including a two-step photocrosslinking wherein the first polymer is crosslinked to form a rigid and brittle network, followed by the diffusion of the second polymer into the first network and photocrosslinking it to form a second soft and ductile network. As such, the initial rigid network is responsible for handling stress throughout the material, whereas the second network prevents fracture [14]. Double networks lacking interconnection have been reported to be stronger than if there is interconnection [31]. The utilization of multiple crosslinking mechanisms also contributes greatly to an increase in mechanical strength. Thermoresponsive polymers undergo rapid physical crosslinking [23] once deposited, allowing for initial shape fidelity [32]. Thereafter, the polymers undergo covalent crosslinking via photopolymerization which encourages added network stability [18]. An interesting example of this involves a decellularized extracellular matrix (dECM) bioink that mimics the native environment [33]. Improving mechanical properties was achieved through covalent crosslinking through adding vitamin B2 (riboflavin) – a biocompatible photocrosslinker [34]. The study also employed a dual mechanism by implementing further physical crosslinking, through thermally triggered collagen fibrillogenesis [35] and this dual crosslinking exhibited a stable storage modulus superior to that of singularly crosslinked constructs. It was also observed that an increase in vitamin B2 decreased the stiffness of constructs, demonstrating that each crosslinking step contributes to the overall mechanical profile and constructs can be tailored as required [36] (Fig. 4).

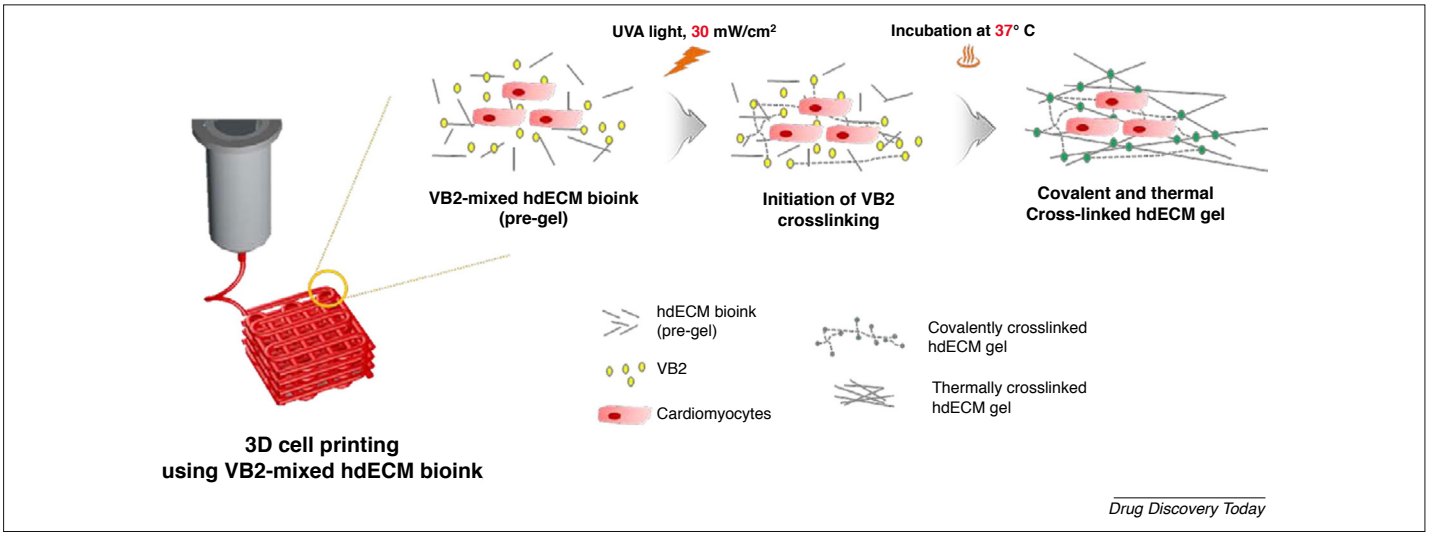

FIGURE 4

Schematic illustration of a two-step crosslinking mechanism that applies concurrent crosslinking of vitamin-B2-induced covalent crosslinking and thermal crosslinking. Reproduced, with permission, from Ref. [36].

Some crosslinkers only partially crosslink polymers and, as such, the degradation rates of such materials might still be too fast to be clinically applicable. Thus, by utilizing the concept of secondary crosslinking, constructs produced can be further stabilized resulting in stronger more-durable constructs with longer degradation times [37]. Utilizing secondary materials as binders has also proven to be an effective manner of enhancing mechanical integrity. Zhang *et al.* [38] used polyvinyl alcohol (PVA) as a binder and effectively decreased the brittleness of the scaffolds, thus enhancing their compressive strength. The degree of polymerization of the PVA binder also resulted in a low solubility at physiological temperatures, which enables the scaffolds to retain their mechanical integrity for longer [38].

Other materials have been studied to determine their effect on mechanical properties. Addition of hyaluronic acid has resulted in an enhanced compressive modulus [39]. The addition of silk fibroin to carboxylated agarose/hydroxyapatite composites improved the mechanical strength to supersede that of cancellous bone in the human body [40]. Carboxymethyl chitosan, through ionic bonding of functional groups, proved to improve mechanical properties of the resulting construct [41]. 4-arm poly(ethylene glycol)-tetra-acrylate (PEGTA) was shown to enhance mechanical stability owing to its enhanced crosslinking density through a branched tetravalent structure and numerous active crosslinking sites [25].

Functionalization for improving printability

Printability dictates the ultimate structure and functionality of the printed construct and is based on the printing process as well as alterable bioink properties – including flow properties, elastic modulus, shear thinning behavior and viscoelasticity [42]. Generally, printability can be improved through an increase in the ink's viscosity, decreasing the gelation time or both [36]. Shear thinning behavior can be identified by a decrease in viscosity with increased shear stress [18] and is desirable because it limits chain entanglement, thus allowing smooth extrusion [43] with quick recovery. Spreading, deformation, reduced porosity and collapse of subsequent layers are risked when printing materials with too low a viscosity; however, a formulation that is too viscous can result in a blocked nozzle [44], disjointed extrusion and constructs with limited integration [45]. Materials must be printable and be sufficiently viscoelastic that they are extrudable, followed by rapid recovery so that the printed layers remain self-sustaining [46], distinct and possess high shape fidelity [16]. The viscosity of materials can be adjusted through polymer density [47] and polymer concentration. High concentrations are generally the key to producing good printing fidelity [25], with low concentrations presenting with poor printing quality.

There are many materials that can be added to improve viscosity and induce shear thinning behaviors as well as improve shape fidelity. For example, nanocellulose provides shear thinning with impressive shape fidelity and good resolution, when added to a solution [48]. Methylcellulose [49], hyaluronic acid [12,44] and hydroxyapatite [44] facilitate printing through an increase in viscosity. PCL is characterized by its non-water solubility, which, when used in conjunction with other materials, could prevent setting reactions in a syringe environment. PCL also contributes toward printability because of its high viscosity, which allows the extrusion of materials [50]. PVA is added to crosslinking solutions and increases the viscosity of the solution, thereby preventing printed strands from floating and thus allowing stable structures to form [51].

Gelation can be controlled through adjusting the concentration of the polymer and its crosslinking solution [28], and also through the addition of a retardation agent that dictates the extent of crosslinking and its subsequent rheological properties. This must be undertaken carefully, because it can yield formulations that are too viscous for printing and will lead to poor-quality scaffolds. A solution to bioinks that do not rapidly solidify upon extrusion could be the addition of a thermoresponsive polymer that gels under temperature-related conditions. Thus, such a polymer, once co-extruded with another polymer, rapidly gels upon deposition

under certain temperature conditions, allowing the other polymer to solidify while maintaining the desired structure [27]. Combination bioinks have proved to be advantageous in optimizing properties, as shown by Wst *et al*. [44] who employed a blend of three different materials (alginate–gelatin–hyaluronic-acid) to establish mechanical stability during and after the printing process. The viscosity of the compound was found to be directly dependent on the concentration of one of the polymers (hyaluronic acid) [39], thus altering the printability of the bioink. Another component (gelatin) contributed through the instantaneous, albeit weak, solidification of the construct once it was printed – owing to its thermal gelation process. The last component (alginate) allowed the construct to be chemically crosslinked, post-printing, to maintain long-term stability [44].

Enzymatic crosslinking of gelatin methacryloyl was studied using a highly specific, nontoxic crosslinker: Ca^2-independent microbial transglutaminase (MTGase). Results showed that MTGase concentration affects the viscosity of the solution [13] and that the addition of the MTGase enzyme also catalyzed the crosslinking in gelatin, thus resulting in an increased crosslinking degree [52], which has been shown to correlate to viscosity as well. Highly viscous bioinks that do not possess shear thinning behaviors can make extrusion impossible. Chemical modifications, such as sulfation of the polymer, can partially degrade the structure, which decreases the molecular weight and thus the viscosity of the solution [53]. Utilizing proportions of low molecular weight polymers also results in a lower viscosity. Such properties can be tuned by blending a higher ratio of high molecular weight polymers [24], increasing the concentration or weakly chemically crosslinking solutions [37], should a higher viscosity be desired.

Functionalization for enhancing biocompatibility and bioactivity

Bioactivity is an important functionality of any printed scaffold because they form the platform for new tissue growth and should be able to simulate an environment similar to the native tissue. Thus, scaffolds must not only be biocompatible but should also encourage cellular function and tissue integration [1], as well as possess adequate porosity to enable nutrient and cell diffusion [54]. Processes such as UV light exposure must be appropriately timed and modified because longer periods decrease cell viability but increase stiffness and decrease the degradation rate [55]; but shorter periods decrease the mechanical integrity of the construct and thus the constructs exhibit less structure for cell attachment and spreading [25].

The chemical crosslinking process has proven to have an effect on water uptake with an increase in crosslinker concentration causing a reduction in swelling [56] and water uptake [28], which could compromise cellular viability and proliferation. The presence of excessive crosslinker could lead to loss of cell adhesion, thus lower concentrations should be used to encourage better cell adhesion and growth [28]. Highly crosslinked densities inhibit the secretion and formation of new tissue, whereas lower crosslinking densities encourage matrix formation [12]. Additionally, ionic crosslinking has proven to be less damaging to cell viability than chemical crosslinking [16] and should be considered as an alternative where possible.

It is important to note the effect that crosslinkers can have on cell viability. Crosslinkers such as ethylene glycol diglycidyl ether (EDGE) and glutaraldehyde, although effective, are known to be highly toxic and the produced scaffolds require intensive detoxifying strategies, whereas crosslinkers such as genipin and citric acid have emerged as significantly more cytocompatible, as well as citric acid being cost-effective, and should be considered [57]. With regard to photocrosslinking, some chemical photogenerators are cytotoxic and to dissolve them can require toxic solvents [58]. A way forward could be to combine chemical and physical crosslinking methods, which could make the crosslinking process more effective, as well as less cytotoxic [57].

Materials can undergo modifications, encouraging bioactivity and one such method involved alginate, which is considered bioinert [59] but, upon modification by introduction of sulfur groups, permitted the binding of growth factors [60] and also encouraged cellular proliferation [61]. Alginate sulfate has also been proven to possess mitogenic activity which exhibits superior cellular proliferation [53]. Another method of encouraging bioactivity is through the removal of materials post-printing. Although alginate is important in the printing process, it is bioinert and its removal post-printing can drastically improve the spreading and proliferation of cells, without compromising the density of the cells [25]. Thermoplastics used in blends, like alginate, often provide valuable support to a material during printing and facilitate the solidification of constructs. However, their removal can also increase cell viability, owing to the creation of a more open and porous network that increases diffusion of nutrients and cells [27].

Various materials can be added to bioinks to induce or promote bioactivity. Fibrin gel is known as the gold standard for enhancement of bioactivity, owing to its peptide sequences [62]. The incorporation of an inorganic phase, such as calcium phosphate, into constructs has been shown to improve bioactivity [63]. Silicon-substituted hydroxyapatite, which can work even at low concentrations, exhibits enhanced bioactivity with improved tissue growth. Nguyen *et al*. [64] observed that cells bioprinted in noncytotoxic nanocellulose-based environments exhibit high cell viabilities. Carboxymethyl chitosan is highly porous with large pore definition, which is considered a factor in providing a conducive cellular environment [41]. Polyethylene oxide and polycation polyethyleneimine encourage cellular attachment, differentiation and proliferation [39].

The addition of 4-arm PEGTA also produces constructs with a useful porous structure that induces increased cell growth and spreading [25]. Hyaluronic acid contributes toward a supportive cellular environment through its anabolic effect on ECM synthesis [18] and ability to improve chondrogenesis, but this effect only works at low concentrations [12]. The addition of mesoporous bioactive glass (MBG) stimulates cellular differentiation, as well as cell proliferation which is directly dependent on the concentration of MBG [50]. Positively charged polylysine (PLL) is widely used to promote cell adhesion via enhancing electrostatic interactions with negatively charged ions of the cell membrane [65].

An interesting modification involves nanostructuring (inducing nanopores) [66] gels. Pluronic® lacks the ability to maintain long-term cell viability [67]. Unmodified Pluronic® was mixed with diacrylated Pluronic® and printed. The subsequent elution of

the unmodified Pluronic® led to a nanostructured construct with a low polymer concentration, thus encouraging bioactivity and long-term cell viability [68]. The molecular weight of bioinks also affects bioactivity. Low molecular weight inks enable better mass transfer, cell migration and proliferation; however, low molecular weight scaffolds lack well-defined features [24]. Therefore, bioinks prepared in a ratio of 2:1 (high molecular weight to low molecular weight) presented with enhanced cell viability and proliferation, without compromising the printability and structure [24].

Concluding remarks

Bioinks exhibit a promising role in tissue engineering and regenerative medicine but the field is still in its infancy and requires a lot more R&D to be fully understood and exploited to its full potential. Despite great strides in technological advances, the development of bioinks remains insufficient and, as yet, there has been no report of a singular bioink that fulfils all of the properties required to be bioprinted for tissue engineering. There is also a dearth of existing materials, and research should be dedicated toward discovering and developing novel bioinks. An alternative is to work with what is currently available by dedicating research toward understanding, improving and optimizing the properties of existing materials, so as to enhance their applicability. The 'ideal' bioink, as mentioned above, exhibits a range of properties such as good printability, structural fidelity and mechanical strength, as well as biocompatibility and bioactivity. It is essential to quantify the properties of biomaterials and compare them to those of the 'ideal' bioink, so that their strengths can be exploited and weaknesses combatted through functionalizations and modifications – many of which have been expanded on in this review.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

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