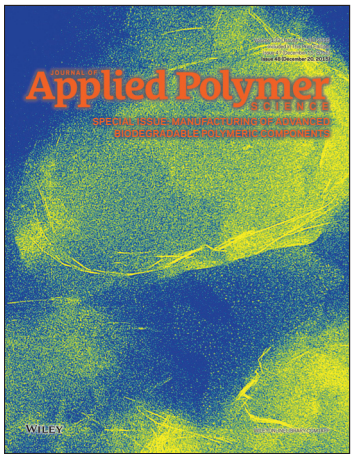
JOURNAL OF Applied Polymer SCIENCE

Special Issue: Manufacturing of Advanced Biodegradable Polymeric Components

Guest Editors: Prof. Roberto Pantani (University of Salerno) and
Prof. Lih-Sheng Turng (University of Wisconsin–Madison)



EDITORIAL

REVIEWS

ARTICLES

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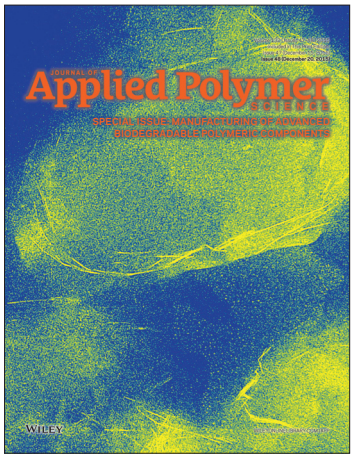

Rotational molding of biodegradable composites obtained with PLA reinforced by the wooden backbone of opuntia ficus indica cladodes
A. Greco and A. Maffezzoli, *J. Appl. Polym. Sci.* 2015, **DOI:** 10.1002/app.42447

Foam injection molding of poly(lactic) acid: Effect of back pressure on morphology and mechanical properties
V. Volpe and R. Pantani, *J. Appl. Polym. Sci.* 2015, **DOI:** 10.1002/app.42612

Modification and extrusion coating of polylactic acid films
H.-Y. Cheng, Y.-J. Yang, S.-C. Li, J.-Y. Hong and G.-W. Jang, *J. Appl. Polym. Sci.* 2015, **DOI:** 10.1002/app.42472

Processing and properties of biodegradable compounds based on aliphatic polyesters
M. R. Nobile, P. Cerruti, M. Malinconico and R. Pantani, *J. Appl. Polym. Sci.* 2015, **DOI:** 10.1002/app.42481

Journal of Applied Polymer Science

REVIEW

3D bioprinting of photocrosslinkable hydrogel constructs

Rúben F. Pereira,[1,2,3,4] Paulo J. Bártolo[5,6]

[1]Centre for Rapid and Sustainable Product Development (CDRsp), Polytechnic Institute of Leiria, Marinha Grande 2430-028, Portugal

[2]Instituto de Investigação e Inovação em Saúde, (I3S) Universidade do Porto, Porto 4200-393, Portugal

[3]Instituto Nacional de Engenharia Biomédica (INEB), Universidade do Porto, Porto 4150-180, Portugal

[4]Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Porto 4050-313, Portugal

[5]School of Mechanical, Aerospace and Civil Engineering, University of Manchester, Manchester M13 9PL, United Kingdom

[6]Manchester Institute of Biotechnology, University of Manchester, Manchester M1 7DN, United Kingdom

Correspondence to: P. J. Bártolo (E-mail: paulojorge.dasilvabartolo@manchester.ac.uk)

ABSTRACT: Three-dimensional (3D) bioprinting comprises a group of biofabrication technologies for the additive manufacturing of 3D constructs by precisely printing biocompatible materials, cells and biochemicals in predesigned spatial positions. These technologies have been successfully applied to fabricate biodegradable 3D constructs with intricate architectures and heterogeneous composition, assuming a pivotal role in the field of tissue engineering. However, the full implementation of bioprinting strongly depends on the development of novel biomaterials exhibiting fast crosslinking schemes and appropriate printability, cell-compatibility and biomechanical properties. Photocrosslinkable hydrogels are attractive materials for bioprinting as they provide fast polymerization under cell-compatible conditions and exceptional spatiotemporal control over the gelation process. Photopolymerization can also be performed during the bioprinting to promote the instantaneous formation of hydrogel with high well-defined architecture and structural stability. In this review paper, we summarize the most recent developments on bioprinting of photocrosslinkable biodegradable hydrogels for tissue engineering, focusing on the chemical modification strategies and the combination of photocrosslinking reactions with other gelation modalities. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42458.

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INTRODUCTION

The ultimate goal of tissue engineering (TE) relies on the development of clinically effective strategies to promote the full restoration of injured or dysfunctional tissues and organs.[1,2] The classical approach in TE involves the fabrication of porous scaffolds through either traditional or additive biofabrication techniques, followed by the seeding of cells onto such scaffolds, and subsequent implantation into the defect.[3–7] This approach allows the fabrication of three-dimensional (3D) scaffolds capable of promoting the *in vivo* regeneration of certain tissues like bone,[8,9] though it faces important limitations on the effective regeneration of highly complex and heterogeneous tissues due to the difficulty in precisely seeding scaffolds with multiple cells, heterogeneous distribution of cells throughout the scaffold, limited cell densities, and insufficient vascularization.[1,10] As scaffold-based therapies do not consider the incorporation of cells and biological entities within the scaffold during biofabrication, they are unable to adequately reproduce the cellular microenvironment *in vivo*, in which cells are surrounded by a highly hydrated extracellular matrix (ECM) composed of insoluble macromolecules, soluble signals and cell-adhesive proteins.[11,12] To address these shortcomings, and better recapitulate the architectural and compositional features of natural ECM, recent approaches in TE evolved from the seeding of cells onto pre-fabricated solid scaffolds towards the fabrication of highly organized cellular constructs. The basic concept underlying this approach lies on the ability of cells to undergo self-assembly and self-organization when placed in appropriate positions within a suitable microenvironment, forming new tissues without need of rigid porous scaffolds.[10,13] In recent years, these principles have been applied to engineer multicellular and multimaterial 3D constructs showing promising results in the *in vivo* regeneration of multilayered and complex tissues, such as the skin.[14–16]

Among the wide range of biofabrication techniques currently available to generate cellular constructs for TE, 3D bioprinting

Rúben F. Pereira is researcher at the Centre for Rapid and Sustainable Product Development (CDRsp) and PhD student in the University of Porto (Portugal). His research interests focuses on the design of *in situ* biofabrication strategies for skin tissue regeneration, and the synthesis of hydrogels for tissue regeneration and cell encapsulation.

Paulo J. Bártolo is Chair Professor in Advanced Manufacturing at the School of Mechanical, Aerospace and Civil Engineering, University of Manchester (UK); Director of the Manchester Biomanufacturing Centre, University of Manchester (UK), Principal Investigator at the Manchester Institute of Biotechnology, University of Manchester (UK), Visiting Professor at the Nanyang University (Singapore) and Polytechnic Institute of Leiria (Portugal), Fellow of the International Academy of Production Engineering and Portuguese Representative at the Global Alliance of Rapid Prototyping Associations. He is also Editor-in-Chief of both Virtual and Physical Prototyping Journal and Biomanufacturing Reviews.

is one of the most attractive owing its ability to print multiple biomaterials, cells and biochemicals (termed as "bioinks") in precise spatial locations with high resolution, accuracy and reproducibility.[4,17,18] Bioprinting technologies enable the automated biofabrication of cell-laden constructs through the layer-by-layer deposition of bioinks in both *in vitro* and *in vivo*.[19–23] In addition, these technologies are controlled by computer and can be combined with medical image systems (e.g., computed tomography, magnetic resonance imaging) and computer-aided design and computer-aided manufacturing (CAD/CAM) tools to generate personalized constructs organized at different length scales.[17,24] In contrast to the classical TE approach, bioprinting allows the direct fabrication of complex 3D constructs containing spatial variations of biomaterials, cells and biochemicals in the same structure, which significantly increases the level of biomimicry regarding the compositional, architectural and biochemical characteristics of the cell niche within the body. The increased complexity of the generated constructs is relevant not only for applications in tissue regeneration, but also for the development of *in vitro* models for cell biology, drug development and study of diseases.[25–28]

Despite the numerous advantages of bioprinting, the specificity of the printing process together with the processing of cells and sensitive biomolecules, result in new challenges regarding the biomaterial's properties, crosslinking pathways and printing fidelity. Hydrogels are gold standard materials for bioprinting as they provide an elastic and hydrated crosslinked network similar to the natural ECM, in which cells can be viable and functional.[1,17,18,29] An important feature of these materials is the ability to be rapidly formed *in situ* and in the presence of cells through a variety of physical and chemical crosslinking methods, including photopolymerization, ionic interaction or Michael addition reactions.[30–33] The crosslinking scheme is of prime importance for bioprinting as it determines the mechanical properties of the constructs, the gelation kinetics, the shape fidelity post-printing, and the viability of encapsulated cells. Photopolymerization is emerging as a promising crosslinking reaction for bioprinting because it enables the rapid formation of hydrogels immediately after printing through the incidence of light energy at appropriate wavelengths.[18,29,31,34,35] By simply adjusting the light intensity, exposure time and the illuminated area, photopolymerization provides an exceptional control over the spatiotemporal formation of the hydrogel and its network properties, including the crosslinking density and matrix stiffness.[36,37] This paper describes the recent progress on the bioprinting of photocrosslinkable hydrogels for tissue regeneration. A brief overview about the operating principles and main characteristics of 3D bioprinting technologies is provided, paying special attention to the printing requisites of each modality. Finally, the photocrosslinking biomaterials explored for bioprinting are presented, highlighting the progress in the field.

OVERVIEW OF 3D BIOPRINTING TECHNOLOGIES

Bioprinting technologies can be categorized according to several criteria, including the stimuli used to assist the deposition of bioinks, the printing modality (drop-based or extrusion-based bioprinting) or the mode of ejection from the reservoir (orifice-free or orifice-based). Here, the classification of bioprinting technologies is based on the stimuli employed to assist the printing process for inkjet bioprinting (thermal and piezoelectric effects), laser-assisted bioprinting (light energy) and extrusion bioprinting (pressurized air and mechanical effects). Despite specific differences among these technologies according to the mechanism used to assist the deposition of cells and biomaterials, the typical apparatus includes a computer controlled reservoir containing the bioink to print, a system or device to generate the ejection of bioink from the reservoir, and a receiving stage that collects the printed material (Figure 1).

Inkjet Bioprinting

Inkjet bioprinting is a non-contact technology that deposits small droplets of a bioink (1–100 picolitres) onto a receiving platform

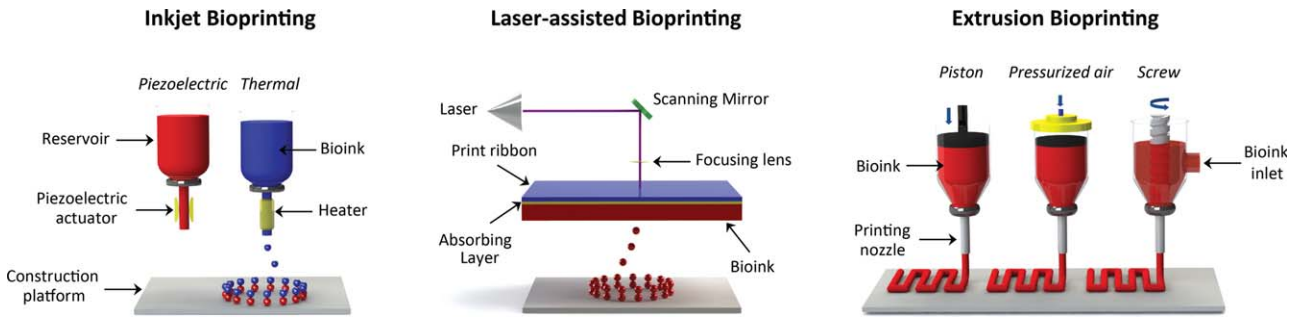

Figure 1. Illustration of 3D bioprinting processes and its main components. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with micrometer resolution.[38] In drop-on-demand printing, droplets with an average diameter of 10–50 μm are quickly generated through the action of either thermal or piezoelectric effects, and subsequently ejected through a tiny orifice placed in the extremity of the reservoir.[18,39] Thermal inkjet uses a heating element to induce the vaporization of a small volume of bioink inside the reservoir, with the consequent formation and ejection of a small droplet. As a result, this method subjects the cells to high temperatures (~300°C) for few microseconds (~2 μs) during the printing process, which may result in the formation of transient pores within the cell membrane.[17,40] However, several works established that thermal heating has minimal deleterious effects in the viability of several cell types.[40–42] In piezoelectric inkjet, the droplet generation and ejection is achieved through the mechanical deformation of a piezoelectric transducer controlled by the application of external voltage, which precludes the temperature increase to supraphysiological levels.[39,43,44] Even though inkjet bioprinting has been successfully applied to generate 2D/3D patterns of mammalian cells with minimal reduction on cell viability,[22,40] its orifice nature severely restricts the processing window. To prevent clogging issues, bioinks for inkjet bioprinting must exhibit low viscosity (1–10 mPa/s) and limited cell densities (typically <106 cells/mL), which causes additional constrains in the printing process, such as cell settling and sedimentation, droplet spreading, loss of resolution and limited fabrication of 3D constructs with controlled architectures.[17,18,45,46]

Laser-Assisted Bioprinting

Laser-assisted bioprinting (LAB) evolved from the conventional laser-induced forward transfer process originally developed to the direct writing of metals.[47] Its operation principle involves the application of a high-energetic pulsed laser (often a near infra-red laser) onto a donor ribbon coated with the bioink to be printed to generate the local ejection of small droplets. The incident laser light is primarily focused on a laser-transparent substrate (e.g., glass or quartz substrate), which is coated with a thin metal (e.g., gold and titanium) layer that absorbs the light energy and promotes its transfer to the bioink. At this moment, a high-pressure bubble is generated, and a small droplet is propelled towards a receiving platform.[17,23,48] The mechanisms of droplet formation and the effects of biofabrication parameters on the printing resolution and cell functions are well documented in the literature.[14,23,49–51] Deleterious effects to the cells mainly result from the thermal heating, interaction with laser light and impact with the receiving substrate.[48,49] LAB is one of the most promising bioprinting modalities for TE due to its unique resolution (10–100 μm), high-throughput, and ability to produce heterogeneous tissue constructs containing high cell densities. Contrary to the inkjet printing, LAB is an orifice-free technology, which precludes clogging issues and affords the deposition of bioinks with a large range of viscosities (1–300 mPa/s) and cell concentration (10^8 cells/mL).[17] However, to print 3D constructs retaining the prescribed resolution in the placement of cells and biomaterials, LAB requires the development of biomaterials exhibiting fast crosslinking mechanisms compatible with the light-wavelengths emitted by the laser sources. The biofabrication of clinically relevant constructs through LAB takes long periods of time and eventually the preparation of multiple ribbons containing multiple materials and cell types.

Extrusion Bioprinting

Extrusion bioprinting is the most popular method to fabricate 3D cell-laden constructs for tissue regeneration. In a typical setup, cells suspended in prepolymer solutions are loaded within disposable medical grade syringes or reservoirs, and subsequently printed onto a platform driven by pressurized air or mechanical forces generated by either a piston or a rotating screw.[17,18] Temperature controlled modules are often employed to adjust the temperature of both the bioink and the construction platform during fabrication, which is particularly relevant to control the bioink viscosity and to induce the *in situ* gelation of temperature-sensitive polymers.[52] Light sources and spraying nozzles can also be coupled to the bioprinting system to provide additional crosslinking schemes, improving the printing fidelity and the structural stability of tissue constructs.[24,53,54] Compression forces and shear stresses generated during the printing are the major sources of cell damage, requiring a careful optimization of the processing parameters (e.g., cellular density, bioink viscosity, temperature, and air pressure) to prevent cell apoptosis. Although there are several works indicating how these effects can be controlled and optimized to increase the printing accuracy and cell viability,[55–58] there is a need for systematic studies detailing the functionality of cells after printing. Instead of printing small droplets onto a platform, extrusion bioprinting produces 3D constructs through the sequential deposition of hydrogel filaments with diameters in a range of 150–300 μm.

The great advantage of this approach lies in the ability to print viscous biomaterials containing very high cell densities into 3D constructs with clinically relevant dimensions, which is impracticable using inkjet bioprinting and LAB. On the other hand, major limitations are related to the reduced resolution (approximately 200 μm), nozzle clogging, and difficulties in fabricating 3D constructs maintaining the predesigned shape and providing a suitable environment for the cells.[17,18] Actually, the printing of 3D constructs with complex and intricate microarchitectures still remains a huge challenge in bioprinting. Until now, the most common approach to address this issue involves the use of highly concentrated polymer solutions, which imposes severe restrictions to the cell mobility within the polymer network and might ultimately lead to poor cell adhesion, and even cell death.[59] More recently, alternative approaches based on the combination of multiple crosslinking pathways (e.g., photocrosslinking and thermal gelation),[52,60] integration of bioprinting with melt extrusion,[61–64] and the use of partial crosslinked hydrogels[54,59] have been explored with promising outcomes.

PHOTOCROSSLINKABLE BIOMATERIALS FOR BIOPRINTING

The development of advanced biomaterials simultaneously exhibiting appropriate printability, biomechanical properties, and providing a suitable microenvironment within which encapsulated cells can migrate, proliferate and synthetize new ECM still remains the major challenge in bioprinting. Hydrogels are the most commonly explored materials for the fabrication of cell-laden constructs through bioprinting, as they provide a soft and porous matrix for encapsulated cells and their properties can be easily tuned.[12,18,29,65] For example, the biological and degradation properties of hydrogels can be tailored by the incorporation of cell-adhesion motifs (e.g., Arg-Gly-Asp-RGD)[66,67] and cell-proteolytic domains into the polymer backbone,[68,69] whereas the biomechanical characteristics can be adjusted by varying the polymer molecular weight or the crosslinking density.[70,71] The wide spectrum of crosslinking schemes and the large range of network properties also give hydrogels high versatility for application in different tissues, including cartilage, bone, cardiovascular and skin.[33,72]

The formation of 3D hydrogels through photopolymerization is well documented in literature with several works focusing on the effects of light parameters and hydrogel precursors on cell fate and *in vivo* tissue regeneration.[73–76] This crosslinking reaction has been used in conventional mold-based approaches and additive biofabrication techniques, such as single- or two-photon stereolithography and micro stereo-thermal-lithography for many years.[34,77–79] However, over the past 5 years, the application of photopolymerization reactions in bioprinting has been receiving a great deal of interest due to its unique spatiotemporal control over the hydrogel formation and fast polymerization under cytocompatible conditions. Photopolymerization can be employed during the bioprinting[80] or just after the deposition[56] to induce the rapid establishment of crosslinks between the polymer chains, this way addressing current challenges in the field, including the fabrication of structurally stable 3D constructs with intricate architectures, enhanced spatial resolution and printing fidelity.

Photopolymerization Reactions for Bioprinting

Free radical photopolymerization is the most popular method to create chemically crosslinked cellular hydrogels by bioprinting through a multistep process of initiation, propagation and termination reactions. In the first step, a photosensitive system containing unsaturated prepolymers, cells and photoinitiators is irradiated with a sufficient dosage of light energy to excite the photoinitiators and trigger the formation of free radicals. Then, these reactive species propagate across vinyl moieties on prepolymers in solution, resulting in both generation of new free radicals and the establishment of crosslinks between the polymer chains. As the reaction proceeds, the number of crosslinks in the system increases and a highly crosslinked network structure is obtained *via* a chain-growth mechanism.[34,81] Despite free radical photopolymerization enables the rapid fabrication of natural and synthetic hydrogels under biocompatible conditions, it suffers from drawbacks including the oxygen inhibition, lack of control over the crosslinking kinetics, and the generation of heterogeneities within the hydrogel network.[81,82] The presence of inhomogeneities in the network resulting from the random chain polymerization have a significant impact in the mechanical integrity and swelling behavior of the constructs.[83]

Recently, photopolymerization reactions based on bioorthogonal click reactions are emerging as promising alternatives to the free radical photopolymerization counterpart.[84,85] The thiol-norbornene photopolymerization is one example of a click reaction that is triggered by an external light source, proceeding in a step-growth mechanism with the formation of structurally uniform hydrogels with minimal network defects, which provides better control over the gel crosslinking density and hydrogel properties.[81,82] Under ultraviolet (UV) or visible light irradiation and in the presence of low amounts of a photoinitiator, thiol-ene reaction promotes the rapid radical-mediated addition of thiols to carbon-carbon double ene bonds of functionalized prepolymers, yielding thiol-ether bonds.[82,86] Thiol-norbornene photopolymerization has numerous advantages, as it is not inhibited by oxygen, proceeds in a faster magnitude than chain-growth photopolymerization, and can be performed in the presence of cells.[81,86] This photopolymerization reaction has successfully been applied for the fabrication of cell-laden hydrogels using natural and synthetic polymers functionalized with norbornene moieties.[87–89] Photocrosslinkable hydrogels can also be formed from mixed-mode polymerizations (e.g., thiol-acrylate).[81,90]

Major concerns with photopolymerization reactions for cell encapsulation arise from three major issues: potential deleterious effects of UV light irradiation, cytotoxicity of radicals generated by the dissociation of photoinitiators, and local inflammation due to unreacted double bonds. For cell encapsulation applications, light sources emitting either in the UV-A range or visible light are usually employed to reduce deleterious effects on the biological systems.[91] Despite several studies have shown that the cell damage caused by UV irradiation can be significantly reduced or even eliminated by choosing appropriate light wavelengths, intensity and irradiation time, great efforts are currently being done towards the development of photosensitive systems (hydrogel precursors and photoinitiators)

capable of undergoing photocrosslinking upon exposure to safe visible light wavelengths.[90,92] In parallel to the light source, photoinitiators are a key element for photopolymerization, as they are responsible for the generation of free radicals that promote the formation of crosslinks between the polymer chains. However, these radicals can react with cellular components during the photopolymerization either *via* direct contact or the formation of reactive oxygen species, which may compromise the cell viability and ultimately induce DNA damage.[73,74] To prevent cell damage and ensure enough cell viability for new tissue formation, the wavelength of emitted light must overlap the absorption spectra of photoinitiator, and its concentration must be carefully optimized, taking into account the compromise between the crosslinking time and cell viability. Water soluble type I initiators such as Irgacure 2959 (1-[4-(2-Hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one), LAP (lithium phenyl-2,4,6-trimethylbenzoylphosphinate) and VA-086 (2,2′-Azobis[2-methyl-*N*-(2-hydroxyethyl)propionamide]) are the most commonly used for cell encapsulation because of their superior initiation efficiency and cytocompatibility.[56,93] The selection of the most appropriated photoinitiator can be partially determined by the number and the reactivity of functional groups in the polymer backbone. The functionalization of natural and synthetic polymers with reactive side groups such as acrylates, methacrylates, fumarates and vinyl esters, is an indispensable requisite for the photopolymerization. These reactive groups are introduced in the polymer chain through chemical reactions with functional groups already present in the native polymer structure in either homogeneous[93] or heterogeneous[94] conditions. The type and the number of reactive groups introduced in the polymer backbone is often determined by the cytotoxicity and reactivity of the functional group (reactivity: acrylate > vinyl ester > vinyl carbonate > vinyl carbamate > methacrylate > fumarate), being very important to ensure a maximal consumption of reactive groups during the photopolymerization to prevent local inflammation and non-specific side reactions with surrounding proteins.[81]

Photocrosslinkable Bioinks for Bioprinting

In addition to the classical properties that biomaterials should present in the context of TE,[4,95] bioinks for bioprinting of biological substitutes must fulfill additional requirements such as the printing under cell-compatible conditions, exhibit fast crosslinking reaction, allow the material to maintain its structural integrity after the deposition process, and provide an interactive microenvironment for the encapsulated cells.[17,18,59] Rather than simply provide a suitable environment that maintains the cells viable and support their functions, advanced bioinks must be capable of providing specific spatiotemporal cues to the embedded cells towards direct the cell adhesion, proliferation, differentiation and morphogenesis. As modern biomaterials are already modified to incorporate cell-adhesion sites for cell anchorage, and proteolytically sensitive domains for cell-mediated matrix remodeling,[12,65,96] these approaches can now be applied for the design of advanced bioinks. Despite the bioprinting field is still in the beginning, recently there has been great advances in the development of novel bioinks addressing some of the current challenges. Several natural and synthetic hydrogels have been specifically modified to fulfill the printing requisites of each bioprinting process, undergoing crosslinking through a variety of crosslinking pathways, including ionic interactions, photopolymerization and thermal gelation.[21,30,32,37,45,52,56,59,60,97–104]

Naturally Derived Bioinks. Natural polymers are widely used to engineer bioinks for bioprinting due to their inherent biochemical similarities with the natural ECM, biodegradability and biological recognition.[72,105] These materials are also readily available in nature and contain a myriad of functional groups in their chemical structure, which affords several functionalization strategies for photopolymerization. However, the main concerns with their use are related to the batch-to-batch variability, potential immunogenicity, narrow processing window, and limited mechanical properties.[34,105] Even though there is a wide range of natural polymers currently available for biomedical applications, photocrosslinkable bioinks for bioprinting are mainly developed using the hyaluronic acid (HA) and gelatin.

Hyaluronic Acid. Hyaluronic acid or hyaluronan is an anionic, non-sulfated, linear polysaccharide consisting of alternating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine linked together by β-1,4 and β-1,3 glycosidic bonds.[72] It can be extracted from animal products or produced by bacterial fermentation (e.g., Bacillus subtilis) to obtain a reproducible and controlled molecular weight. HA is an important glycosaminoglycan in synovial fluid and natural ECM, being a major constituent of connective, epithelial, and neural tissues.[106] It is a biocompatible and hydrophilic polymer that forms viscous solutions at relatively low concentrations, playing a key role in wound healing by promoting cell motility and proliferation. Moreover, HA is biodegradable in mammals by the enzyme hyaluronidase and reactive oxygen species, yielding the formation of low molecular weight hyaluronic acid and oligosaccharides. The useful ability of HA to interact with several cell surface receptors like CD44, CD54 and CD168 also makes this material very appealing for biomedical applications, including TE, wound healing and controlled delivery.[106,107] The main limitations of HA rely on slow degradation and the inability to bind key adhesion receptors such as integrins.[12] To address these pitfalls, HA-based hydrogels are usually decorated with cell-adhesion ligands and matrix metalloproteinase (MMP)-sensitive sites, which allow enhanced control over the cell adhesion and degradation rate.

An important characteristic of HA for photopolymerization lies on the presence of many free sites in its native chemical structure [glucuronic acid carboxylic acid, primary and secondary hydroxyl groups, *N*-acetyl group (following deamidation)],[107] which afford a myriad of functionalization strategies to render HA crosslinkable upon the light exposure in presence of a photoinitiator. Photocrosslinkable HA hydrogels can be obtained by reacting the polymer with functional groups, like methacrylates and norbornenes, under homogeneous (e.g., water) or heterogeneous (e.g., water/DMSO) reaction conditions, resulting in the covalent derivatization of the carboxylic acid or hydroxyl groups.[60,87,94] To improve the modification efficiency and reduce the hydrolysis of functionalizing agents, HA can be converted into a tetrabutylammonium (TBA) salt to allow dissolution in organic solvents.[60,108]

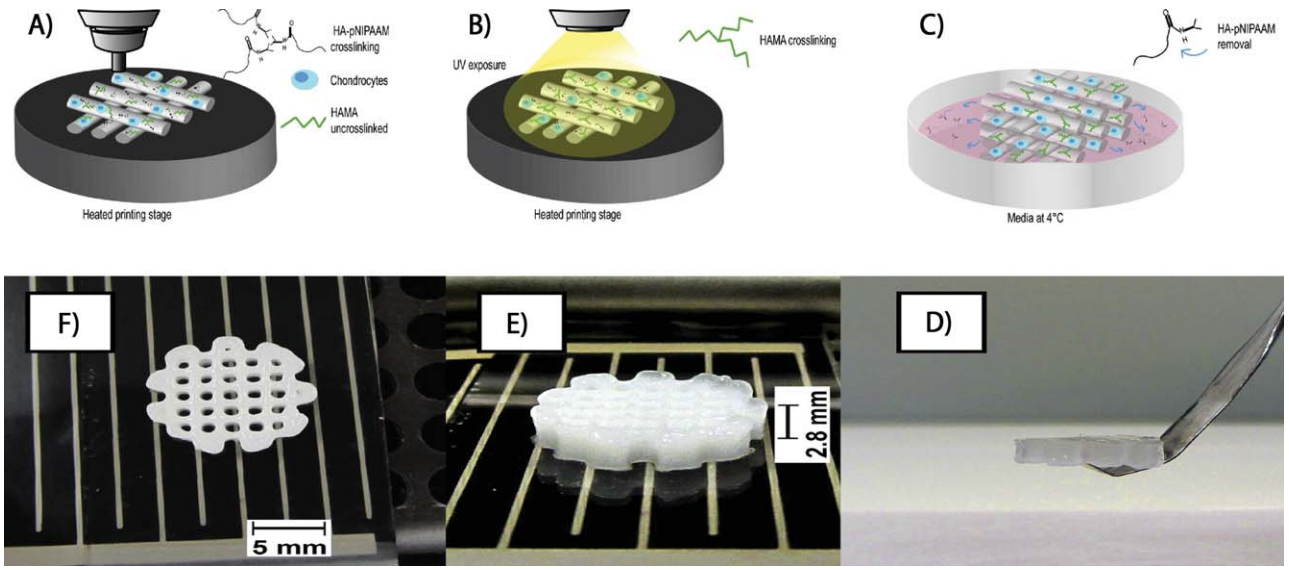

Figure 2. Illustration of the biofabrication procedure to fabricate 3D constructs: (A) thermal gelation of the bioink to maintain the printed shape, (B) secondary crosslinking through UV photopolymerization, and (C) removal of the supporting HA-pNIPAAM. Top (D) and perspective (E) view of MA-HA-HA-pNIPAAM scaffolds after printing and at 37°C (F). Reproduced from Ref. 60 with permission from Elsevier. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.].

Chemically hydrogels exhibiting tunable mechanical, swelling and biodegradation properties can be obtained by varying the degree of substitution achieved during the chemical functionalization, the polymer concentration or the light parameters.[94,109]

Photocrosslinkable HA-based hydrogels have been widely investigated for the bioprinting of 3D cell-laden hydrogels. Skardal *et al.*[59] synthetized a new biocompatible, photocrosslinkable hydrogel consisting of methacrylated HA (here referred as MA-HA) and gelatin ethanolamide methacrylate (gelatin methacrylate is herein termed as gelMA) for the fabrication of tubular constructs by extrusion bioprinting. MA-HA was synthetized by reaction with an excess of methacrylic anhydride, while gelMA was obtained through a two-step procedure encompassing the conversion of the carboxylic acid groups to ethanolamide derivatives, followed by the methacrylation of primary hydroxyl groups with methacrylic anhydride. Before bioprinting, the MA-HA:gelMA (4:1) blend containing HepG2 C3A cells (25×10^6 cells/mL) was partially crosslinked by exposure to UV light irradiation for 120 seconds to facilitate the deposition. The printing procedure of tubular constructs was carried out in a Fab@Home printing system through the sequential deposition of a central cell-free MA-HA hydrogel, followed by a cell-containing MA-HA:gelMA hydrogel, and finally an outer cell-free MA-HA hydrogel. After the printing procedure, the constructs were further crosslinked with UV light to consolidate the polymer network. After 3 weeks of *in vitro* culture, cells were adhered only in the hydrogels containing gelatin, remodeled the printed ECM and secreted collagen. Recently, Duan *et al.*[109] developed photocrosslinkable hydrogel formulations based on MA-HA and gelMA to print heart valve conduits containing encapsulated human aortic valvular interstitial cells. The composition of the bioink was firstly optimized regarding the matrix stiffness, viscosity, cell spreading and printing accuracy. The most promising polymer formulation (4% MA-HA/10% gelMA) containing the photoinitiator Irgacure 2959 (0.05 wt %) was loaded in syringes of a Fab@Home printing device, and extruded into a receiving platform to produce a 3D cellular trileaflet heart valve model. After photocrosslinking with UV light, the constructs maintained the structural integrity and supported high cell viability ($92.1 \pm 2.5\%$) for up 7 days of *in vitro* culture. It was also found that encapsulated cells also remodeled the initial matrix by depositing collagen and glyosaminoglycans. Recently, Kesti *et al.*[60] added MA-HA and non-modified HA to the thermoresponsive polymer poly(*N*-isopropylacrylamide) (pNIPAAM) in order to develop a bioink that undergoes fast thermal gelation immediately after printing, followed by the photopolymerization of MA-HA to consolidate the structure (Figure 2). In this study, HA-pNIPAAM was employed as a temporary matrix that provides immediate thermal gelation post-printing, being washed out from the construct (4°C/30min) after UV photocrosslinking. The bioink (15% HA-pNIPAAM, 2% MA-HA, 0.05 wt % LAP as photoinitiator) containing bovine chondrocytes (6×10^6 cells/mL) was printed into 3D cellular constructs with defined architectures using a BioFactory extrusion system containing a heated substrate and temperature controlled syringes. After 7 days of printing, the viability of chondrocytes within the 3D constructs containing HA-pNIPAAM was very low due to the more close porous structure and reduced diffusion of nutrients to the construct. Despite the removal of HA-pNIPAAM from the constructs significantly increased the cell viability from 34% to 91%, cell death was noticed in the edges of the filaments probably due to the drying during the printing. The combination of photopolymerization with thermal gelation allowed the printing of 3D cellular constructs with high structural fidelity and long-term stability without compromising the viability of embedded cells.

Photocrosslinkable MA-HA was also used to engineer a composite hydrogel containing gelMA and chondroitin sulfate methacrylate for cartilage TE.[110] Despite bioprinting being not

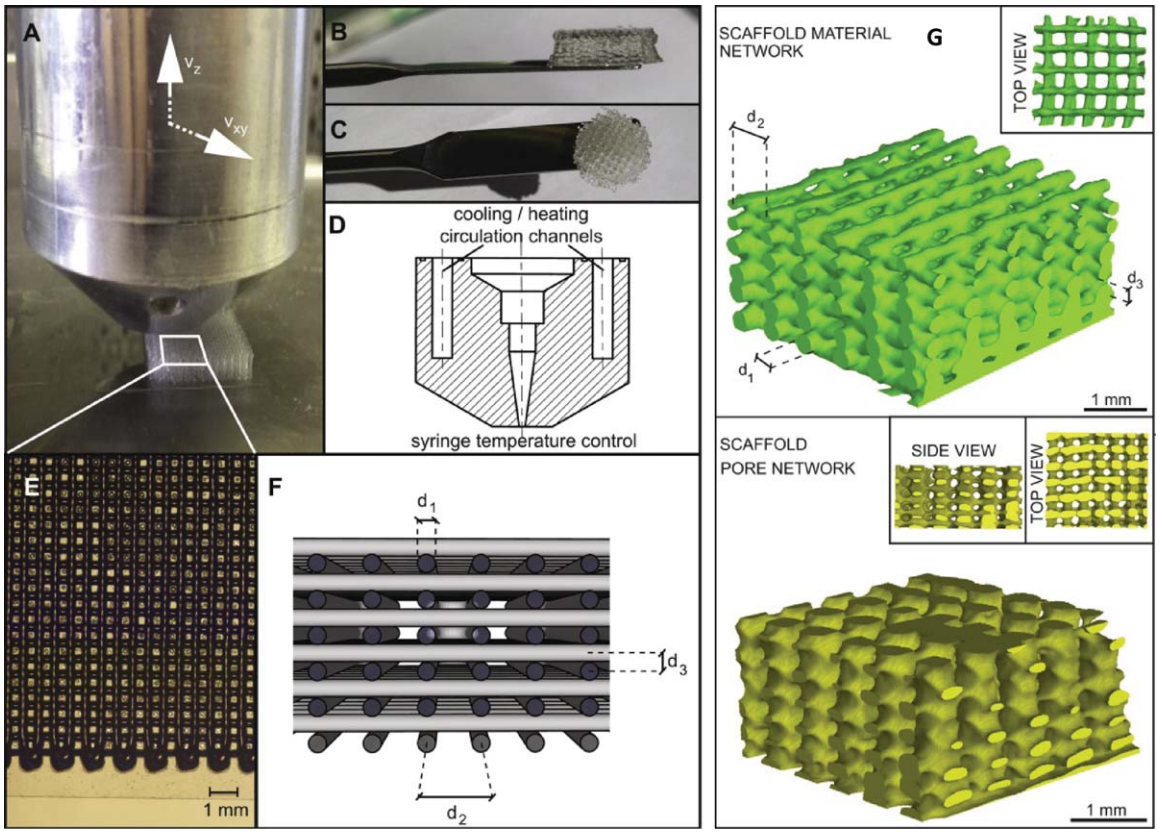

Figure 3. Macroscopic images of the printed 3D gelMA construct (A–C); Cross-section of the heating mantle around the tip to ensure stable printing (D); Light microscopy image of the porous scaffold (E); Illustration of the theoretical construct dimensions (F); Microcomputed tomography reconstructions of the hydrated scaffold representing the gelMA portion (green) and the scaffold pore network (yellow) (G). Reproduced from Ref. 56 with permission from Elsevier. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

demonstrated in this work, the addition of MA-HA improved the chondrogenesis and increased the quantity and distribution of new ECM, which provides new perspectives for the formulation of novel bioinks for bioprinting. In another strategy, non-modified HA was combined with hydroxyethyl-methacrylate-derivatized dextran to obtain a biocompatible formulation with appropriate viscosity and viscoelastic properties for extrusion bioprinting. The bioink was printed into 3D acellular scaffolds through a Bioscaffolder system and photocrosslinked by exposure to UV light (10 min, 6 mW/cm^2), yielding the formation of a semi-interpenetrating hydrogel network.[102]

Gelatin. Gelatin is a mixture of peptide sequences obtained by the denaturation of collagen through acid or alkaline treatment, which results in gelatins with different negative charges.[72] The acid treatment does not significantly affect the amide groups, whereas the basic treatment hydrolyzes the amide groups of asparagine and glutamine into carboxyl groups.[111] Gelatin is a water-soluble and thermoresponsive protein that undergoes a reversible sol-gel transition when cooled upper its critical solution temperature (25–35°C), which is below the temperature of the human body.[37] One efficient approach to prevent the dissolution of gelatin at physiological temperature involves the formation of chemical crosslinks through photopolymerization.[56] Gelatin has been widely explored for TE applications owing its biocompatibility, biodegradability and ability to support cell adhesion and spreading due to the presence of cell adhesion domains (e.g., RGD). However, as gelatin is derived from collagen, the main component of the ECM and most abundant protein in humans, it is a potential source of disease transmission and immunogenic reactions.[12] Another drawback of gelatin is the weak mechanical strength, which limits the range of applications. One strategy to address this issue, while preserving its inherent cell-adhesive properties, relies on the combination of gelatin with melt-extruded polymers. This approach was recently explored to engineer mechanically robust 3D constructs for cartilage TE through the alternated deposition of either gelMA or gelMA/HA hydrogels between the strands of melt extruded ε-caprolactone scaffolds, followed by the UV irradiation of gelMA to promote the formation of chemical crosslinks.[37] In a similar work, gelMA was deposited within the pores of thermoplastic scaffolds composed of poly(hydroxymethylglycolide-co-e-caprolactone)/poly(e-caprolactone) functionalized with methacrylate groups (pMHMGCL/PCL). Covalent crosslinks established between the pMHMGCL/PCL and gelMA resulted in a significant increase in the interface-binding strength, while the chondrocytes within the hydrogel were capable of synthetizing glycosaminoglycans and collagen type II both *in vitro* and *in vivo*.[112]

Photocrosslinkable gelatin hydrogels are usually formed by the modification of primary amines with methacrylate groups

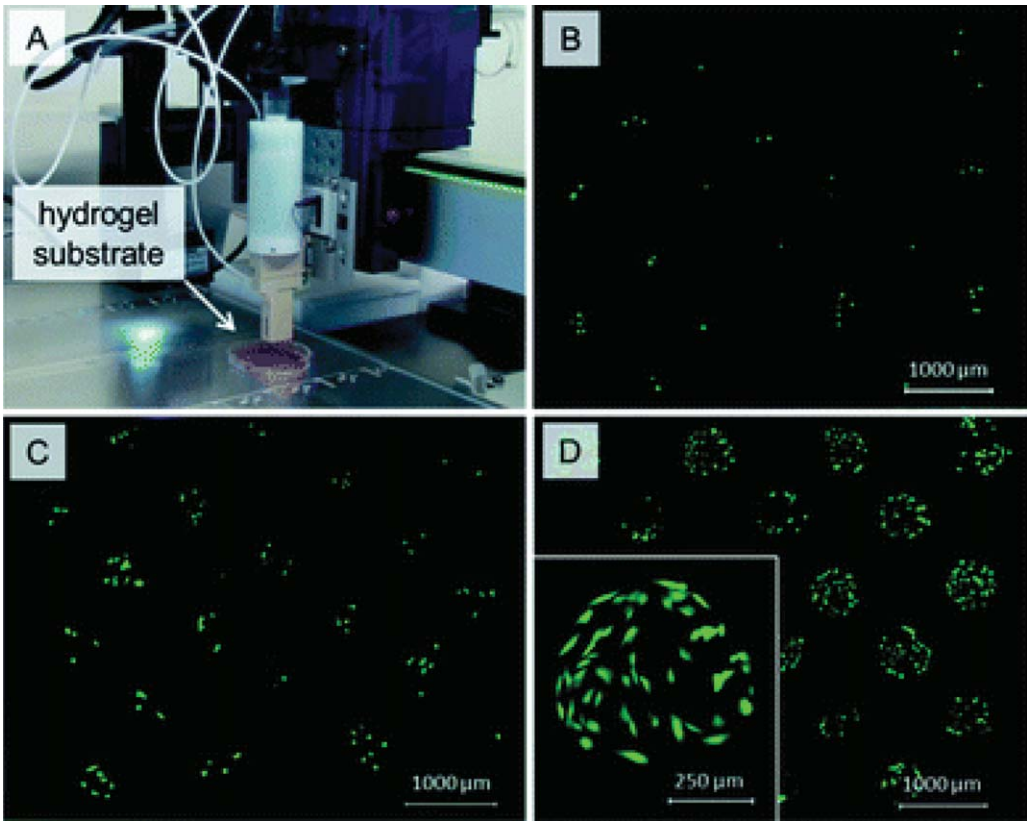

Figure 4. Inkjet bioprinting of porcine articular chondrocytes embedded in gelMA onto swollen gelMA hydrogel substrates (A); Live/dead staining showing the cell viability and morphology of printed cells after 3 hours, (C) 24 hours, and (D) 72 hours post-printing. Reproduced from Ref. 45 with permission from RCS Publishing. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(often methacrylic anhydride), yielding a light-sensitive polymer that undergoes free radical polymerization in the presence of a photoinitiator upon exposure to UV light. Recently, a great number of gelatin-based bioinks have been developed for 3D bioprinting, enabling the fabrication of cell-laden 3D constructs with reproducible architectures.[37,54,56,101,103,113] The extrusion bioprinting of photocrosslinkable gelatin was elegantly demonstrated by Billiet *et al.*,[56] who formulated and optimized a bioink composed of gelMA, VA-086 as photoinitiator, and hepatocarcinoma cell line (HepG2, 1.5×10^6 cells/mL). They started by carrying out a systematic evaluation about the effects of the bioink properties (e.g., viscosity, concentration, cell density, photoinitiator, curing kinetics) and the operating parameters (e.g., pressure, plotting speed, needle type and diameter) on the architecture of bioprinted scaffold, including the filament diameter, pore geometry and structural integrity. After the establishment of optimal parameters, the bioink was printed into 3D cell-laden constructs with interconnected pores (Figure 3) and excellent cell viability after 14 days post-printing (98.92%). Encapsulated cells maintained the proliferative capacity and the ability to express liver specific functions, as observed by hematoxylin and eosin staining. In a different bioprinting approach, Bertassoni *et al.*[54] developed a three-step biofabrication strategy to print cell-laden gelMA constructs encompassing the (i) aspiration of the hydrogel precursor, followed by (ii) the photocrosslinking inside a glass capillary, and (iii) the dispensing *via* mechanical extrusion. They firstly accessed the influence of polymer concentration, UV irradiation and cell concentration on the printability and mechanical properties of gelMA hydrogels. Then, they showed the ability of their strategy to print gelMA hydrogels with different architectures, containing HepG2 cells that remained viable (>80%) for at least 8 days after the bioprinting process. This strategy allows the fabrication of hydrogels with complex architectures without clogging due to the deposition of pre-polymerized cell-laden gels from a glass capillary, though the length of each deposited filament is limited to 65mm, which prevents the fabrication of constructs with higher dimensions.

Hoch *et al.*[45] developed a photocrosslinkable gelatin bioink to fulfill the requisites for inkjet bioprinting of mammalian cells. Gelatin was rendered photocrosslinkable through the reaction of methacrylic anhydride and the effects of the methacrylation degree, viscosity and printability of gelatin bioink were carefully accessed to preclude nozzle clogging. The gelMA was further modified through the acetylation of free amino groups to reduce the solution viscosity and prevent the formation of physical gels during the printing procedure. A piezoelectric inkjet bioprinting system was used to print porcine articular chondrocytes embedded in gelMA (1×10^6 cell/mL) at 25°C, as this temperature led to reduced evaporation, nozzle clogging, and better bioink stability. Both the bioink and the printing

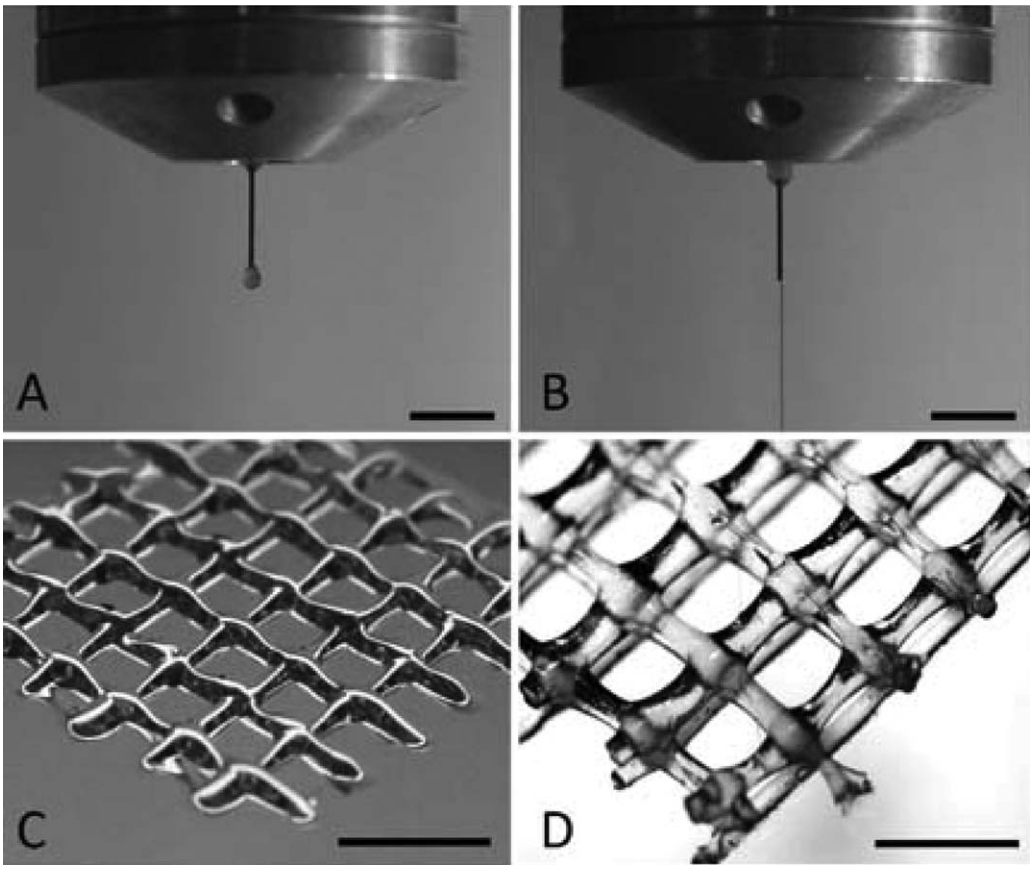

Figure 5. Extrusion bioprinting of gelMA bioink forming droplets at the nozzle (A) and printed in flat strands that spread out on the surface (C). Addition of HA to gelMA allows the formation of strands that could be printed from the nozzle (B), allowing the fabrication of a construct composed of 4 layers (D). Scale bars represent 5 mm in A–C and 2 mm in D. Reproduced from Ref. 37 with permission from Wiley-VCH.

parameters demonstrated to be cytocompatible, allowing the printing of hydrogels capable of supporting cell adhesion and proliferation (Figure 4). In another work, Gurkan *et al.*[27] used a valve-based droplet ejector system to print nanoliter droplets of gelMA loaded with human mesenchymal stem cells (hMSCs), bone morphogenetic protein 2 (BMP-2), and transforming growth factor β1 (TGF-β1) to engineer an anisotropic biomimetic fibrocartilage microenvironment. The authors demonstrated the capabilities of the bioprinting process to generate biochemical gradients by printing two cell-laden gelatin bioinks, one containing BMP-2 and the other loaded with TGF-β1, and carried out a comprehensive phenotypic evaluation of the tissue model.

Melchels *et al.*[101] combined gelMA with high-molecular weight gellan gum to develop a novel bioink that affords the fabrication of reproducible 3D constructs through a custom-made bioprinter. The addition of small concentrations of gellan gum and variable salt (Na^+, K^+) concentrations to the gelMA resulted on the formation of ionic crosslinks between the gellan chains, which allowed the formulation of a bioink exhibiting suitable rheological properties (pseudo-plasticity and yield stress) for bioprinting. The gelMA/gellan gum bioink was printed in 3D constructs that retained the shape after fabrication and were further stabilized by the photocrosslinking of gelMA. To demonstrate the cell printing, a 3D cellular construct was fabricated from a bioink composed of 1.0% gellan gum, 10% gelMA and chondrocytes (0.5×10^6 cells/mL) in isotonic mannose, and subsequently crosslinked by UV irradiation for 15 minutes. Neither the printing process nor the bioink composition was cytotoxic to the cells, though the resuspension of cells at a supraphysiological temperature (50°C) led to a significant decrease on cell viability (~50%). In a similar work, 2.4% HA was added to a solution of 20% gelMA to promote an increase the viscosity and allow the reproducible fabrication of 3D scaffolds with interconnected pores and dimensions of 20 mm × 20 mm × 1.2 mm (Figure 5).[37]

3D bioprinting technologies, in particular extrusion bioprinting, together with photopolymerization are also assuming a pivotal role in the fabrication of functional vessel networks within hydrogels to supply the embedded cells with nutrients and remove the waste products, which is essential for cell survival and new tissue formation.[114–116] Bertassoni *et al.*[114] used an extrusion bioprinting system to create perfusable vessels within 3D gelMA hydrogels. In this approach, perfusable microvascular vessels were created by printing agarose template fibers, followed by casting gelMA to cover the deposited filaments (Figure 6). After gelatin photocrosslinking, the filaments were removed from the construct, leaving functional and perfusable vessels capable of improving the viability and differentiation of encapsulated osteogenic cells.

Synthetic-Derived Bioinks. Synthetic polymers are assuming a pivotal role in the design of functional biomaterials for tissue regeneration due to their superior mechanical properties, reproducible composition and controlled properties.[11,105,117] Although they often exhibit limited biocompatibility, biodegradability and cell adhesiveness, synthetic polymers can be modified using several functionalization strategies to provide cell-mediated degradation and greater control over the cell functions, including adhesion, proliferation and morphogenesis.[11,117] In the context of 3D bioprinting, Polyethylene glycol (PEG) is the most commonly used synthetic polymer because of its hydrophilicity, presence of functional groups in the polymer backbone for chemical modification and adjustable properties.

Polyethylene Glycol. PEG is a non-ionic polyester that has been widely used for several biomedical applications owing its hydrophilicity, low immunogenicity and antigenicity.[81,117,118] Its basic chemical structure is PEG diol with two hydroxyl end groups, but it can be modified in an easy and reproducible manner into other functional groups, such as acrylate, amine, thiol, azide or vinyl sulfone.[117] PEG is usually referred as a "blank slate" material due to the absence of cell-adhesion domains, lack of protein binding sites and low degradability.[12] However, these properties can be easily modified in a reproducible and controlled manner by a variety of chemistries, including the co-polymerization with other macromolecules and the introduction of functional moieties to allow the cell-anchorage, cell-proteolysis and photodegradation.[81,119–121] PEG macromolecules can also be easily functionalized through the hydroxyl end groups to yield a variety of hydrogels precursors for photopolymerization. PEG acrylates are the most used macromers for photopolymerization, including PEG diacrylate (PEGDA), PEG dimethacrylate (PEGDMA) and multiarmPEG(n-PEG). The later has been functionalized with norbornene terminal groups for photopolymerization through a step-growth mechanism.[73,80,117,120]

Photocrosslinkable PEG has been widely used in bioprinting either alone or combined with other polymers, providing a soft and hydrophilic environment for the encapsulated cells. Censi *et al.*[97] developed a biodegradable triblock copolymer hydrogel for bioprinting based on PEG and methacrylate-modified poly(*N*-(2-hydroxypropyl)methacry-lamide lactate) that undergoes crosslinking by means of thermal gelation and UV light exposure. The hydrogel formulation solidifies by thermal gelation after printing, and is then consolidated through UV photopolymerization. The bioink was successfully deposited in predesigned patterns by extrusion bioprinting, affording the additive biofabrication of stable 3D constructs with reproducible vertical porosity, internal design and dimensional stability, though the transversal pores suffered fusion during the printing. 3D constructs exhibited an elastic modulus of 119 ± 4 kPa and were completely degraded in approximately 190 days of incubation in phosphate buffer at pH 7.4 and 37°C. Cell encapsulation studies also show a good viability of chondrocytes ($85 \pm 7\%$) embedded within photocrosslinked solid constructs after 3 days of *in vitro* culture, which reveals promising properties for 3D bioprinting. Hockaday *et al.*[24] combined PEGDA with alginate to fabricate heterogeneous aortic valve hydrogel scaffolds through extrusion bioprinting with simultaneous photopolymerization (Figure 7). Alginate was added to the PEGDA to achieve suitable viscosity for bioprinting, being washed out upon photocrosslinking. Micro-CT analysis revealed that the printing strategy allows the fabrication of 3D constructs with high geometric precision, but the accuracy decreases with the reduced size. For cell studies, collagen I was added to the PEGDA bioink in an attempt to enhance cell adhesion and spreading. Results showed that porcine aortic valve interstitial cells seeded onto the 3D constructs populated the scaffold surface and remained viable throughout 3 weeks, though collagen did not significantly affected the cell behavior. Although this work showed the capabilities of bioprinting combined with photopolymerization to generate anatomically complex 3D valve conduits at relevant scale lengths, the functionality of the printed construct was not yet demonstrated.

Rather than forming composite bioinks by the addition of other polymers, PEGDMA was also used as a sole biomaterial to encapsulate human articular chondrocytes for the fabrication of cellular constructs for cartilage TE.[80,122] Cui *et al.*[80] used a modified thermal inkjet bioprinter to print a PEGDMA bioink containing chondrocytes (5×10^6 cells/mL) in a 4-mm-diameter full-thickness cartilage lesion created in the center of an osteochondral plugs harvested from bovine femoral condyles, serving as a biopaper. Cells printed with simultaneous photopolymerization maintained the initial positions, exhibiting high viability (89.2–3.6%, 24 hours) and ability to express collagen type II and aggrecan after 6 weeks of *in vitro* culture. Results also showed firmly integration between the printed construct and the native cartilage with the production of proteoglycans at the interface. Recently, the same research group demonstrated the one-step inkjet bioprinting of acrylated peptides (RGD and MMP-sensitive), acrylated-PEG and hMSCs to promote the conjugation of peptides to the modified PEG hydrogel through photopolymerization during the printing procedure. The peptide conjugation occurred in biocompatible conditions and significantly enhanced the bone and cartilage differentiation.[123] In one of the few examples about the bioprinting of biological substitutes for bone tissue, PEGDMA and bone marrow-derived human mesenchymal stem cells (hMSCs) were printed together with nanoparticles of bioactive glass (BG) and hydroxyapatite (HA) to stimulate the osteogenesis of hMSCs.[124] A thermal inkjet bioprinter was employed to produce 3D cell-laden bone constructs through the layer-by-layer printing of the composite bioink with simultaneous photopolymerization to promote the chemical crosslinking of PEGDMA. The presence of BG nanoparticles led to a reduced viability of hMSCs ($63.80 \pm 7.54\%$) encapsulated within the hydrogels when compared with the HA nanoparticles ($86.62 \pm 6.02\%$), which indicates possible cytotoxicity. Printed HA nanoparticles also resulted in increased expression of ECM, collagen production and alkaline phosphatase activity, indicating the osteogenic differentiation of encapsulated hMSCs.

CONCLUSIONS AND FUTURE PERSPECTIVES

3D bioprinting is assuming a key role in the fabrication of advanced 3D cellular constructs for tissue repair and

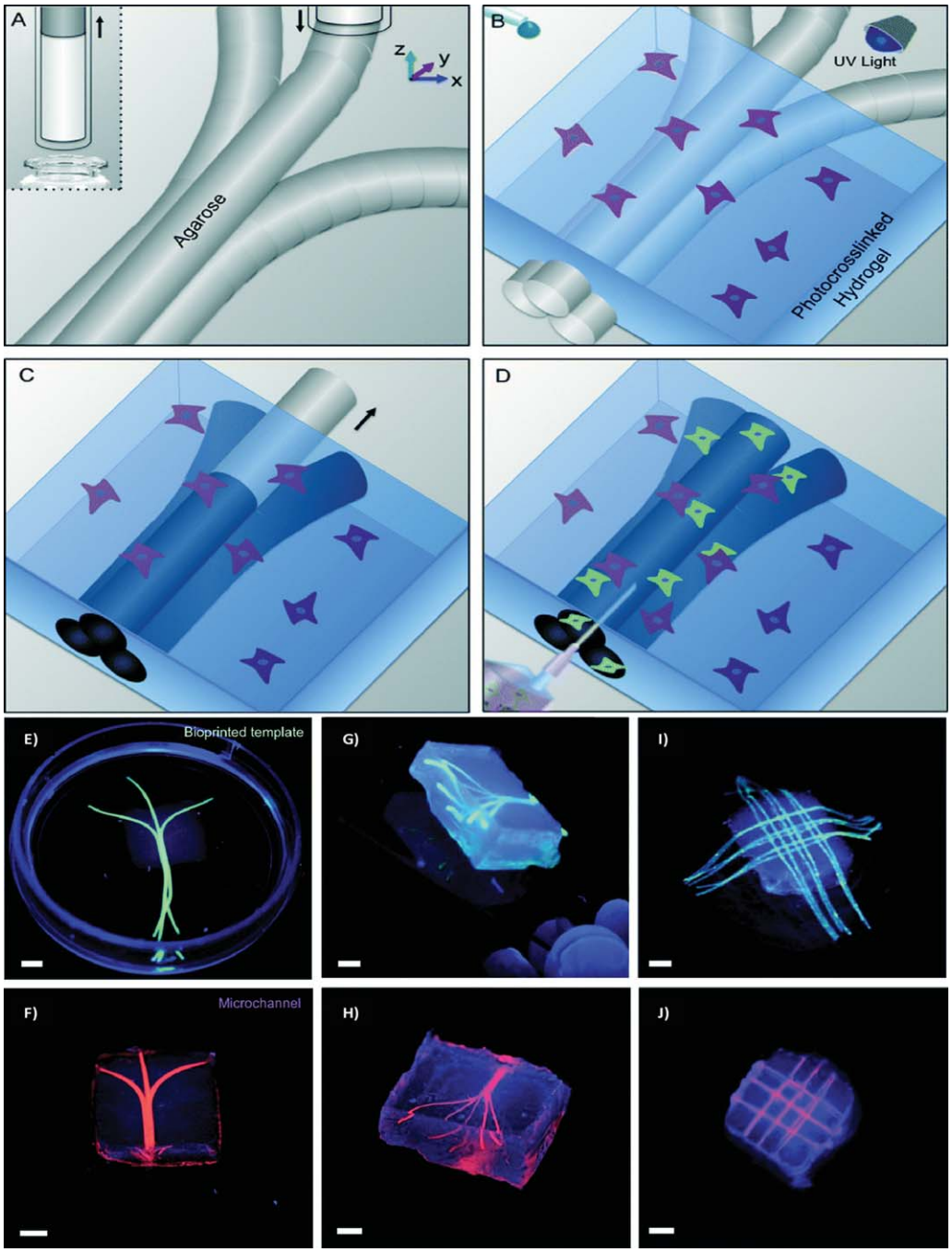

Figure 6. Schematic illustration of the bioprinting procedure: (A) bioprinter equipped with a piston fitted inside glass capillary deposits agarose fibers after gelation at predesigned locations; (B) gelMA hydrogel precursor is casted over the bioprinted mold and UV photocrosslinked; (C) agarose fibers are removed from the hydrogel; (D) perfusable vessel is formed. Photographs of planar bifurcated agarose fibers (green) within the gelMA construct (E) and respective network after perfusion (F); 3D branched agarose templates embedded in the gelMA hydrogel (G) and resulting 3D branched network after perfusion (H); 3D lattice template within the gelMA network before (I) and after perfusion (J). Scale bars represent 3 mm. Reproduced from Ref. 114 with permission from The Royal Society of Chemistry. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

regeneration. In recent years, its unique capabilities of high resolution and precise printing of a myriad of biomaterials, cells and therapeutics have been explored for the fabrication of highly complex 3D constructs containing multiple materials and cells arranged in specific spatial locations. The complexity of the generated biological structures is still limited when compared with the heterogeneity of natural tissues, though these structures showed promising outcomes for the *in vivo* regeneration of several tissues, including the skin and bone. These encouraging results have attracted the interest of many researchers and companies working in the biomaterials, TE and regenerative medicine fields, resulting in significant technological and

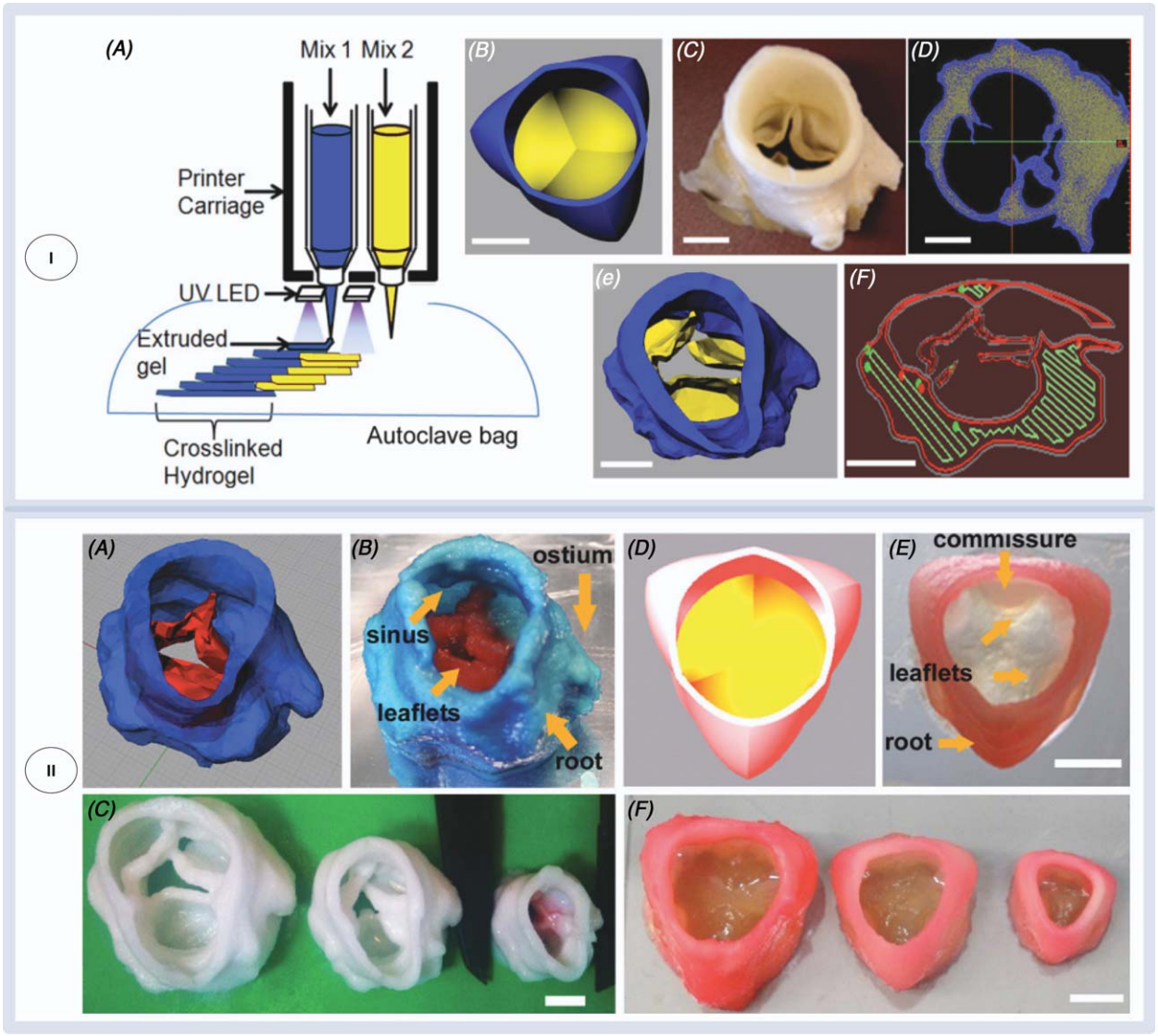

Figure 7. (I) Illustration of the bioprinting setup (A); axisymmetric valve STL file designed by a CAD software (B); micro-CT scan of the porcine aortic valve (C); representative micro-CT image of the valve with leaflet and root regions thresholded based on tissue density (D); thresholded regions were reconstructed into printable STL geometries (E); printing software with sliced the geometries (F) (scale bar represents 1 cm). (II) Printing of heterogeneous valve constructs at different scales: porcine aortic valve model (A) and respective constructs (B, C); axisymmetric valve model (D) and respective constructs (E, F) (scale bar represents 1 cm). Reproduced from Ref. 24 with permission from IOP Publishing. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

scientific advances regarding the commercialization of dedicated bioprinting technologies and the development of novel biomaterials for bioprinting. Most of the bioprinting systems currently used in laboratory evolved from technical modifications of available technologies, however, there are now several bioprinters in the market that were specifically designed to print biological materials under sterile conditions, following best manufacturing practices. In parallel, researchers have been investigating the applicability of several biomaterials for bioprinting to solve the lack of suitable bioinks for the fabrication of larger 3D constructs in some extent similar to the natural tissue organization. Most of these materials are inappropriate due to the narrow rheological, mechanical and biological boundaries of bioprinting, nevertheless they allowed a better comprehension about the printing requisites. Based on this knowledge, several photocrosslinkable biomaterials have been explored for bioprinting to address the current need for bioinks exhibiting fast crosslinking schemes, appropriate printability, biomechanical properties, structural stability after printing, and suitable microenvironment in which embedded cells can reside and synthetize new tissue. Photocrosslinkable bioinks fulfill the major physical, mechanical and biological requisites of bioprinting, allowing the rapid formation of hydrogels during the printing procedure upon the exposure to light energy, which allows the fabrication of 3D constructs with complex internal architectures, structural stability and printing accuracy, the major pitfalls of other crosslinking pathways under investigation for bioprinting. In addition, cells and biochemical entities (e.g., growth factors) can be

added to photocrosslinkable bioink and printed into 3D hydrogels with simultaneous photopolymerization without inducing significant reduction on the viability of encapsulated cells. Even so photopolymerization has emerged as a promising crosslinking scheme for bioprinting, systematic studies detailing the combined effects of functionalized biomaterials, printing procedure, photoinitiators and light parameters on the function of printed cells are necessary to elucidate and prove the safety of this biofabrication strategy. Efforts should also be concentrated in the research of more efficient and cytocompatible photoinitiators to promote the formation of hydrogels under UV light and visible light. Simultaneously, *in vivo* studies should also be conducted to evaluate the interaction between the biological tissues and the bioprinted photocrosslinked hydrogels, and their efficacy in promoting the repair and regeneration of complex tissues. The potential of photocrosslinkable hydrogels for LAB should also be investigated to access their feasibility to generate 3D constructs with relevant resolution for clinical applications.

ACKNOWLEDGMENTS

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