Annals of Biomedical Engineering, Vol. 44, No. 6, June 2016 (© 2016) pp. 2090–2102
DOI: 10.1007/s10439-016-1638-y

Emerging Trends in Biomaterials Research

Advanced Bioinks for 3D Printing: A Materials Science Perspective

DAVID CHIMENE,[1] KIMBERLY K. LENNOX,[1] ROLAND R. KAUNAS,[1] and AKHILESH K. GAHARWAR[1,2,3]

[1]Department of Biomedical Engineering, Texas A&M University, College Station, TX 77843, USA; [2]Department of Materials Science and Engineering, Texas A&M University, College Station, TX 77843, USA; and [3]Center for Remote Health Technologies and Systems, Texas A&M University, College Station, TX 77843, USA

(*Received 9 March 2016; accepted 3 May 2016; published online 16 May 2016*)

Associate Editor Michael S. Detamore oversaw the review of this article.

Abstract—Advanced bioinks for 3D printing are rationally designed materials intended to improve the functionality of printed scaffolds outside the traditional paradigm of the "*biofabrication window*". While the biofabrication window paradigm necessitates compromise between suitability for fabrication and ability to accommodate encapsulated cells, recent developments in advanced bioinks have resulted in improved designs for a range of biofabrication platforms without this tradeoff. This has resulted in a new generation of bioinks with high print fidelity, shear-thinning characteristics, and crosslinked scaffolds with high mechanical strength, high cytocompatibility, and the ability to modulate cellular functions. In this review, we describe some of the promising strategies being pursued to achieve these goals, including multimaterial, interpenetrating network, nanocomposite, and supramolecular bioinks. We also provide an overview of current and emerging trends in advanced bioink synthesis and biofabrication, and evaluate the potential applications of these novel biomaterials to clinical use.

Keywords—3D printing, Bioinks, Hydrogels, Interpenetrating networks (IPNs), Nanomaterials, Supramolecular.

INTRODUCTION

The recent emergence of 3D printing technology in tissue engineering has resulted in the development of bioprinted scaffolds loaded with cells for engineering complex tissue structures.[44,46,49,59] A vital yet limiting aspect of the design and implementation of a bioprinting is the selection of materials to be used as bioinks.[43] Polymeric hydrogels, highly hydrated three-dimensional polymeric networks, are one of the most viable classes of bioink materials, as they can mimic, augment or replace the native tissue microenvironment and control cell fate.[17,20,30,60,65] In addition, hydrogel networks can also facilitate matrix remodeling, cell migration, and cell–cell adhesions necessary for normal development of a functional tissue. The behavior of cells within these printed matrices is regulated by the physical and chemical properties of the hydrogel networks. In the past decade, biomimetic, responsive, and smart hydrogels that mimic the native microenvironment have been developed.[8,13,17,40]

Significant progress has been made in designing single-component hydrogels for bioprinting applications, but these hydrogels suffer from serious limitations since properties that enhance cell viability and function are at odds with those that facilitate printing.[44,50,61] Cells generally thrive in porous networks with cell binding domains to facilitate cell spreading and proteolytic cleavage sites to allow cell migration. Single component hydrogels are typically optimized for bioprinting by increasing polymer concentration and crosslink density. While these changes improve print fidelity, they are detrimental to encapsulated cells because they reduce porosity and thus prevent cell spreading and migration, and limit nutrient diffusion.[5,15,44,47,48]

Traditional hydrogels for bioprinting and bioprinting techniques have already been covered in recent, well-written reviews.[7,38,52,69] In this review, we will instead highlight four advanced bioink design strategies currently under development—multimaterials, IPNs, nanocomposites, and supermolecular networks. These innovations in advanced hydrogel design provide high print fidelity, cytocompatibility, mechanical strength, and desirable cell-scaffold interactions. We will focus on the materials science aspects of bioink development, provide a critical overview of these

Address correspondence to Akhilesh K. Gaharwar, Department of Biomedical Engineering, Texas A&M University, College Station, TX 77843, USA. Electronic mail: gaharwar@tamu.edu

emerging bioink designs, and evaluate their potential for engineering complex tissue structures. Finally, we will identify promising new research directions in the field of advanced hydrogels for bioprinting applications.

DESIGN PARAMETERS FOR ADVANCED BIOINK DEVELOPMENT

3D bioprinting is a process that uses computer-controlled deposition of biologically relevant materials to create 3D tissue constructs. 3D bioprinting is gaining prominence in tissue engineering because it offers a straightforward method for fabricating 3D constructs containing complex geometric distributions of cell types, materials, and biochemical cues, which makes it a promising tool for the development of functional tissues.[49] Multiple bioprinting strategies, including inkjet, extrusion, stereolithography, and laser induced forward transfer (LIFT), are being pursued with the goal of developing functional tissue constructs.[56,59] Each of these modalities relies on a bioink that contains cells; however, specific bioink requirements vary depending on printing modality. For example, inkjet bioprinting requires low viscosities to avoid clogging and low thermal conductivity to prevent heat damage to the cells. In contrast, extrusion bioprinting can accommodate much higher viscosities but shear thinning materials are often necessary to prevent mechanical damage to the cells.[7,44,49] Despite these considerations, most developments in bioink design originate with extrusion 3D bioprinting, a modality that places very high demands on the rheological properties of bioinks.

In extrusion bioprinting, a bioink filament is continuously extruded through a deposition nozzle.[48,49] A low viscosity is generally desirable during extrusion to avoid excessive fluid shear stress and potential for jamming. Upon deposition, a high viscosity or solidification rate is needed so that the filament retains its shape in order to maintain high print *fidelity*, i.e., the precision of the printed structures. For example, thermoresponsive gelation of gelatin can be employed in bioprinting since it aids in retaining shape of printed constructs.[4,29] However, gelatin has not often been used alone for bioprinting because its reversible sol–gel transition poses difficulties in optimizing printing temperature and viscosity.[66] Similarly, poly(ethylene glycol) (PEG) pre-polymer solution have low viscosity and are too soft to maintain their shape after printing.[21,55] Bioinks have the additional constraint that the cells must remain viable during extrusion and solidification phases.[35,44] Many extrudable hydrogels, such as agarose, maintain their structural integrity through high polymer concentrations.[5] The resulting high viscosity of bioinks is detrimental to cell viability and thus agarose hydrogels are mostly used in 3D printing as sacrificial structures.[44]

The *biofabrication window* is a concept that describes the compromises that have traditionally been made to design bioinks that have suboptimal, yet passable, print fidelity while maintaining cell viability (Fig. 1a). A range of physical, chemical and biological characteristics can influence the application of bioinks for 3D printing applications. These properties include viscosity, shear-thinning, viscoelasticity, cytocompatibility and biocompatibility, gelation kinetics, biodegradation, and hydration degree (Fig. 1b). Low-viscosity bioinks that are cytocompatible can be used if printed with another sacrificial bioink. The rate of gelation, which can rely on conformation changes or crosslinking of polymer network, also affects print fidelity by determining how quickly the bioink can be crosslinked after printing.[44] In addition to the print fidelity and cytocompatibility requirements, bioinks play a significant role in controlling cell functions including adhesion, migration, proliferation and differentiation. Cell–matrix interactions facilitate matrix remodeling and extracellular matrix (ECM) synthesis,[25] which are important characteristics of bioactive bioinks. Some natural polymers such as gelatin and fibrin are intrinsically bioactive and contain cell attachment molecules.[64] Synthetic hydrogels have also been functionalized with arginyl-glycyl-aspartic acid (RGD) to integrate bioactivity.[65] Another approach to integrate bioactive characteristics is to incorporate therapeutic drugs and biologics (e.g., bone morphogenetic proteins, fibroblast growth factors).[13] These therapeutic biomolecules can be physically or chemically incorporated within the polymer network. Nanomaterials offer new approaches of controlling the release kinetics of these biomolecules and increases therapeutic efficacy and reduces doses.[8,20,36] Beyond biochemistry cues, biophysical cues from bioinks can also influence cell fate. ECM stiffness has been shown to direct cell differentiation, with stiffer ECMs directing stem cells toward osteogenic lineage, while softer ECMs promote chondrogenic lineage.[16,25] The stiffness of hydrogel network can be modulated by employing interpenetrating networks (IPNs) or reinforcing with nanoparticles.[26,32,33]

Another important consideration for bioink development is the ability of the hydrogel network to respond to cell-mediated matrix remodeling.[60] Biodegradation of bioinks can occur enzymatically (e.g., natural polymers such as collagen or gelatin), hydrolytically (e.g., synthetic polymers such as polyesters) and through ion exchange (e.g., alginate and carrageenans).[2,23] The degradation kinetics of bioinks can

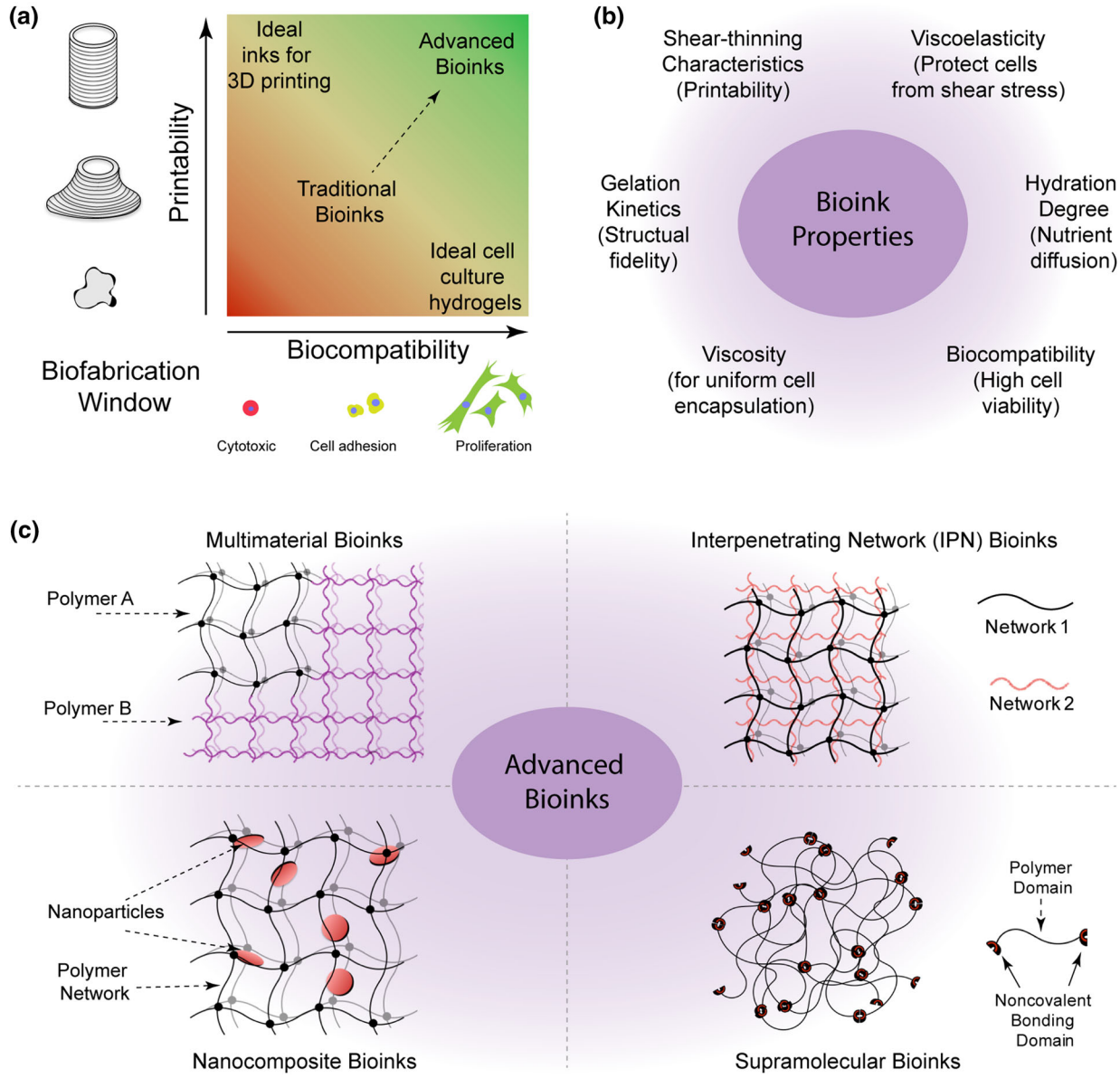

FIGURE 1. Advanced bioinks for 3D printing. (a) Biofabrication window for rational design of bioinks requires compromise between printability and biocompatibility. (b) Ideal bioink characteristics require interplay between different materials properties. (c) Advanced bioinks can be classified into four major categories.

modulate ECM production and remodeling.[23] Interplay between these parameters should be carefully considered in the design and development of bioink compositions.

Advanced bioinks (Fig. 1c) use multiple strategies to improve printability and cytocompatibility. For example, bioinks designed with shear thinning properties have lower viscosities at the high shear rates generated during extrusion. After extrusion, viscosity increases result in high print fidelity and cell viability. Interpenetrating network, nanocomposite, multimaterial, and supramolecular hydrogels can all exhibit shear thinning characteristics. Functional groups can also be added to accelerate solidification upon exposure to UV irradiation. Further, functional groups and nanoparticles can provide bioactive properties to the bioink to direct cell function.

MULTIMATERIAL BIOINKS FOR 3D PRINTING

Multimaterial hydrogels are the most widely investigated bioinks to overcome the limitations of single component hydrogels.[34] For example, alginate has been used as a single component hydrogel in tissue engineering because it is biocompatible and can be

ionically crosslinked using calcium ions to obtain mechanically robust hydrogels.[57] Biocompatibility is defined as the ability of a material to be implanted *in vivo* without causing deleterious local or systemic reactions.[30] Unfortunately, alginate is largely bioinert, meaning that it does not interacts with cells. For example, cells cannot remodel or adhere to alginate matrix. The lack of cell adhesion moieties on the alginate backbone can induce apoptosis of the encapsulated cells *via* anoikis.[1,5,31] To overcome these limitations, Chung *et al*. incorporated gelatin to increase the viscosity and cytocompatibility of alginate bioink.[12] Gelatin is denatured collagen, which is capable of reversible thermal gelation and has cell adhesive arginyl-glycyl-aspartic acid (RGD) domains. Addition of gelatin increased the viscosity of the alginate and significantly increased the storage modulus when the composite was cooled below the gelation temperature of gelatin, resulting in improved print fidelity. The compression modulus of alginate and alginate-gelatin hydrogels were similar after ionic crosslinking of the alginate. However, due to ionic crosslinking, the bioprinted structure lost its mechanical integrity after 3–4 days. Thus, while the com posite improved printing performance, the long-term mechanical properties remained sub-optimal.[12]

Covalent crosslinking is an effective method for improving the physiological stability of printed structures. Kesti *et al*. developed a dual crosslinked bioink consisting of methacrylated hyaluronan (HA-MA) and thermoresponsive polymer poly(*N*-isopropylacrylamide) (pNIPAAM) grafted hyaluronan (HA-pNIPAAM) for enhanced mechanical integrity.[37] HA-MA is a promising bioactive hydrogel for tissue engineering and can be covalently crosslinked after UV exposure, but is not suitable alone for printing because of its low viscosity. They first conjugated pNIPAAM to HA-MA to obtain a quickly gelling thermoresponsive component as a temporary support, making it suitable for 3D bioprinting. The thermoresponsive nature of the HA-pNIPAAM component provides rapid gelation and post-printing structural fidelity. This bioink was able to print strands down to 620 μm wide and 200 μm in height from a 300 μm needle. After the HA-MA had been crosslinked, the HA-pNIPAAM was rinsed away, leaving only an intact HA-MA scaffold. The presence of only HA-MA significantly increased the viability of encapsulated cells. This strategy may have the potential to be generalized to other hydrogels to improve the pre-crosslinking storage modulus.[37]

In a similar experiment, Duan *et al*. developed bioink from HA-MA and gelatin methacrylate (GelMA) to print 3D trileaflet heart valves.[14] GelMA was incorporated to improve cell adhesion characteristics of the composite network. HA-MA increased bioink viscosity and the resulting hydrogel stiffness, while GelMA also enhanced viscosity and maintenance of a fibroblastic phenotype of encapsulated human aortic valve interstitial cells (HAVIC). Seven days after printing, the encapsulated HAVIC showed enhanced production of collagen and glycosaminoglycan, indicating ECM remodeling. This development is particularly important because previous synthetic scaf folds with much higher stiffness than natural heart valves showed limited remodeling.

To improve the hydrogel tunability, Rutz *et al*. designed a bioink from GelMA and multifunctional PEG crosslinkers (PEGX) (Fig. 2a).[58] They used long PEGX crosslinkers to loosely connect the gelatin backbone to provide the necessary viscosity for bioprinting applications with low gelatin concentrations. The increased viscosity of the bioink resulted in high structural fidelity without directly crosslinking gelatin polymers to each other. PEGX crosslinking allowed properties like viscosity and biodegradability to be tuned without compromising cytocompatibility. Taken together, these multimaterial hydrogels are a facile and effective approach for obtaining desirable bioink characteristics without compromising cell viability.

Recently, printing strategies have begun to use multiple bioinks to fabricate large and complex constructs. Kang *et al*. used a multi-head printer to print a complex interwoven scaffold consisting of hydrogel bioinks and polycaprolactone (PCL) and Pluronic F-127 (Fig. 2b).[35] PCL was selected for its biocompatibility and relatively low melting temperature (~60°C), while Pluronic F-127 was selected as a sacrificial material due to its thermosensitive characteristics. The bioinks were synthesized using gelatin, fibrinogen, hyaluronic acid (HA), and glycerol. Fibrinogen was used to provide cell adhesion properties, while gelatin was used to improve print fidelity. HA and glycerol acted as plasticizers. Interweaving these materials resulted in a support structure for the mechanically weak bioink component. After printing, the fibrinogen was crosslinked using thrombin and the sacrificial components (Pluronic F-127, gelatin, HA, and glycerol) were rinsed away.

Finally, some recent strategies seek to alter bioink printability by extruding into hydrogel support baths. Hinton *et al*. developed a bioink containing collagen, Matrigel, fibrinogen, and hyaluronic acid.[29] They used this multimaterial bioinks to build complex structures by embedding the printed structure within a secondary "sacrificial" hydrogels (gelatin slurry). After printing the structure, the gelatin support bath was removed by heating the bath to physiological temperature. Models of complex structures such as a human right coronary arterial tree and explanted an embryonic chick heart

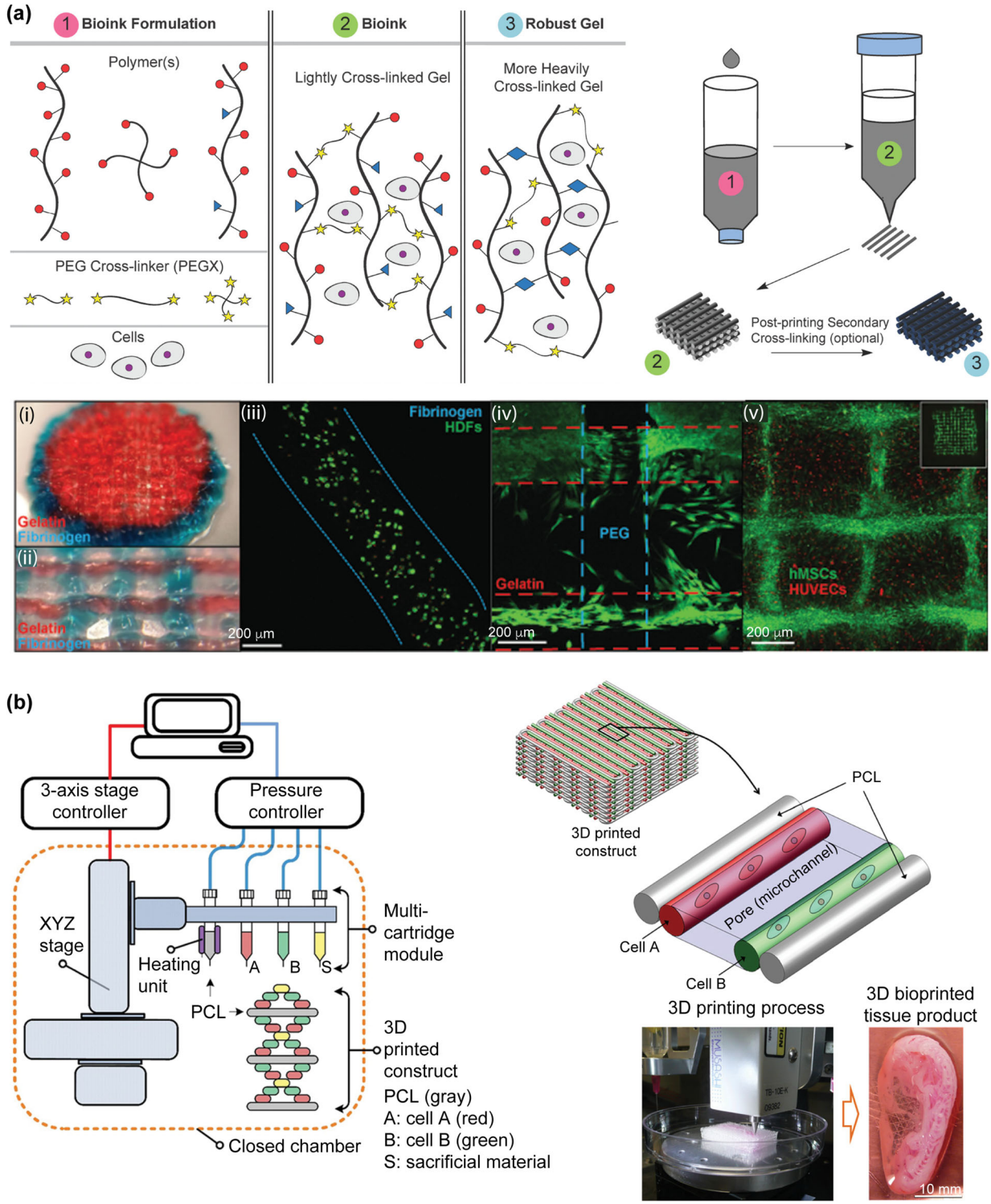

FIGURE 2. Multimaterial bioinks for 3D bioprinting. (a) Bioinks consist of GelMA and PEG crosslinker. The length of PEG crosslinker can be modulated to control the mechanical properties of printed structures. Cells can be incorporated within the bioink prior to printing. 3D printed structures show high cell viability and support cell proliferation. (b) Multi-head printer used to print a complex interwoven scaffold consisting of hydrogel bioinks, polycaprolactone(PCL) and Pluronic F-127. Adapted and reproduced by permission from Wiley[58]© 2015 and Nature American Inc.[35]© 2016.

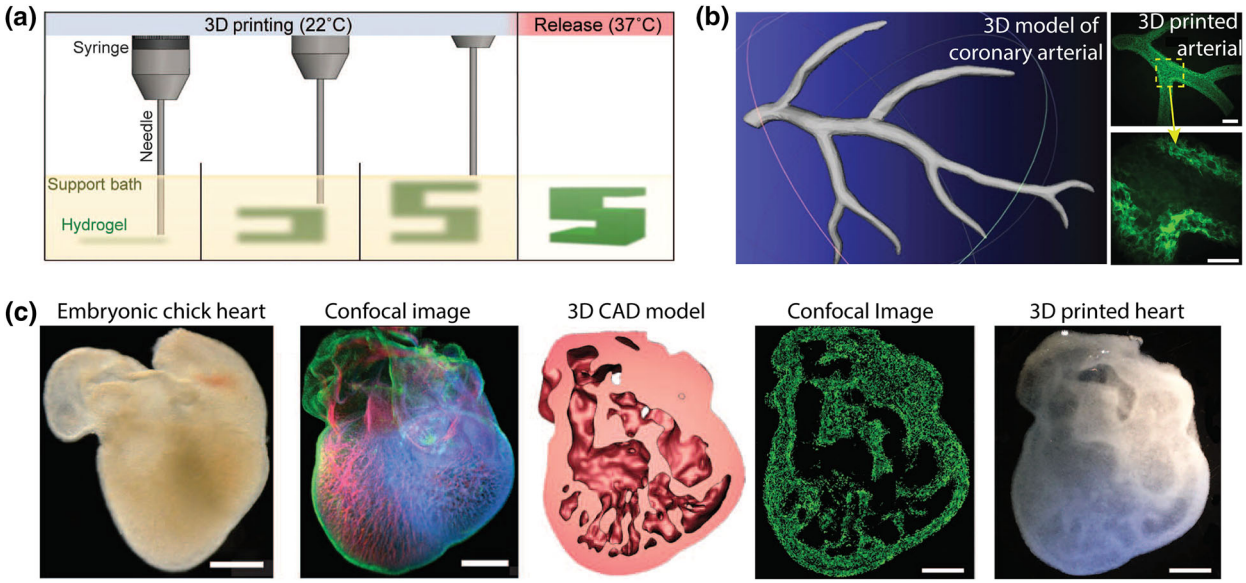

FIGURE 3. Multimaterial bioinks for 3D bioprinting using a sacrificial support bath. (a) 3D printing of a multimaterial bioink within a thermoreversible support bath. A range of complex tissue structures such as (b) a human right coronary arterial tree and (c) an explanted embryonic chick heart can be printed using computer models (Scale bar = 1 mm). Adapted and reproduced by permission from American Association for the Advancement of Science[29]© 2015.

can be printed with high structural fidelity (Fig. 3). In another approach, Bhattacharjee *et al.* used a similar method to print thin rings of fluorescently labeled endothelial cells into a Carbopol granular gel medium.[6] This printing process relies on the bingham plastic and thixotropic flow of the support materials. Although cell-containing bioinks were only printed into simple flat structures, the studies demonstrated that this technique can also be used to achieve very high fidelity with biocompatible materials like alginate. This technique represents a promising new approach to improving the printability of bioinks without sacrificing biocompatibility.

INTERPENETRATING NETWORKS BIOINKS FOR 3D PRINTING

Interpenetrating Networks (IPNs) are composite hydrogels in which each polymer network has limited interactions with the other.[3,9,26,39,62] Unlike multimaterial hydrogels, where different constituent polymers may be crosslinked together, IPNs are composed of separate polymer networks that are physically entangled within each other. The IPNs are often crosslinked using different chemistries to encourage each polymer network to only crosslink with itself.[9] Limited unintentional inter-network crosslinking may occur depend ing on the type of polymers and crosslinking reactions used, which is believed to be significant in semi-IPNs where only one of the polymer networks has been crosslinked.[3,9,26,39,62] IPNs have been shown to have enhanced toughness and fracture strength relative to the single component networks of either of its constituent polymers. Generally, the primary network is composed of a flexible & elastic polymer, while the secondary network consists of a high-stiffness, brittle polymer at much lower concentration.

Double network (DN) hydrogels are a subset of IPNs that have been synthesized through a two-step polymerization. In the first step of traditional DN network preparation, polymer chains are covalent crosslinked to obtain the primary hydrogel network. The secondary polymer monomers are then dispersed throughout the primary network to be later crosslinked to obtain the DN hydrogel. Although this method has been widely used for DN hydrogel formation, it is too slow to be suitable for 3D bioprinting. Ionic-covalent entanglement (ICE) gels are now being developed that are both physically and chemically crosslinked and form at a sufficient rate to facilitate their use in 3D printing.[9,10]

Recently, Bakarich *et al.* demonstrated the use of ICE hydrogels containing acrylamide and alginate for bioprinting.[3] The acrylamide solution loaded with alginate maintained the printed shape and allowed formation of a covalently crosslinked acrylamide network that was then physically crosslinked with calcium chloride solution. The physical crosslinking of the gel was shown to restrict hydrogel swelling in water and

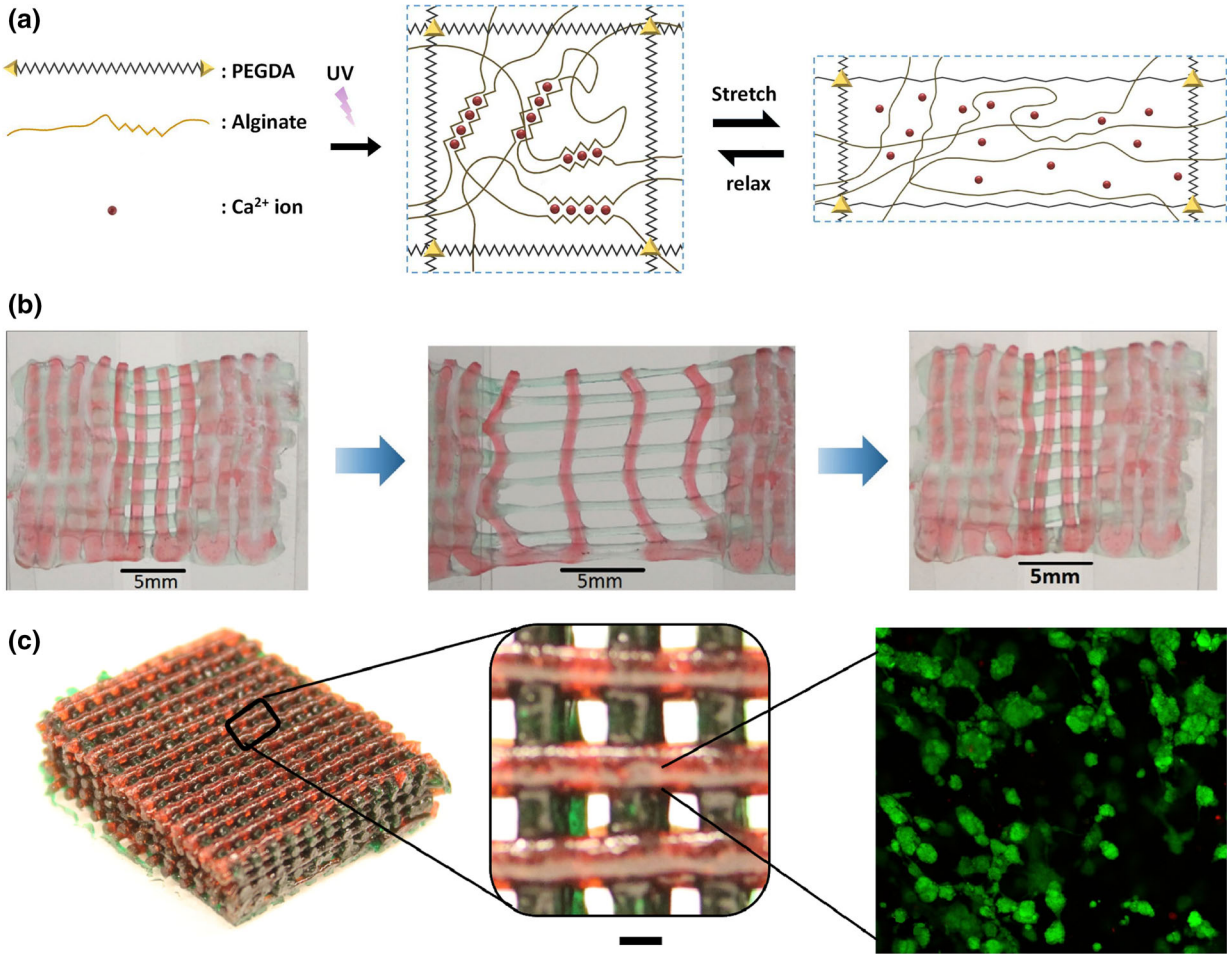

FIGURE 4. Interpenetrating network (IPN) bioinks for 3D printing. (a) IPNs were synthesized by covalently crosslinking PEG and ionically crosslinking alginate. (b) A mesh printed with the tough and biocompatible hydrogel can be subjected cyclic mechanical deformation. (c) Encapsulated cells show high cell viability. Adapted and reproduced by permission from Wiley[31] © 2015.

increased both stiffness and failure stress by roughly an order of magnitude: from 23 to 260 kPa, and from 11 to 130 kPa, respectively. Additionally, soaking in calcium chloride increased strain at failure for the gels from ~23 to 90%. This experiment demonstrated the utility of ICE bioinks as well as the improved mechanical properties that can be achieved with the addition of a biocompatible secondary network to create IPNs.[3]

In another study, Hong *et al.* fabricated elastomeric ICE hydrogels from poly(ethylene glycol) diacrylate (PEGDA) and alginate (Fig. 4).[31] The addition of calcium ions to ICE hydrogels increased fracture strength from ~200 J/m^2 to over 1500 J/m^2, comparable to native cartilage. Moreover, the hydrogel networks were able to sustain mechanical stress without significant plastic deformation. This behavior was mainly attributed to the elastomeric characteristics of the PEGDA network and the reversible crosslinking of the alginate that can reconfigure during deformation. The encapsulated cells within these ICE hydrogels showed high cell viability (75.5 ± 11.6%) over a period of 7 days. IPNs could be further modified with nanomaterials, as discussed in the following section, to obtain high print fidelity. Overall, this study showed that ICE hydrogels can be used to fabricate mechanically tough 3D-printed structures for regenerative engineering.

NANOCOMPOSITE BIOINKS FOR 3D PRINTING

Nanoengineered hydrogels have been investigated for a range of biomedical and biotechnology applications.[8,20,24,54] Small amounts of nanoparticles added to polymeric hydrogels can result in significant alterations in various physical and chemical characteristics including increased stiffness, shear-thinning characteristics,

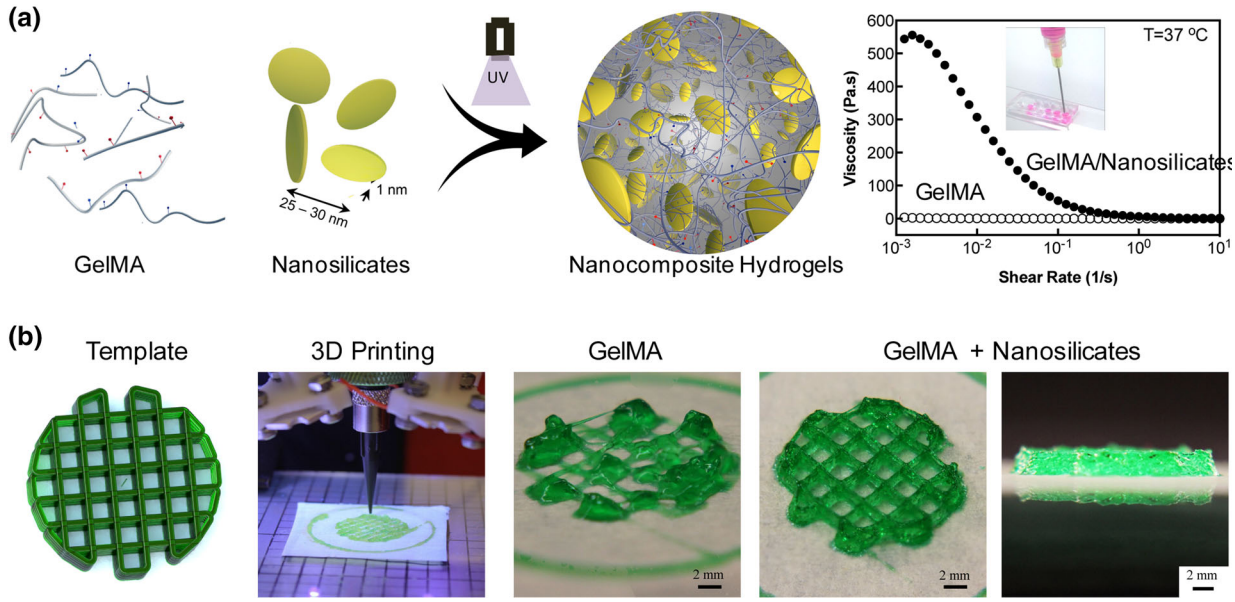

FIGURE 5. Nanoengineered bioinks for 3D printing. (a) Shear-thinning hydrogels were prepared by combining synthetic nanosilicates with gelatin methacrylate (GelMA). (b) The addition of nanosilicates to GelMA results in high print fidelity and structural stability. After UV crosslinking, printed hydrogels showed high physiological stability. Adapted and reproduced by permission from American Chemical Society[66]© 2015.

and resistance to degradation under physiological conditions.[33,36,63] Depending on the type of nanoparticles used to reinforce the hydrogel network, unique properties such as bioactivity, controlled drug release, electrical conductivity, photoresponsiveness, and magnetism can be incorporated.[20] Although several nanocomposite hydrogels have been developed for tissue engineering applications, very few studies have investigated their potential for 3D bioprinting.[18,20,51]

In a recent study, Gao *et al*. explored the bioactive potential of hydroxyapatite nanoparticles (nHAp) for bone tissue engineering by combining poly(ethylene glycol) dimethacrylate (PEGDMA) with nHAp (~200 nm) and/or bioactive glass (BG) (~20 μm) for 3D printing.[22] Although the addition of nHAp to PEGDMA increases mechanical strength, their effects on shear-thinning characteristics and print fidelity were not measured. Human mesenchymal stem cells (hMSCs) were printed with bioinks consisting of nHAp and BG using layer-by-layer assembly, resulting in uniform cell distribution within the hydrogel and high cell viability (>80%). The addition of nHAp resulted in significant increases in ECM deposition and upregulation of bone-related gene expression (collagen I, osteocalcin, collagen X, and MMP13) compared to PEGDMA scaffold alone. This study demonstrates that addition of nHAp to PEG bioinks increases compressive modulus and promotes osteogenic differentiation of hMSCs.

In another approach, a shear-thinning bioink was developed by combining nanofibrillated cellulose with alginate for printing soft tissue structures.[45] They showed that the designed bioinks can be printed with high structural fidelity at room temperature. Anatomically shaped models of cartilage tissues such as meniscus and ear can be printed with high structural fidelity using MRI and CT images. The printed structures were ionically crosslinked by exposure to cations, and their mechanical stiffness could be modulated by alginate concentration. As a proof-of-concept, they showed that human chondrocytes can be encapsulated within the bioink and printed with high cell viability (>70%). In the future, it would be interesting to investigate the effect of nanofibrillated cellulose on the mechanical characteristics of bioinks and production of cartilage ECM.

Other types of nanoparticles, such as synthetic silicate clays, are extensively used for bioprinting applications. These clays are 2D coin-shaped nanomaterials characterized by a high surface-to-volume ratio and an unusual charge distribution (negatively charged flat surfaces and positively charged edges).[11] These characteristics result in strong, reversible, non-covalent interactions with both natural and synthetic polymers and overall shear-thinning mechanical properties. Shear-thinning is an important characteristic for bioprinting applications as high fidelity structures can be obtained through reversible changes in the shear viscosity of bioinks. For example, Xavier *et al*. synthesized bioactive bioinks using these synthetic nanosilicates and GelMA (Fig. 5).[66] While GelMA hydrogels provide cell adhesion sites, their poor

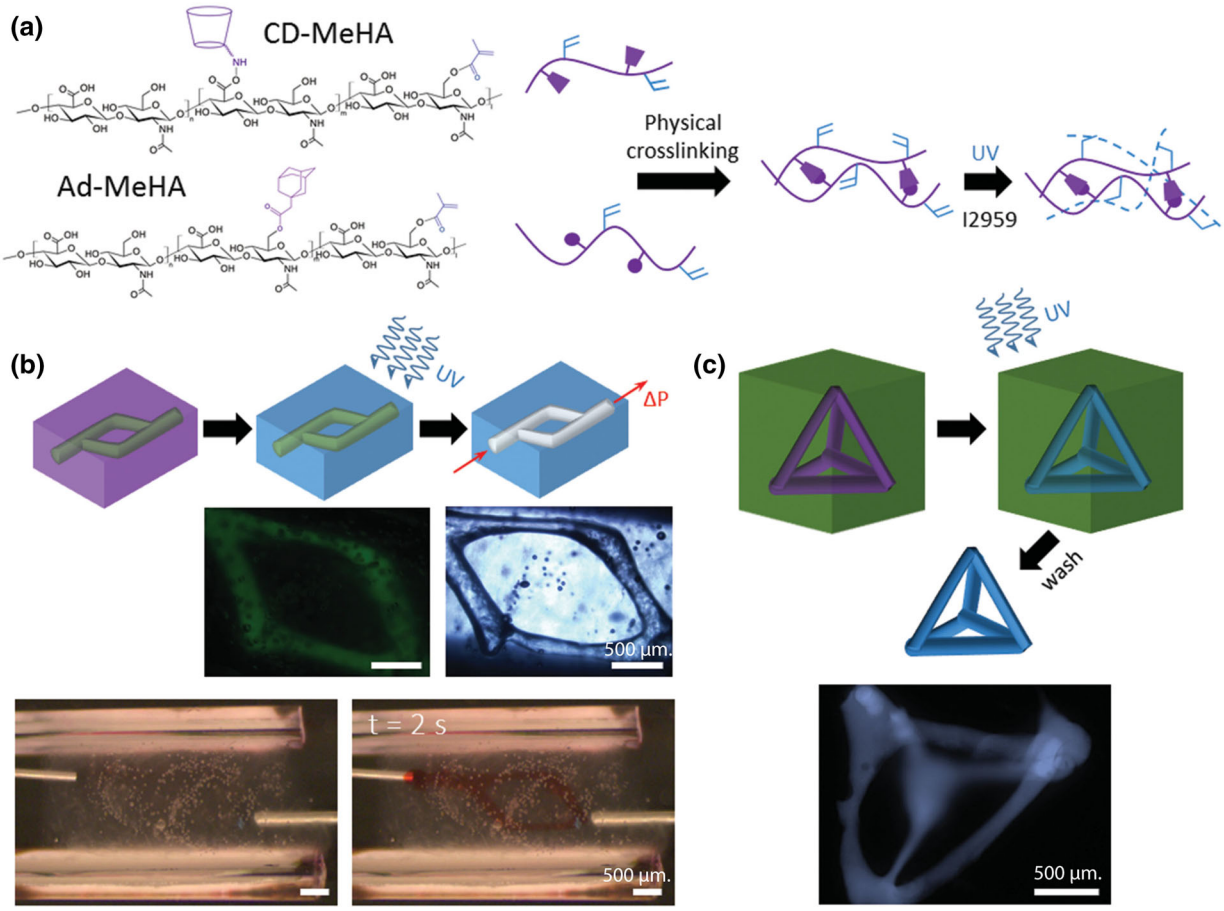

FIGURE 6. Supramolecular bioinks for extrusion-based 3D bioprinting applications. (a) Hydrogels were fabricated by combining CD-MeHA with Ad-MeHA to obtain physically crosslinked bioinks. (b) After exposing the printed structure to UV light, covalently crosslinked supramolecular hydrogels were obtained. (c) The printed structure shows high mechanical integrity and can be used to print complex structures. Adapted and reproduced by permission from Wiley[28]© 2015.

mechanical strength limits their utility as a bioink. The addition of nanosilicates to GelMA increased viscosity at low shear rates, while it exhibited similar viscosity to pure GelMA at high shear rates, which facilitated the printing of complex shapes with high shape fidelity. Hong *et al.* showed similar behavior upon incorporation of silicate nanoparticles to ICE hydrogels (PEGDA/alginate).[31] Xavier *et al.* also showed that addition of nanosilicates to GelMA promotes osteoblast differentiation and induces production of mineralized ECM.[66] Taken together, this research exemplifies the potential of new nanocomposite bioinks for both enhancing the 3D printing process and providing bioactive cues to encapsulated cells.

SUPRAMOLECULAR BIOINKS FOR 3D PRINTING

Hydrogels for tissue engineering applications should be mechanically tough and capable of surviving repeated mechanical deformation. When subjected to repeated stress, bonds in conventional hydrogels can break, resulting in progressive loss of mechanical integrity. To overcome this drawback, supramolecular bioinks are currently under investigation.[27,68] Supramolecular polymers are composed of short repeating units with functional groups that can interact non-covalently with other functional units, forming large, polymer-like entanglements. Under high stress, these non-covalent bonds are reversibly broken to dissipate energy. The reversibility of these bonds also leads to shear-thinning properties that facilitate their use in bioprinting.

In a recent study, Highley *et al.* described a straightforward method for fabricating shear-thinning and mechanically resilient hydrogels for 3D bioprinting applications using a cytocompatible hyaluronic acid (HA)-based supramolecular hydrogel (Fig. 6).[28] HA was modified with either adamantane or β-cyclodextrin functional groups that can interact with each other through guest–host interactions and can

rapidly form a supramolecular polymer. The reversible nature of the non-covalent bonds in the hydrogel caused the gel to exhibit low viscosity under mechanical deformation (or at high strain) and recovery of mechanical integrity after cessation of stress. The rapid increase in viscosity after strain cessation prevents the bioink from continuing to flow after printing, resulting in high structural fidelity and integrity. The bioink was shown to be highly cytocompatible (>80% cell viability). HA macromers were also ~25% methacrylated in order to allow for UV crosslinking after 3D printing, as the supramolecular bonds themselves lacked the mechanical strength for long-term stability.

DNA hybridization represents another approach to fabricating supramolecular hydrogels. Li *et al.* developed supramolecular polypeptide–DNA hydrogel for rapid *in situ* 3D bioprinting by designing two bioinks—one containing a polypeptide–DNA conjugate and the other containing the complementary DNA linker (Fig. 7).[37] DNA hybridization between the complementary DNA molecules led to rapid crosslinking and gelation within one second. The rigidity of DNA polymers allows for the printing of structures on the millimeter scale with high structural integrity. These scaffolds were shown to have high cytocompatibility and could be selectively biodegraded using either proteases or nucleases.

EMERGING TRENDS AND FUTURE OUTLOOK

The current bottleneck in designing complex tissue structures using 3D printing is the limited availability of versatile bioinks. While multimaterial bioinks have been the most extensively explored solution, many promising combinations of innovative polymers have yet to be evaluated. For example, PEG hydrogels functionalized with RGD or other binding moieties can provide cell adhesion to otherwise bioinert hydrogels.[64] Combinatorial screening of biomaterials using high-throughput techniques such as 3D biomaterial microarrays might provide optimum hydrogel combinations to support and direct cell fate.[18] Combinations of different strategies described in this review can lead to development of the next generation of bioinks. For example, mechanical improvement of multicomponent polymeric bioinks can be obtained by incorporation of shear-thinning nanoparticles.[19] Incorporation of bioactive components such as growth factor-loaded nanoparticles or microparticles within polymeric network will provide additional tools to control cell fate.[36]

We have described some minimal criteria for developing advanced bioink formulations. They should be able to print complex, high-resolution tissue structures such as vascularized tissues and biomimetic architectures. The printed structure should have high structural fidelity and should facilitate a cell-initiated remodeling process. The bioink should be able to modulate cell phenotype within the printed structure. We can expect to see an increased number of strategies to better meet these requirements, especially the development of new bioresponsive inks to control and direct cellular process.[3] Breakthroughs in related fields such as polymer chemistry, nanomaterials, stem cell technology, and 3D printing equipment will certainly facilitate the development of hydrogel bioink technologies in unexpected ways. For example, current research into nanomaterials is primarily in the fundamental research stage, with relatively few studies applying this technology to biomedical engineering.[11] While current bioink research has so far been confined primarily to traditional polymeric hydrogels, promising categories of nanoparticles including stimuli-responsive nanomaterials and two-dimensional nano materials have the promise to add additional functionalities to bioinks.[11]

The combination of new strategies to control stem cell differentiation is expected to play a prominent role in designing advanced bioinks. Bioinks with sustained release of growth factors will not only provide favorable conditions for directed cell fate, but local release will also reduce the amount of growth factor required. The use of controlled and stimuli-responsive release of immunomodulators and growth factors has the potential to add another level of control of the bioactivity of bioinks.[11,67] In addition, stem cell fate can also be directed towards different lineages using mechanical cues. Cells respond to cyclic strain on GelMA nanocomposites by aligning to an extent dependent on stiffness of the hydrogel.[34] While the role of mechanical cues in tissue engineering fields are relatively well established,[25,66] they have not been applied to 3D printed constructs. In the near future, we can expect to see incorporation of these modalities within 3D printing.

Bioprinters with multiple printing heads are also needed to rapidly produce complex, heterogeneous tissue structures.[35] 3D bioprinters with multiple heads have the ability to deposit multiple formulations simultaneously to fabricate complex and biomimetic tissue structures including vascularized tissue. Multi-head 3D printers with the ability to control different types of bioink to be printed will be crucial. Different types of bioinks are needed to design intricate geometries, as the ultimate goal of bioinking is to obtain biomimetic tissue structures. Different bioink formulations with a range of mechanical characteristics, gelation mechanisms and bioactivities need to be developed.[53] Multi-head printers would provide greater control over the spatial distribution of biochemical cues. Recently, studies have explored the use of multiple polymeric bioinks for printing complex

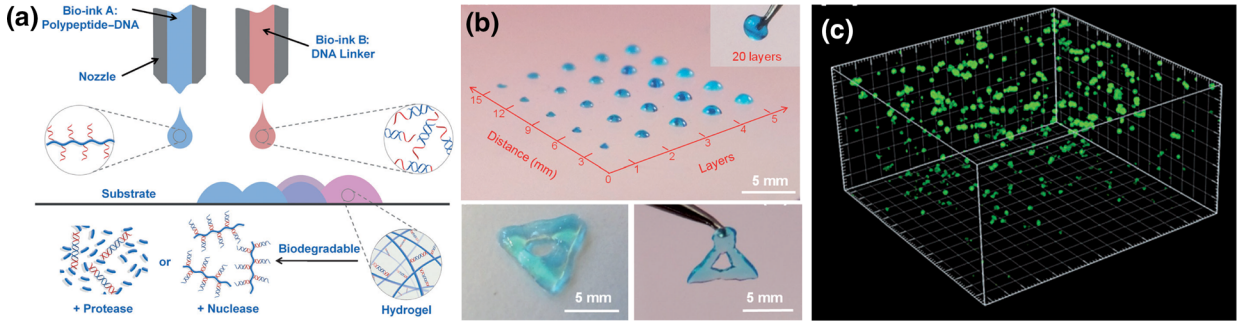

FIGURE 7. 3D bioprinting of supramolecular bioinks. (a) Polypeptide–DNA hydrogels were synthesized by using two bioinks [Bioink A (blue): polypeptide–DNA, and Bioink B (red): DNA linker]. Hybridization of these two bioinks result in crosslinking, leading to hydrogel formation. (b) Hydrogels with different sizes and complex structures can be obtained. (c) Encapsulated cells showed high viability. Adapted and reproduced by permission from Wiley[37] © 2015.

tissue structures.[41,42] In the future we expect to see a significant development in advanced bioink compositions for printing complex structures that are currently not feasible due to technological limitations.

CONCLUSION

3D bioprinting is a promising solution to some of the most daunting obstacles facing the field of tissue engineering, including vascularization of tissue constructs, creation of complex architectures, and directing stem cell differentiation. However, the lack of suitable bioinks has emerged as one of the most significant obstacles to the advancement of 3D bioprinting research. Traditional single component hydrogels have lacked one or more of the characteristics desired in a bioink, including high structural fidelity and printability, high mechanical strength post-printing, and bioactivity and biodegradability. Attempts to rectify mechanical and rheological shortcomings of bioinks through increased polymer and crosslink density tend to reduce the cytocompatibility of single component bioinks. Recent developments in advanced bioinks avoid these tradeoffs without sacrificing cell viability.

Multimaterial hydrogels are gaining popularity as a facile and effective method for obtaining desirable bioink characteristics. Due to their high viscosities and shear-thinning characteristics, multimaterial bioinks have desirable printability and high structural fidelity. Meanwhile, IPN bioinks have been shown to combine the physical and chemical characteristics of multiple polymeric hydrogels into a single hydrogel. This is particularly evident in IPN mechanical properties, which combine the stiffness of the ionically crosslinked networks with the elasticity and strain recovery characteristics of covalently crosslinked networks. A range of IPNs have been formulated that have higher stiffness at lower polymer concentrations than single component hydrogels, resulting in a useful blend of stiffness and cytocompatibility that is likely to continue to be explored for bioinks development. Nanocomposite hydrogels provide a facile method to combine multiple functionalities within 3D printed structures by incorporating nanoparticles with unique characteristics. These nanoparticles physically and chemically interact with polymer chains to result in shear-thinning, and sometimes even bioactive, hydrogels, which are highly desirable characteristics for bioprinting applications. Finally, supramolecular hydrogels have favorable shear-thinning characteristics and offer high shape fidelity for millimeter-sized structures. However, these bioinks have limited mechanical strength, which will need to be improved to make this a viable strategy for bioprinting. Despite these drawbacks, supramolecular hydrogels are promising candidates for bioink applications due to their ability to create reversible non-covalent bonds. Overall, advances in bioink design promise to bring 3D bioprinting and tissue engineering closer to clinical applications in treating a wide range of tissue ailments, from lower hanging fruit, such as arthritis and burn treatments, towards eventual complex organ replacement.

ACKNOWLEDGMENTS

This research was supported by the National Science Foundation Award No. HRD-1406755 and CBET-1264848 and NIH R01 AR066033-01.

REFERENCES

[1]Augst, A. D., H. J. Kong, and D. J. Mooney. Alginate hydrogels as biomaterials. *Macromol. Biosci*. 6:623–633, 2006.

[2]Azagarsamy, M. A., and K. S. Anseth. Bioorthogonal click chemistry: an indispensable tool to create multifaceted cell culture scaffolds. *ACS Macro Lett*. 2:5–9, 2012.

[3]Bakarich, S. E., R. Gorkin, M. I. H. Panhuis, and G. M. Spinks. 4D printing with mechanically robust, thermally actuating hydrogels. *Macromol. Rapid Commun*. 36:1211–1217, 2015.

[4]Bertassoni, L. E., J. C. Cardoso, V. Manoharan, A. L. Cristino, N. S. Bhise, W. A. Araujo, P. Zorlutuna, N. E. Vrana, A. M. Ghaemmaghami, and M. R. Dokmeci. Direct-write bioprinting of cell-laden methacrylated gelatin hydrogels. *Biofabrication* 6:024105, 2014.

[5]Bertassoni, L. E., M. Cecconi, V. Manoharan, M. Nikkhah, J. Hjortnaes, A. L. Cristino, G. Barabaschi, D. Demarchi, M. R. Dokmeci, and Y. Yang. Hydrogel bioprinted microchannel networks for vascularization of tissue engineering constructs. *Lab Chip* 14:2202–2211, 2014.

[6]Bhattacharjee, T., S. M. Zehnder, K. G. Rowe, S. Jain, R. M. Nixon, W. G. Sawyer, and T. E. Angelini. Writing in the granular gel medium. *Sci. Adv*. 1:e1500655, 2015.

[7]Billiet, T., M. Vandenhaute, J. Schelfhout, S. Van Vlierberghe, and P. Dubruel. A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering. *Biomaterials* 33:6020–6041, 2012.

[8]Carrow, J. K., and A. K. Gaharwar. Bioinspired polymeric nanocomposites for regenerative medicine. *Macromol. Chem. Phys*. 216:248–264, 2015.

[9]Chen, Q., H. Chen, L. Zhu, and J. Zheng. Fundamentals of double network hydrogels. *J Mater Chem B* 3:3654–3676, 2015.

[10]Chen, Q., L. Zhu, L. Huang, H. Chen, K. Xu, Y. Tan, P. Wang, and J. Zheng. Fracture of the physically cross-linked first network in hybrid double network hydrogels. *Macromolecules* 47:2140–2148, 2014.

[11]Chimene, D., D. L. Alge, and A. K. Gaharwar. Two-dimensional nanomaterials for biomedical applications: emerging trends and future prospects. *Adv. Mater*. 27:7261–7284, 2015.

[12]Chung, J. H., S. Naficy, Z. Yue, R. Kapsa, A. Quigley, S. E. Moulton, and G. G. Wallace. Bio-ink properties and printability for extrusion printing living cells. *Biomater. Sci*. 1:763–773, 2013.

[13]Discher, D. E., D. J. Mooney, and P. W. Zandstra. Growth factors, matrices, and forces combine and control stem cells. *Science* 324:1673–1677, 2009.

[14]Duan, B., E. Kapetanovic, L. A. Hockaday, and J. T. Butcher. Three-dimensional printed trileaflet valve conduits using biological hydrogels and human valve interstitial cells. *Acta Biomater*. 10:1836–1846, 2014.

[15]Durmus, N. G., S. Tasoglu, and U. Demirci. Bioprinting: functional droplet networks. *Nat. Mater*. 12:478–479, 2013.

[16]Engler, A. J., S. Sen, H. L. Sweeney, and D. E. Discher. Matrix elasticity directs stem cell lineage specification. *Cell* 126:677–689, 2006.

[17]Fisher, O. Z., A. Khademhosseini, R. Langer, and N. A. Peppas. Bioinspired materials for controlling stem cell fate. *Acc. Chem. Res*. 43:419–428, 2010.

[18]Gaharwar, A. K., A. Arpanaei, T. L. Andresen, and A. Dolatshahi-Pirouz. 3D biomaterial microarrays for regenerative medicine: current state-of-the-art, emerging directions and future trends. *Adv. Mater*. 28:771–781, 2016.

[19]Gaharwar, A. K., R. K. Avery, A. Assmann, A. Paul, G. H. McKinley, A. Khademhosseini, and B. D. Olsen. Shear-thinning nanocomposite hydrogels for the treatment of hemorrhage. *ACS Nano* 8:9833–9842, 2014.

[20]Gaharwar, A. K., N. A. Peppas, and A. Khademhosseini. Nanocomposite hydrogels for biomedical applications. *Biotechnol. Bioeng*. 111:441–453, 2014.

[21]Gaharwar, A. K., C. P. Rivera, C.-J. Wu, and G. Schmidt. Transparent, elastomeric and tough hydrogels from poly (ethylene glycol) and silicate nanoparticles. *Acta Biomater*. 7:4139–4148, 2011.

[22]Gao, G., A. F. Schilling, T. Yonezawa, J. Wang, G. Dai, and X. Cui. Bioactive nanoparticles stimulate bone tissue formation in bioprinted three-dimensional scaffold and human mesenchymal stem cells. *Biotechnol. J*. 9:1304–1311, 2014.

[23]Gasperini, L., J. F. Mano, and R. L. Reis. Natural polymers for the microencapsulation of cells. *J. R. Soc. Interface* 11:20140817, 2014.

[24]Goenka, S., V. Sant, and S. Sant. Graphene-based nanomaterials for drug delivery and tissue engineering. *J. Control. Release* 173:75–88, 2014.

[25]Guilak, F., D. M. Cohen, B. T. Estes, J. M. Gimble, W. Liedtke, and C. S. Chen. Control of stem cell fate by physical interactions with the extracellular matrix. *Cell Stem Cell* 5:17–26, 2009.

[26]Haque, M. A., T. Kurokawa, and J. P. Gong. Super tough double network hydrogels and their application as biomaterials. *Polymer* 53:1805–1822, 2012.

[27]Hart, L. R., J. L. Harries, B. W. Greenland, H. M. Colquhoun, and W. Hayes. Healable supramolecular polymers. *Polymer Chemistry* 4:4860–4870, 2013.

[28]Highley, C. B., C. B. Rodell, and J. A. Burdick. Direct 3D printing of shear-thinning hydrogels into self-healing hydrogels. *Adv. Mater*. 27:5075–5079, 2015.

[29]Hinton, T. J., Q. Jallerat, R. N. Palchesko, J. H. Park, M. S. Grodzicki, H.-J. Shue, M. H. Ramadan, A. R. Hudson, and A. W. Feinberg. Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels. *Sci. Adv*. 1:e1500758, 2015.

[30]Hoffman, A. S. Hydrogels for biomedical applications. *Adv. Drug Deliv. Rev*. 64:18–23, 2012.

[31]Hong, S., D. Sycks, H. F. Chan, S. Lin, G. P. Lopez, F. Guilak, K. W. Leong, and X. Zhao. 3D printing of highly stretchable and tough hydrogels into complex, cellularized structures. *Adv. Mater*. 27:4035–4040, 2015.

[32]Huang, T., H. G. Xu, K. X. Jiao, L. P. Zhu, H. R. Brown, and H. L. Wang. A novel hydrogel with high mechanical strength: a macromolecular microsphere composite hydrogel. *Adv. Mater*. 19:1622–1626, 2007.

[33]Jaiswal, M. K., J. R. Xavier, J. K. Carrow, P. Desai, D. Alge, and A. K. Gaharwar. Mechanically stiff nanocomposite hydrogels at ultralow nanoparticle content. *ACS Nano* 10:246–256, 2016.

[34]Jakab, K., C. Norotte, F. Marga, K. Murphy, G. Vunjak-Novakovic, and G. Forgacs. Tissue engineering by self-assembly and bio-printing of living cells. *Biofabrication* 2:022001, 2010.

[35]Kang, H.-W., S. J. Lee, I. K. Ko, C. Kengla, J. J. Yoo, and A. Atala. A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nat. Biotechnol*. 34:312–319, 2016.

[36]Kerativitayanan, P., J. K. Carrow, and A. K. Gaharwar. Nanomaterials for engineering stem cell responses. *Adv. Healthc Mater*. 4:1600–1627, 2015.
[37]Kesti, M., M. Müller, J. Becher, M. Schnabelrauch, M. D'Este, D. Eglin, and M. Zenobi-Wong. A versatile bioink for three-dimensional printing of cellular scaffolds based on thermally and photo-triggered tandem gelation. *Acta Biomater*. 11:162–172, 2015.
[38]Kirchmajer, D. M., R. Gorkin, III, and M. I. H. Panhuis. An overview of the suitability of hydrogel-forming polymers for extrusion-based 3D-printing. *J. Mater. Chem. B* 3:4105–4117, 2015.
[39]Kirchmajer, D. M., and M. I. H. Panhuis. Robust biopolymer based ionic-covalent entanglement hydrogels with reversible mechanical behaviour. *J. Mater. Chem. B* 2:4694–4702, 2014.
[40]Kloxin, A. M., C. J. Kloxin, C. N. Bowman, and K. S. Anseth. Mechanical properties of cellularly responsive hydrogels and their experimental determination. *Adv. Mater*. 22:3484–3494, 2010.
[41]Lee, V. K., D. Y. Kim, H. Ngo, Y. Lee, L. Seo, S.-S. Yoo, P. A. Vincent, and G. Dai. Creating perfused functional vascular channels using 3D bio-printing technology. *Biomaterials* 35:8092–8102, 2014.
[42]Lee, V., G. Singh, J. P. Trasatti, C. Bjornsson, X. Xu, T. N. Tran, S.-S. Yoo, G. Dai, and P. Karande. Design and fabrication of human skin by three-dimensional bioprinting. *Tissue Eng. Part C Methods* 20:473–484, 2013.
[43]Malda, J., and J. Groll. A step towards clinical translation of biofabrication. *Trends Biotechnol*. 34:356, 2016.
[44]Malda, J., J. Visser, F. P. Melchels, T. Jüngst, W. E. Hennink, W. J. Dhert, J. Groll, and D. W. Hutmacher. 25th anniversary article: engineering hydrogels for biofabrication. *Adv. Mater*. 25:5011–5028, 2013.
[45]Markstedt, K., A. Mantas, I. Tournier, H. C. Martínez Ávila, D. Hägg, and P. Gatenholm. 3D Bioprinting human chondrocytes with nanocellulose-alginate bioink for cartilage tissue engineering applications. *Biomacromolecules* 16:1489–1496, 2015.
[46]Melchels, F. P., M. A. Domingos, T. J. Klein, J. Malda, P. J. Bartolo, and D. W. Hutmacher. Additive manufacturing of tissues and organs. *Prog. Polym. Sci*. 37:1079–1104, 2012.
[47]Mironov, V., N. Reis, and B. Derby. Review: bioprinting: a beginning. *Tissue Eng*. 12:631–634, 2006.
[48]Mironov, V., R. P. Visconti, V. Kasyanov, G. Forgacs, C. J. Drake, and R. R. Markwald. Organ printing: tissue spheroids as building blocks. *Biomaterials* 30:2164–2174, 2009.
[49]Murphy, S. V., and A. Atala. 3D bioprinting of tissues and organs. *Nat. Biotechnol*. 32:773–785, 2014.
[50]Murphy, S. V., A. Skardal, and A. Atala. Evaluation of hydrogels for bio-printing applications. *J. Biomed. Mater. Res. A* 101A:272–284, 2013.
[51]Parani, M., G. Lokhande, A. Singh, and A. K. Gaharwar. Engineered nanomaterials for infection control and healing acute and chronic wounds. *ACS Appl. Mater. Interfaces* 8:10049–10069, 2016.
[52]Pati, F., J. Gantelius, and H. A. Svahn. 3D bioprinting of tissue/organ models. *Angew. Chem. Int. Ed*. 55:4650–4665, 2016.
[53]Pati, F., J. Jang, D.-H. Ha, S. Won Kim, J.-W. Rhie, J.-H. Shim, D.-H. Kim, and D.-W. Cho. Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink. *Nat. Commun*. 5:3935, 2014.
[54]Paul, A. Nanocomposite hydrogels: an emerging biomimetic platform for myocardial therapy and tissue engineering. *Nanomedicine* 10:1371–1374, 2015.
[55]Peak, C. W., J. K. Carrow, A. Thakur, A. Singh, and A. K. Gaharwar. Elastomeric cell-laden nanocomposite microfibers for engineering complex tissues. *Cell. Mol. Bioeng*. 8:404–415, 2015.
[56]Pereira, R. F., H. A. Almeida, and P. J. Bártolo. Drug delivery systems: advanced technologies potentially applicable in personalised treatmentBiofabrication Hydrogel Constr., Dordrecht: Springer, pp. 225–254, 2013.
[57]Rowley, J. A., G. Madlambayan, and D. J. Mooney. Alginate hydrogels as synthetic extracellular matrix materials. *Biomaterials* 20:45–53, 1999.
[58]Rutz, A. L., K. E. Hyland, A. E. Jakus, W. R. Burghardt, and R. N. Shah. A multimaterial bioink method for 3D printing tunable, Cell-compatible hydrogels. *Adv. Mater*. 27:1607–1614, 2015.
[59]Skardal, A., and A. Atala. Biomaterials for Integration with 3-D Bioprinting. *Ann. Biomed. Eng*. 43:730–746, 2015.
[60]Slaughter, B. V., S. S. Khurshid, O. Z. Fisher, A. Khademhosseini, and N. A. Peppas. Hydrogels in regenerative medicine. *Adv. Mater*. 21:3307–3329, 2009.
[61]Stanton, M., J. Samitier, and S. Sánchez. Bioprinting of 3D hydrogels. *Lab Chip* 15:3111–3115, 2015.
[62]Suekama, T. C., J. Hu, T. Kurokawa, J. P. Gong, and S. H. Gehrke. Double-network strategy improves fracture properties of chondroitin sulfate networks. *ACS Macro Lett*. 2:137–140, 2013.
[63]Thakur, T., J. R. Xavier, L. Cross, M. K. Jaiswal, E. Mondragon, R. Kaunas, and A. K. Gaharwar. Photocrosslinkable and elastomeric hydrogels for bone regeneration. *J Biomed Mater Res A* 104:879–888, 2016.
[64]Thiele, J., Y. Ma, S. Bruekers, S. Ma, and W. T. Huck. 25th Anniversary article: designer hydrogels for cell cultures: a materials selection guide. *Adv. Mater*. 26:125–148, 2014.
[65]Tibbitt, M. W., and K. S. Anseth. Hydrogels as extracellular matrix mimics for 3D cell culture. *Biotechnol. Bioeng*. 103:655–663, 2009.
[66]Xavier, J. R., T. Thakur, P. Desai, M. K. Jaiswal, N. Sears, E. Cosgriff-Hernandez, R. Kaunas, and A. K. Gaharwar. Bioactive nanoengineered hydrogels for bone tissue engineering: a growth-factor-free approach. *ACS Nano* 9:3109–3118, 2015.
[67]Xu, Y., and X. Wang. Application of 3D biomimetic models in drug delivery and regenerative medicine. *Curr. Pharm. Des*. 21:1618–1626, 2015.
[68]Yang, L., X. Tan, Z. Wang, and X. Zhang. Supramolecular polymers: historical development, preparation, characterization, and functions. *Chem. Rev*. 115:7196–7239, 2015.
[69]Zhu, W., X. Ma, M. Gou, D. Mei, K. Zhang, and S. Chen. 3D printing of functional biomaterials for tissue engineering. *Curr. Opin. Biotechnol*. 40:103–112, 2016.