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Review

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Evolution of Bioinks and Additive Manufacturing Technologies for 3D Bioprinting

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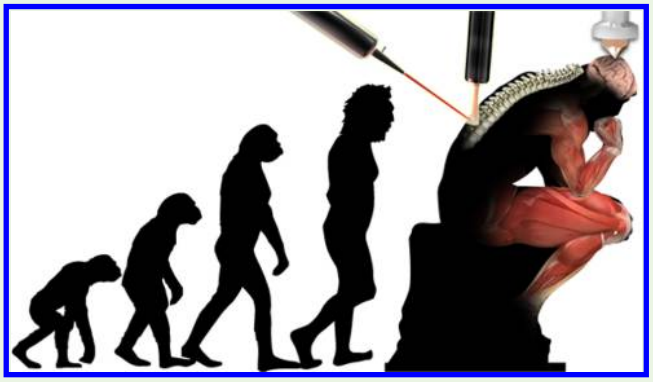
ABSTRACT: 3D printing is an additive manufacturing (AM) technique that has quickly disrupted traditional design and manufacturing strategies. New structures can be manufactured that could not be fabricated using other methods. These new capabilities are considered by many to hallmark a historic shift representative of a new industrial revolution. Exciting utilities of this evolving technology are the fields of biomedical engineering and translational medicine, particularly in applying three-dimensional (3D) printing toward enabling on-demand fabrication of customized tissue scaffolds and medical device geometries. AM techniques are promising a future where on-demand production of patient-specific living tissues is a reality. In this review, we cover the rapid evolution and widespread concepts of a bio-"ink" and bioprinted devices and tissues from the past two decades as well as review the various additive manufacturing methods that have been used toward 3D bioprinting of cells and scaffolds with a special look at the benefits and practical considerations for each method. Despite being a young technology, the evolution and impact of AM in the fields of tissue engineering and regenerative medicine has progressed rapidly. We finish the review by looking toward the future of bioprinting and identify some of the current bottlenecks facing the blossoming industry.



KEYWORDS: *additive manufacturing, bioprinting, bioinks, regenerative medicine, tissue engineering*

1.1. THE ORIGINS AND GOALS OF BIOPRINTING

The turn of the century saw important concurrent events that solidified 3D bioprinting as a new direction in biomedical engineering (Figure 1). In 1986, Charles Hull patented the liquid photopolymer based technology termed stereo lithography.[1] Later that same year a graduate student from the University of Austin Texas, Carl Deckard, developed the selective laser sintering (SLS) printing process.[2] Together these two techniques are considered the birth of 3D printing, which has since been adopted as a biotechnology technique with widespread use in tissue engineering and regenerative medicine.[1] In 1999, Anthony's Atala's team at the Wake Forest Institute for Regenerative Medicine used molding techniques to produce a synthetic human bladder scaffold that was later coated with cells taken from the patient.[3] Although no AM techniques were used to manufacture the scaffold, this "lab grown organ" set the stage for future engineered organs via bioprinting techniques.

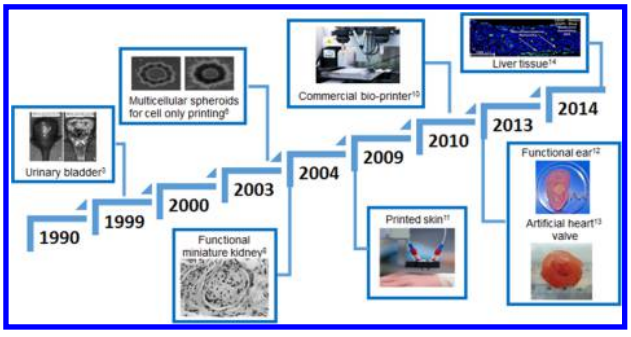


Figure 1. Timeline of influential events considered major milestones in bioprinting. Multicellular spheroids adapted from with permission from ref 5. Copyright 2004 National Academy of Sciences. Functional ear adapted with permission from ref 10. Copyright 2013 American Chemical Society.

In 2003, Tom Boland,[4] then at Clemson, received the first patent for a bioprinting technique, which was based on inkjet technology. The same year, Garbor Forgacs and his team in the University of Missouri created multicellular spheroids for 3D printing and this technique is often regarded as the first step toward scaffold free printing of cells.[5] The following year again proved to be important in biological additive manufacturing as Douglas Chisey's team at the Naval Research Laboratory applied laser technology to print bioinks and mammalian cells into 3D structures[6] and the first international workshop was conducted to review the state of the art of bioprinting and to

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Table 1. AM Technologies Assembled per ASTM Standard F2792.[39]

manufacturing process		technology	benefits	limitations	unique features	refs
			Cell Compatible			
inkjet based	material jetting	inkjet printing	2-D resolution	Z resolution limited	small amounts of material used	40−45
			low cost, fast	low viscosity	cell compatible	
		acoustic ejection droplet	ultra-high throughput	inverted substrates	touchless handling	
			high precision	Z resolution limited		
laser based	VAT Polymerization	stereo lithography (SLA)	3D resolution	cell photo induced damage	compatible with most photo-polymerizable materials	46−48
				photosensitive materials		
extrusion based	material extrusion	fused deposition modeling (FDM)	high accuracy	thermoplastic materials only	commercially available	49,50
			reproducible well established methods	only cell compatible when processed at physiological temperatures		
		direct write	variety of materials	low mechanical strength	complex 3D structures with cell compatible process	51−53
					free form structures possible	
			Not Cell Compatible			
inkjet based	binder jetting	powder bed and inkjet head printing (3D powder)	bioactive components can be incorporated	powdered thermos responsive materials only	mechanical strength	54−57
laser based	powder bed fusion	selective laser sintering (SLS)	mechanical strength	thermos responsive powdered materials	FDA approved product (OsteoFab)	58−60,61
			faster and higher resolution than other powder methods			

establish a network for collaboration.[7] Discussions led by Brian Derby, Doug Chrisey and Vladimir Mironov defined bioplotting or bioprinting as "the use of material transfer processes for patterning and assembling biologically relevant materials—molecules, cells, tissues, and biodegradable biomaterials—with a prescribed organization to accomplish one or more biological functions."[7]

The next major step for the industry occurred in 2009 when Organovo and Invetech created the first commercial bioprinter[8] and researchers led by Anthony Atala at Wake Forest University produced printed skin constructs that are considered by some to be the bioprinting endeavor closest to being functional tissue replacements.[9] Other recent achievements in bioprinting have come in the form of ear-shaped constructs[10] and heart valve models.[11] Finally in 2014, Organovo applied bioprinting techniques to produce the first commercially available liver tissue model.[12]

The use of these advanced technologies led to the construction of complex 3D multicellular constructs. The ability to plot cells and other biologicals as desired enables more sophisticated investigation of cellular behavior and disease modeling. These techniques provide a high throughput method with potential to in part displace more traditional *in vitro* techniques using molded or cast scaffolds. Since the design of optimal scaffolds remains to be identified, rapid prototyping (RP) is uniquely suited to scaffold fabrication due to its capability to incorporate a rapid series of programmable variables for study of generated suitable scaffolds.

The ability to design tailored implant and scaffold geometries using 3D patient scans and computer-aided design currently exist.[13] However, in order to translate designs into tangible cellularized constructs, further development of accurate high-resolution fabrication techniques is needed. Over the past decade, several AM technologies originally developed for nonbiomedical applications have demonstrated potential for bioprinting and biofabrication.[14−17] Computer controlled RP is capable of producing small to large physiologically relevant structures with the aim of replicating biomedical implants or organ geometry. Some RP paradigms and techniques have become standardized, such as the use of G programming language as the most widely used numerical control programming language for creating machine instructions for computer-aided engineering in automation.[18,19] Programmable microcontrollers and high-resolution stepper motors now enable RP to generate precisely modulated variables such as geometry, porosity, mechanical properties, and structure or biochemical moieties.[20,21]

AM builds a 3D object from the bottom-up by adding a series of cross-sections in a layer by layer fashion which does not require milling or molding. The field has evolved a variety of methods for creating 3D objects from ceramics, metals, and polymers. AM methods and capabilities vary according to the materials used, cross-linking mechanisms and extrusion techniques. Each technique supports a particular range of control over matrix architecture, mechanical properties, degradation, and biological components. Exploitation of these features used in conjunction with the application-specific ink formulations creates a platform for fabricating patient customized materials and devices. A summary of these techniques is presented in Table 1 and these techniques will be described in detail in the following sections. The AM techniques are divided between those that are cell compatible (Sections 1.4−1.11) and those that are not cell compatible but utilize naturally derived materials for scaffold fabrication (Sections 1.12 and 1.13).

Starting from a biomaterials perspective from the late 1990s to the early 2000s, work had been developed with ceramics and colloidal mixtures using direct-write techniques to create two-dimensional microscale structures.[22−25] These direct write techniques such as robocasting and ink jet printing were developed for microfabrication processes to directly deposit or pattern materials in specific geometries.[22] These were mainly two-dimensional processes that required materials with tailored chemistries or rheological properties. The microscale resolution

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of these technologies was used to construct complex scaffolds for tissue engineering,[26] microvascular networks[27] and sensors.[28,29] This early work set the stage for formulation of biomaterials that could be suitable for AM technologies.

Reviews in the past decade have followed the development of the emerging field of 3D bioprinting. As summarized in 2008, at its initial stages bioprinting depended on retrofitting technologies that were being developed for industrial processes.[15] These came at a time where there was a transition from the high end systems being developed by industry leaders such as 3D Systems and Stratasys to user-friendly low-cost printers pioneered by Bowyer in 2007.[30] These low-cost printers were based on the RepRap concept of an open source user environment, bringing accessible printing technology to the hands of research laboratories and the general public. The same trend in 3D bioprinting occurred as use shifted away from cumbersome high cost systems which were not designed to be used with biological materials or cells, into customizable, biocompatible devices evolving at both research and commercial scales. A 2014 review[31] summarized the advances developed during this time period that saw 3D bioprinting emerge as a viable field.

As the technology has become mainstream, the influence that it can have in medical and biological fields is becoming evident for specific applications. Recent reviews summarized the growing use of these technologies for clinicians, specifically in the fields of radiology[13] and cardiovascular[32] and reconstructive surgery.[33] In the research environment, the technologies are revolutionizing tissue engineering[34] with emphasis on bone,[35,36] skin,[37] and myocardial[32,38] tissue models. This review will provide a big picture view of the development of the field from its inception to the current commercial and widespread laboratory applications. Furthermore, the diversity of methods for 3D patterning of biological materials will be explored as well as the wide assortment of materials and bioinks used from the onset of the field up until the present.

1.2. BIOPRINTING APPLICATIONS

Applications for bioprinting are quite broad. The versatility of robotic deposition and rapid prototyping equipment requires little augmentation to enable the fabrication of different materials and products that can range from large reconstructive bone scaffolds[57] to printing single cells[4] or nanoparticles.[17] The prospect of printed organs, while often publicized, seems relatively far off in terms of approved medical options. Despite the excitement surrounding the development of printable tissues and organs, there are numerous other applications for bioprinting. For example, printed tissue mimics can be relevant as disease models[62−64] and acellular constructs can function as structured scaffolding for bone grafting materials.[36] Bioprinting is also efficient for the development of high-throughput assays and drug discovery.[65−67]

In biomedical research, bioplotting is useful for the controlled deposition of biologicals such as pharmaceuticals and small molecule screens. For example, this approach has been applied to pattern drugs and factors toward the development of printable medicines, bioactive coatings, affinity membranes, and for high-throughput screening.[68] In tissue engineering, bioplotting has been used to develop cytocompatible or cell-laden constructs for use in *in vitro* tissue modeling. Even single cells can be printed into finely organized 2D and 3D patterns. This enables the high-throughput generation of micro cultures or intricately patterned cocultures that cannot be easily generated using other methods.[68] Clinical interests substantially drive the development of this type of research toward surgical reconstruction, with an ultimate goal of printing whole-organ replacements.

1.3. GENERAL BIOPRINTING TECHNIQUES

3D printing of biological components has encompassed two separate areas: acellular functional scaffolds which incorporate biological components, and cell-laden constructs aiming to recapitulate *in vivo* processes. For both of these focus areas traditional AM technologies have been adapted to produce biocompatible, implantable constructs. The terminology that defines these areas has not been consistent across the literature. The terms bioplotting and bioprinting have been used interchangeably and do not indicate whether cells are being printed or involved at any stage of the process. These printing processes do not need to include cells to be categorized as bioprinting; however, cells can be incorporated into the process. Bioplotting and bioprinting can be performed without cells, as long as there is some biological component involved, such as drugs, or extracellular matrix (ECM).

Bioplotting refers to the 2D or 3D plotting of any biological component or combination of biological components onto or into any substrate, such as on the surface of Petri dishes or within preformed gels, using robotics. Bioprinting more specifically refers to the printing processes and biomaterial formulations, which possess the components required to generate and cure solid structures, on an initially blank printing surface.

Cell printing or cell plotting refer to the 2D or 3D plotting of cells and media onto or into any substrate using robotics. Cell printing or cell plotting are processes typically capable of robotically deposition with controlled quantities of viable cells ranging 1 to 1 million per each command of a stepper motor. Bioplotting is often used in conjunction with cell plotting in 2 steps in order to generate a rapid prototyped tissue model. Bioprinting is a process capable of accomplishing both the formation and cellularization of a scaffold in one step. This terminology, cell printing or plotting, has been used interchangeably; however, the terminology used in this review is defined as described above.[31,69]

1.4. BIOINKJET PRINTING

What is now known as 3D bioprinting originated from one of the earliest AM techniques in the field, known as inkjet printing, a strategy that is also credited with the first cell-printing patent.[70] In the most basic form, bioinkjet variants are slightly modified versions of common desktop printers. The bioink is stored in a cartridge or reservoir until it is transferred to the ink chamber for ejection from the print nozzle to the print surface (Figure 2).

Bioink ejection is commonly driven by one of two standard mechanisms. The first strategy utilizes a small piezoelectric actuator. Applying a voltage to the actuator induces rapid actuation, which in turn generates the pressure needed to expel ink from the chamber. The second strategy employs a heating element. This element superheats the ink and creates a vapor bubble which expands until it expels ink through the nozzle. The heating can reach 300 °C but is highly localized and only persists on the order of a few microseconds. This results in less than a 10 °C increase in temperature to the bioink and a minimal impact to the viability of printed biologicals such as

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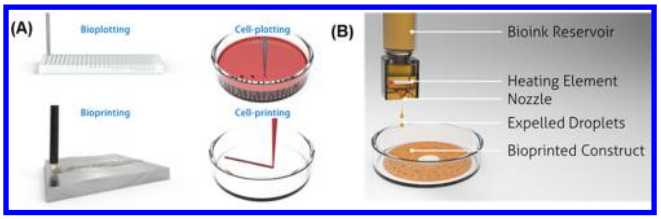

Figure 2. (A) Illustrations emphasizing the differences between bioplotting and bioprinting strategies. (B) Schematic of a bioinkjet style printing strategy.

drugs, DNA, or mammalian cells.[40,71,72] Cell viabilities are quite good using this technique and have been reported to be in the range of 85%.[41,73,74]

In comparison to other bioprinting strategies, bioinkjet printing equipment is widely available and relatively inexpensive and the volume of bioink droplets can be controlled electronically ranging from one to hundreds of picoliters.[75] This allows affective patterning with low quantities of reagents. Small volumes enable high printing resolution. Inkjet printing is capable of fast printing of two-dimensional highly repeatable patterns, with deposition rates as fast as 10 000 droplets per second.[76] The printing of biological gradients is also feasible. Unlike other techniques, bio inkjet printing does not require contact with the printing surface.

A variety of ECM components can be added to the bioink blends. Fibronectin and laminin have been bioinkjet printed in precise patterns and shown to subsequently affect cell organization.[77,78] Fibrin has been printed for cartilage engineering.[55] Growth factors can also be patterned onto culture surfaces with high stability. Fibroblast growth factor-2 (FGF-2) and ciliary neurotrophic factor (CNTF) have been inkjet patterned onto culture surfaces to inhibit or reinforce the differentiation of neural stem cells into astrocytes.[79] Similarly, FGF-2, bone morphogenetic protein-2 (BMP-2) and insulin-like growth factor II (IGF-II) have been printed to influence the differentiation of muscle-derived stem cells toward osteogenic or skeletal muscle lineages.[80] This technique has also been used to generate lipid vesicles,[81] and enzymes, such as glucose oxidase, have been printed.[82] This technique also facilitates the rapid production of combinatorial arrays for high throughput screening, based on rate and the small volumes used.

Cells can also be directly printed using inkjet technology to generate biologically viable constructs.[43,83] However, inkjet printing of cell-inclusions are challenging with these systems. Inkjet systems were originally designed for homogeneous low-viscosity solutions and bioinkjet printing is typically only compatible with solution viscosities below 10 cP.[84] Higher viscosities require excessive force to eject drops which can damage cell membranes.[84] This problem also limits the cell density supported by a printable bioink. Suspensions of cells are prone to sedimentation and aggregation within the reservoir and other components of the system.[72,85] Cell aggregation also leads to clogging within the narrow geometry of the inkjet nozzle. This may lead to increased mechanical stress to cells and the generation of nonuniform droplet sizes. Bioink additives can alleviate this issue; however, effective strategies such as surfactants may harm cell viability.

The low viscosity ink used in inkjet printing also does not often facilitate the rapid generation of 3D structures. However, cross-linking methods via chemical, pH or ultraviolet light stimulation to the printed inks and configurations using multiple nozzles have been applied to address this limitation. Many PolyJet 3D printing strategies employ a photopolymerizing polymer. Photopolymers can be extruded in a continuous bead or deposited as discrete droplets through a series of inkjet heads which are mounted in-line with an ultraviolet (UV) curing lamp. Alternatively, alginate solution can be coprinted or printed in turn with calcium chloride solution as a cross-linker in order to generate 3D systems. However, as discussed later, many cross-linking strategies are toxic to cells and lead to a slower and more complicated printing process.

1.5. ACOUSTIC DROPLET EJECTION

Acoustic droplet ejection (ADE) produces similar results to inkjet printing but in an inverted manner via a nozzleless ejection technology. Here a pulse of ultrasound is used to move low volumes of fluids (typically nanoliters or picoliters) without physical contact. Acoustic droplet ejection utilizes a transducer to generate surface acoustic waves in order to form an acoustic focal point. The acoustic energy of an ultrasound pulse is focused near the surface of a fluid bioink. The focal point induces the formation of a mound of liquid until a picoliter droplet is ejected from the bioink reservoir onto the printing surface. The droplet is ejected upward toward an inverted printing surface positioned overhead (Figure 3). The small

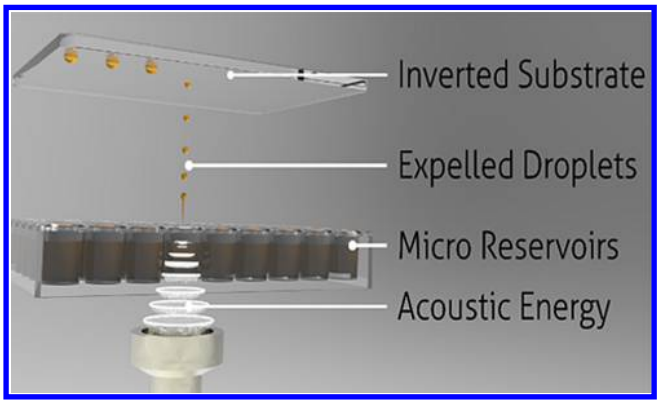

Figure 3. Schematic of acoustic droplet ejection technology. Here a pulse of ultrasound is used to move low.

volumes confer the ability to deposit with a high level of precision. Droplet size can be manipulated by varying the frequency of the acoustic energy; higher frequencies produce smaller droplets. Typically frequencies of 5−300 MHz are used which correspond to droplet diameters from 300−5 μm.[86] In contrast, other printing techniques which rely on droplet formation through a nozzle orifice lose droplet precision as the transfer volume decreases. Acoustic droplet ejection does not suffer these issues to the same degree.[87] Therefore, this technique shows improved reproducibility in the low-nanoliter range deposition, while still being compatible with many life science applications, including cell printing.

ADE technology is theoretically a gentle process, and it can be used to transfer many biologicals without damage or loss of viability. The capability to encapsulate single to few cells with micrometer precision, high viability, and controlled directionality using a gentle acoustic field is applicable in tissue engineering, high throughput screening, and clinical diagnostics.[76] This method has proven valuable in microwell applications such as for RNA, DNA, and protein analysis.

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This technique also allows fine polymer, ECM, or factor deposition and encapsulation of single cells in droplets. The inverted nature of the technique facilitates the fabrication of hanging drop cultures with superior efficiency compared to other RP strategies. This capability can be exploited using patterned multiple cell types for high-throughput modeling of disease tissues such as breast cancer.[76,87] One limitation of this technique is the necessity for inverted substrates for deposition, as not all cell assay systems are amenable to build up via hanging drop methods. This challenge further effects the potential 3D nature of acoustically manufactured constructs as ejected droplets rely on substrate adhesion and surface tension, and face critical size limitations.[83]

1.6. STEREOLITHOGRAPHY (SLA)

Stereolithography (SLA) utilizes an ultraviolet laser to photopolymerize the surface of a bath of liquid polymer. This process is repeated to generate subsequent layers as an additive technique. Mechanical properties are initially weak and can limit applications to bioadhesives, injectables, or cellular scaffolds. However, additional curing in a UV oven improves material strength but often induces shrinking or warping of geometry. Resolution is dependent on laser spot size (75−250 μm) and the occurrence of absorption or scattering of the laser beam.[47] Two-photon polymerization is an improvement, but the process and cost of materials often limits products to a small scale.[48]

There is a limited choice of photopolymerizable biomaterials, however, polymer modification can technically enable any option. Current options include derivatives of polyethylene glycol (PEG) acrylate, polyethylene glycol methacrylate (PEGMA), poly(vinyl alcohol) (PVA), acrylated hyaluronic acid, dextran methacrylate, acrylated capped poly-1-capro-lactone-*co*-trimethylene carbonate, and polypropylene fumarate (Figure 4a).[46]

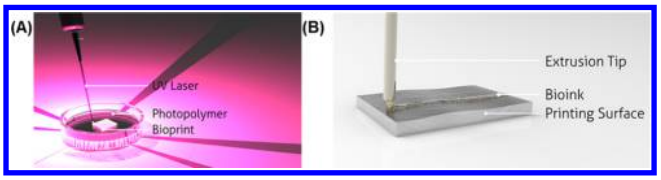

Figure 4. (A) Schematic of the SLA process. (B) Schematic of a basic RD system.

1.7. ROBOTIC DEPOSITION

Robotic deposition (RD) is the foundation of many other bioplotting and 3D bioprinting techniques described here. In its most basic application, this technique is used to dispense discrete volumes of inks into or onto a substrate. Stepper motors are programmed to accurately position the dispensing nozzle along the *x*, *y*, and *z* axes. The positioning is often programmed as a series of sequential layers (Figure 4b)

To build 3D constructs these layers are printed on top of each other. Lead screws or belt drives driven by stepper motors as well as pneumatic actuators are used to drive controlled dispensing through the nozzle. Nano volumes of the dispensed material can be deposited onto a planar surface, or into a liquid or gel. These capabilities increase the throughput of assays and improve assay efficiency by reducing reagent volumes and processing time.[68] This technique is used in pharmaceutical development for the rapid identification of drug candidates. Robotic dispensing is easily adapted to the plotting of cells in media or ECM solution.[88] These systems can be configured to dispense discrete volumes from syringes containing cells in media or ECM solution. A variety of other biopolymers such as poly(lactic-*co*-glycolic acid) (PLGA) can be printed in this manner.[89] Cell-plotting has been performed using RD to position cells and cell aggregates in 3D biomimetic matrices.[68] Controlled deposition in this manner has been shown to affect the response of cells to various drugs such as chemo-therapeutics.[68]

Compared to bioinkjet strategies, these extrusion-based techniques are capable of higher ejection forces and have more tolerance for heterogeneous formulations, allowing the printing of much higher cell densities. This adds the benefit of starting closer to physiologically relevant cell densities, which is a critical improvement in the efficiency for printing tissues. However, cell viability can be lower after extrusion printing compared to inkjet processes. Notably, RD has been used to bioprint endothelial cells with alginate for 3D tissue constructs. This technique is also successful in the fabrication of acellular film-based vascular implants and grafts.[90] RD is also the strategy employed to produce kidney phantoms.[91,92]

1.8. DIRECT-WRITE ASSEMBLY

Direct write can be considered the RD designed to achieve fine feature sizes. Direct-write systems enable 3D prototyping by dispensing beads of material through a microscale orifice directly into an environment which initiates the curing process (Figure 5). The initiation of curing can occur via temperature,

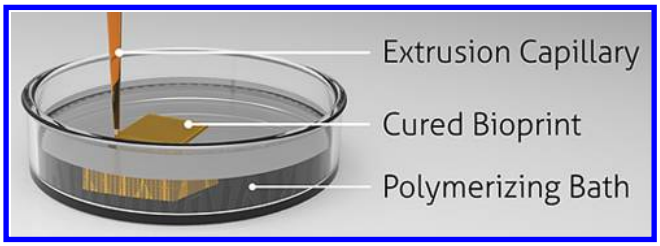

Figure 5. Schematic of a curable bioink being deposited into a bath of polymerizing solution using a direct-write approach.

photopolymerization, humidity gradients, or other chemical polymerization methods.[93,94] Extrusions are polymerized into the desired programmed geometries as they are dispensed into the bath. This technique is compatible with a variety of biomaterials.

Direct-write compatible bioinks have been formulated from biopolymers, hydrogels, metals and ceramics for applications ranging from cellular scaffolding to microstents (Figure 6a).[26,29,95] Cellular inclusions can also be incorporated, however, the biological components must often be added in a separate step because of the toxicity of the curing agents (Figure 6b).[96] This was the case when chitosan−HA was extruded into a sodium hydroxide/ethanol bath and silk was extruded into a methanol bath.[93,94] Recent research has used a jammed soft granular gel medium for direct-write printing, allowing polymeric materials to be cross-linked temporally within the gel close to the time of writing, as well as allowing colloids and cells to be left supported within the granular gel (Figure 6c).[97]

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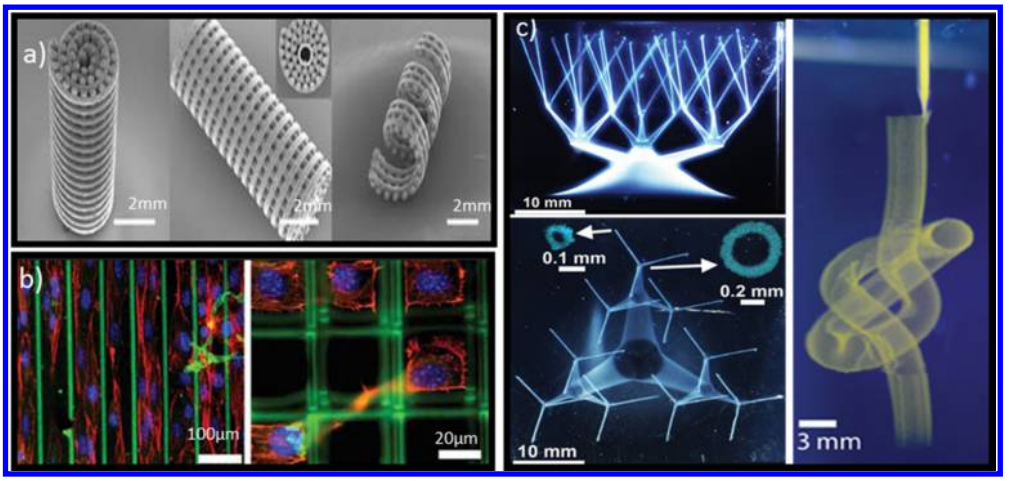

Figure 6. Examples of direct write strategies used to print (a) microstent structures[88] and (b) microscale cellular scaffolds plated with 3T3 fibroblasts.[96] (c) Direct-write printing onto a granular gel medium to make a hollow complex hollow tube and a continuous network of hollow vessels with features spanning several orders of magnitude in diameter and aspect ratio (insets: confocal cross-sections).[97]

Cells can also be written in parallel to collagen, by depositing collagen from a separate reservoir. This technique theoretically allows viable suspension of cells and hydrogels in the granular medium until the medium is washed with water leaving only the cured structure.[97] A similar technique used a blended and centrifuged "gelatin slurry" as a support bath, thus keeping cells and printed biopolymers in aqueous, buffered, cell compatible environments. After printing, the temperature of the bath is raised to 37 °C in order to melt the gelatin slurry support, which allows the print to be surrounded by the appropriate culture media.[52] A major advantage of this technique is the ability to print helical and knotted paths through the *z*-direction as well as other shapes that do not require the standard stacked layer-by-layer additive manufacturing.

1.9. FUSED DEPOSITION MODELING (FDM)

Fused deposition modeling (FDM) and other extrusion-based systems are the most widely used 3D printing strategy. The technique (Figure 7a) often employs thermoset materials that

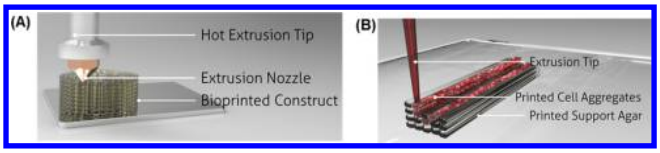

Figure 7. (A) Schematic of a FDM printing process. (B) Schematic of the 3D cell-printing process which directly extrudes cellular aggregates a 3D building blocks.

are heated to a semimolten state before extrusion then allowed to solidify on the printing stage. The printing hardware is a RD system with temperature controllers and heating blocks. Objects are built as a series of layers by directly extruding material as a continuous bead of thermoplastic. Print speed is variable and programmable and dependent on standard motor and drive specifications. Extrusion force is often driven pneumatically or mechanically with a lead screw. These systems are compatible with bioinks with higher viscosities than possible with bioinkjet printing. This process is compatible with a broad range of ink viscosities greater than 6×10^7 mPa/s.[31] This enables a broad selection of materials that can be used to create composite prints composed of many different materials and exhibiting multiple properties. Mechanical properties and culture environments can be patterned in three dimensions.

Traditional FDM printers use plastics that are molten around 250 °C, but synthetic biomaterials such as poly(caprolactone) (PCL), poly(lactic acid) (PLLA), and PLGA have demonstrated adequate thermoplastic performance in these systems at slightly lower temperatures.[89,98] Exploiting thermoplastic biomaterials is advantageous in that the addition of chemical cross-linkers is not required. Although traditional FDM printers use plastics that are molten at 250 °C, biomaterials that can be molten or thermally cross-linked near body temperatures are more suitable for printing with biological inclusions.

Thermoplastic hydrogels must be printable in the range of 37 °C to enable the inclusion of cells. Exploiting low-temperature thermoplastic biomaterials is advantageous in that chemical cross-linkers are avoided, and biologicals can be added as a single step. However, without the cross-linkers, the printed gels may lose integrity and suffer from degradation of printed geometry in culture conditions. In addition to thermoplasticity, materials with shear-thinning properties can be exploited in extrusion-based printing strategies. Several biomaterials such as silk and poly-L-lactide (PLA) exhibit a non-Newtonian decrease in viscosity in response to increases in shear rate.[99,100] The printing hardware can be designed to controllably alter shear rates that are present at the nozzle during extrusion. In response to the high shear rates the viscosity of the bioink will drop significantly, allowing the material to flow through the nozzle.[98,100] After printing onto the substrate, the immediate decrease in shear rate allows restoration of the bioink to the initially higher viscosity state. Compared to bioinkjet strategies, printing hardware capable of high extrusion forces allows printing of much higher suspended cell densities. Starting closer to physiologically relevant cell densities is a critical improvement in the efficiency of printing tissues. However, cell viability can be lower after extrusion printing. Increasing extrusion pressure or decreasing nozzle diameter can affect membrane integrity thereby reducing cell survival rates to the range of 86 to 40%.[73,101] Reducing dispensing pressure as a means to reduce shear stress imparted to cellular inclusions is also prone to increased nozzle clogging. Increasing the nozzle diameter will reduce shear stress at the cost of printing resolution.

Recent research has used a fusion of thermoplastic and high viscosity hydrogel bioink to print an engineered muscle-tendon

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unit where the thermoplastics are used to mimic the biomechanics of the tissue with the cellular bioinks serving as the source cellular source for tissue development. The muscle side of the construct consists of thermoplastic polyurethane with C2C12 myoblasts for the cellular additive, whereas the tendon section contains PCL and NIH/3T3 fibroblasts. Both thermoplastic structural polymers and cell-laden bioinks were printed on the same tri-axis stage with four spatially separated reservoir cartridges.[102] Although it remains to be seen whether similar thermoplastic-bioink blends will enter the mainstream, simpler FDM systems have printed cell types ranging from corneal stromal cells[98] to dermal fibroblasts[98] and have been used to fabricate *in vitro* pharmokinetic[103] and tissue models such as tumor models, aortic valves, and branched vasculature.

1.10. 3D CELL-PRINTING

3D cell-printing is a process in which solid cellular units are directly printed as additive components, in contrast to strategies which use liquid bioinks (Figure 7b).[69] This technique enables scaffold-free bioprinting, free of exogenous biomaterials.[104,105] The cellular aggregates are printed as cylindrical or spherical building blocks with diameters ranging from 260 to 500 μm per aggregate.[69] Fabrication of the aggregates initially requires standard 2D expansion of cells on tissue culture plastic.[106] This has required cells to be passaged and harvested enzymatically using standard protocols; however, novel processing steps are being developed.[69]

After cells are collected and resuspended in culture medium they are shaken for 45 to 120 min in order to initiate cell aggregation.[69] Centrifugation is used to form a pellet of the aggregates which are then aspirated and extruded into molds of desired shapes such as cylinders. The aggregates are maintained in the molds until they exhibit sufficient mechanical integrity as semisolid structures. Afterward, aggregates can be removed from the molds and sectioned into smaller cellular building blocks or returned as is to a suspension culture or printing cartridge. These building blocks are stored in the cartridges until they are directly extruded through the printer head via positive displacement.[106] Cells and cellular aggregates can be deposited in complex geometries. Multiple cell types can be organized in precise coculture patterns. Printing equipment which enable this process, such as the NovoGen MMX Bioprinter (Organovo, San Diego, CA) rely on the concept of tissue liquidity.[69] This concept is based on observations of early developmental biology where the process of morphogenesis appears to be driven by self-assembly.[107] In particular, this technique relies on the innate ability of multicellular aggregates in close proximity to fuse into 3D constructs. Directly after printing, the constructs are fragile and lack cohesive tensile strength. Over time, cells will influence each other by proximity-based cues which subsequently drive maturation into constructs which resemble normal tissues. Successful fusion and maturation of the constructs is dependent on cell type and substrate properties. This approach has been successfully demonstrated using bioprinted liver tissue.[105,108]

A similar technique uses coaxially extruded alginate capsules as casts for cell pellets, which the authors term "tissue strands." These strands are first fabricated from aspirated cell pellets injected into the tubular alginate cast, which are then cultured for 5−7 days in a process facilitating aggregation of cells, followed by de-cross-linking of the alginate by addition of sodium citrate. This technique has thus far shown confluent fibroblast culture with focal-adhesion assembly as well as support for insulin producing βTC-3 cells.[109]

This technique takes advantage of instrinsic cellular development processes from having cells in close contact with neighbors, and has recapitulation of dense native tissue without chemical or enzymatic cross-linkers to facilitate cell aggregation. However, a potential disadvantage of this technique is the comparatively long construct development time, especially with applications where the constructs must be subjected to immediate handling or mechanical stress. Expanding the application to a variety of human cells may also present additional unforeseen challenges.

1.11. LASER GUIDED DIRECT WRITING (LGDW)

Laser guided direct writing (LGDW) is a laser-assisted bioprinting technique capable of depositing cells with micrometer accuracy on diverse surfaces and matrices, including soft gels. This technique utilizes a weakly focused laser beam for cell deposition based on the principles of laser tweezers. The beam is able to control cell deposition via optical forces at a range of a few millimeters. Deposition patterns can be programmed as with other techniques and notably heterotypic interactions between hepatocytes and endothelial cells have been explored in millimeter scale culture viable for up to two months in culture.[110] The major drawback to this approach is the difficulty in applying this technique to the fabrication of 3D constructs, especially with the often limited size of cell arrays, and the current inability to move cells to stack in the *Z*-direction.[111]

1.12. 3D POWDER (3DP)

3DP printing effectively transforms inkjet technology to a true additive manufacturing approach. 3DP printing technology fuses layers of powdered biomaterial such as starch, dextran, gelatin or calcium phosphates.[112] However, rather than depending on sintering, particles are fused using a binder solution such as water or phosphoric acid which is deposited onto the powder bed with a print head (Figure 8).

Figure 8. Illustration of the 3DP process using powdered biomaterials and printable binding solutions.

This technique provides more options for tissue engineering and drug-delivery because incorporated bioactive components are not subjected to deleterious effects of laser-mediated fusion or toxic solvents. However, aqueous binding agents often leave prints water-soluble, and require further postprocessing. A major limitation of powder systems is the difficulty in removing internal unbound powder from desired negative spaces such as hollow chambers. Biomaterial powders such as tetracalcium phosphate and calcium sulfate have been bound with solutions

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such as citric acid to generate bone-like constructs.[113,114] Porous ceramic scaffolds with fully interconnected channels have also been made via 3DP directly from hydroxyapatite (HA) powder for bone replacement.[57] For example, patient-specific replications of skull components for the repair of craniofacial defects have been reported (Figure 9b).[54,112] The initial geometry is accurate but these prints require more than 6 h of postsintering at 1200 °C in a curing oven in order provide sufficient mechanical strength.[54] This approach limits this technique to scaffold construction rather than direct deposition or patterning of living cells.

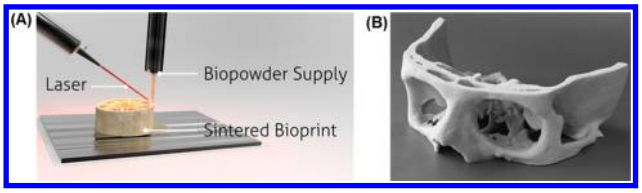

Figure 9. (A) Schematic of the selective laser sintering process applied to rapid biomaterial scaffold prototyping. (B) Tetracalcium phosphate and calcium sulfate with 30 wt % citric acid were used to replicate skull components.[54] Initial prints were treated with 6 h of postsintering at 1200 °C to provide strength.

1.13. SELECTIVE LASER SINTERING (SLS)

Rather than employing the multiple steps of a 3DP strategy, a laser can be used to raise powder temperature beyond the glass transition, generating fusion. Powdered polymeric materials are sintered together into cross-sectional slices onto which subsequent layers can be directly sintered with additional layers of powder (Figure 9a). Because a laser is used to directly and precisely sinter robust biomaterial constructs a preexisting support structure is not needed. The limitations of this technique are chain degradation, oxidation, material shrinkage, and difficulty in controlling porosity. Selective laser sintering is applied to rapid scaffold prototyping in much the same fashion as in the industrial fabrication of metal or plastic components.

In bioprototyping, SLS has most successfully been applied to fabricate bone replacements. The most successful applications have utilized HA to generate ceramic skeletal implants.[61] OsteoFab bone grafts utilize a proprietary process which combines laser sintering technology and a powder formulation (OXPEKK). The OsteoFab patient-specific facial devices (OPSFD) are biocompatible, mechanically similar to bone, radiolucent, and osteoconductive. These devices have recently received FDA approval.[115,116]

1.14. COMMERCIALIZING BIOPRINTING

As the field of bioprinting was only recently defined, characterization of potential products is somewhat ambiguous currently but expected to be well-defined over the next few years. In 2014, the global 3D bioprinting market was valued at $487 million; however, significant growth is expected over the next decade.[117] 3D printing and bioprinting technology were founded in North America, and the region is anticipated to gain on its share of the global market in terms of revenue by growing its 35% market share at a compound annual growth rate of 10.0% through 2022.[117] By then, the global 3D bioprinting market is expected to reach $1.82 billion and include products and materials for dental, medical, analytical, and food applications.[117]

Globally, there are a number of young companies contributing to various niches within the bioprinting field (Figure 10). Some companies are focused on improving printing hardware and/or printing materials; while others are applying bioprinting strategies in the development of high-throughput assays, commercialized tissue models, or grafting products for trauma repair.

The world leading 3D printing company in sales, *Stratasys* (Eden Prairie, MN), has been slow to enter the bioprinting market. Currently, *Stratasys* offers no bioresorbable materials and only a single material (Objet MED610, Stratasys, Eden Prairie, MN) FDA approved for less than 48 h of mucosal membrane contact.[118] The uncertain risks associated with development in a new biomedical market such as bioprinting remain as deterrents to larger scale efforts. In addition,

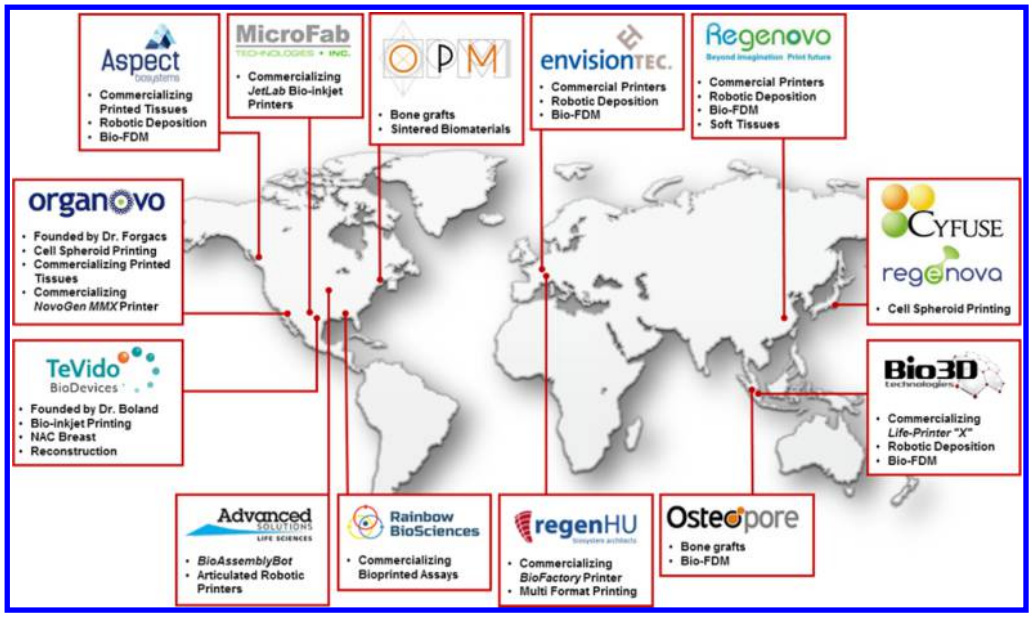

Figure 10. Representative list of major companies involved in bioprinting as of March 2016.

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strategies to overcome regulatory and logistic hurdles associated with future bioprinted products may prove as challenging as the technology itself.

The lack of a clear leader or leading approach leaves opportunity for other companies to develop dominance in bioprinting as an independent 3D printing field, uninfluenced by the current players in traditional plastics. A major competitor, *3D Systems* (Rock Hill, SC), has produced a larger number of biocompatible plastics (VisiJet Series, 3D Systems, Rock Hill, SC), as well a broader and more relevant material palette including direct metal printing (Direct Metal, 3D Systems, Rock Hill, SC). *3D Systems* highly diversified model can 3D-print with metal, sugar, and ceramics, which leaves the company with fewer barriers to enter bioprinting.

Hewlett-Packard (Palo Alto, CA) may be the largest company to enter the biotechnology space. Hewlett-Packard (HP) currently offers a bioplotting platform (HP D300e, Hewlett-Packard, Palo Alto, CA) capable of dispensing pico- to microliters of small molecules and biomolecules in DMSO or aqueous solution. This precision dispensing solution enables production of custom dose−response studies and other assays on-demand. Although the HP product line does not yet include 3D bioprinting technologies, their current experience with the hurdles of producing bioplotting hardware and biomaterials would be a logical fit.

A number of commercially available 3D bioprinters currently exist. The 3D Bioplotter (EnvisionTEC, Gladbeck, Germany) is marketed as a device compatible with a broad list of materials. Commercial systems such as the 3D Bioplotter which employ a more fundamental deposition technique and make use of a standard 3-axis stage may be better suited to laboratories focused on ink development because they do not possess overspecialized components which would narrow material compatibility.

Advanced Solutions (Louisville, KY, US) offers unique features with their 6-axis Bioassemblybot. This unit uses an articulated arm which can apply biomaterials in a number of more complex orientations not possible with standard XYZ platform designs. These features are difficult or expensive to duplicate with commercially available hardware which makes these units attractive. The value of prebuilt systems like the Bioassemblybot is further justified due to the added difficulty of software development compared to standard 3-axis systems. The downside is that these 3D bioprinters tend to carry considerable price tags, typically more than $100 000 per unit. These prices are in the range of current industrial level 3D printers publicly offered by Stratasys or 3D Systems. Since 2014, two small startup companies, CellINK and Biobots, have emerged and sought to bring low cost commercial bioprinters to market. These units are being sold for $5,000 to $10,000 and stand out for their portability and ease-of-use. Biobots, founded in 2014, has developed a desktop bioprinter marketed as an entry-level consumer product and now offers custom inks and support material priced at $1,000 per 100 mL. Swedish based CELLINK entered the market with a similar business model and features affordable and high precision bioprinting systems with advanced features such as clean chamber technology with filtered airflow.

As more sophisticated printing strategies are available, a major bioprinting hurdle still lies with developing bioink formulations, specifically with material properties, printability, and curing strategies matched to specific needs. A focus on polymer chemistry and materials science to achieve non-deleterious processing is needed. There are companies making advancements toward providing bioprinted products such as Oxford Performance Materials (South Windsor, CT). Among other products, Oxford Performance Materials (OPM) currently develops 3D printed patient-specific polymeric implants for cranial prostheses. In 2014, OPM was the first company to receive FDA clearance to manufacture 3D printed patient-specific facial implants.[119] As OPM continues to innovate, they may be considered the leader of acellular 3D bioprinted products. OPM is not the only Biotech company to receive regulatory approval for an acellular product. In 2015, Aprecia Pharmaceuticals (Langhorne, PA) received the first FDA approval for 3D printing Spritam, an epileptic drug treatment.[117]

Because of the state of the market, smaller companies have been granted competitive development space in the field of 3D printing cell-based "tissue" products. Major steps occurred in 2009 when Organovo and Invetech created the first commercial bioprinter, the NovoGen MMX[8] and in 2014, when Organovo applied bioprinting techniques to produce the first commercially available liver tissue model.[12] Organovo Holdings (NYSEMKT:ONVO) has been able to generate revenue (e.g., $328 000 for Q3 2016). Although modest, this revenue is a historic achievement because it can be considered a first for 3D bioprinted cell-based products.

1.15. QUALITY ASSURANCE AND REGULATORY HURDLES

As a technology AM has raised questions because it has put the ability to manufacture complex and functional geometries into the hands of many. The fear that somebody might be able to make a plastic weapon that would bypass metal detectors is an example of the potentially harmful application of these technologies. The regulation of source code CAD files has raised concerns due to a precarious balance between first amendment rights and possible infringement on patented intellectual property. Regulating these source codes would entail restriction and/or censorship of Internet file-sharing capabilities.[120] Many have suggested that a more feasible approach would be the regulation of both AM equipment and the raw materials.[121] In the context of bioprinting, regulations would involve pharmaceuticals, biologicals, and nonbiological raw materials. Each of these materials are governed by different entities and must undergo specific regulatory paths. Finally, the regulation of surgical procedures in the context of bioprinting must also be considered.

The Food and Drug Administration is the regulatory agency that governs medical devices and materials intended for human use in the United States. Currently the FDA does not have a category for bioprinted products, but rather classifies them either as drugs, biologics, medical devices or combination products.[122] Each of the product types is regulated by a different office within the FDA, either the Center for Drug Evaluation and Research (CDER), the Center for Biologics Evaluation and Research (CBER), or the Center for Devices and Radiological Health (CDRH).[123] Products which contain or are derived from cellular therapies are treated as "biologics" and governed by Section 351 of the Public Health Act and the more stringent section 1271, which governs manufacture of human cells, tissues, and cellular- and tissue-based products. New surgical procedures are not governed by the FDA, and can be used as needed by surgeons, with procedural safety regulated by the Health and Human Services. In addition, the final new

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Table 2. Toolkit of Natural and Synthetic Bioink Components Used in the Literature

molecule class	polymer	common gel transition method	limitations	unique property
protein	collagen	physical and chemical cross-linking[135]	potentially immunogenic[136]	collagenase proteolysis[137]
	elastin	self-assembling[138]	hydrophobicity[138]	elastase proteolysis[139]
	fibrin	self-assembling[140]	thrombogenicity[141]	excellent cell-adhesion[142]
	gelatin	physical or chemical gel formation[143]	water-retention[144]	biocompatibility and cell-adhesion[145]
	silk fibroin	physical and chemical cross-linking[146]	need for bulking agent[147]	shear thinning and tunable mechanics[148]
	Matrigel	self-assembling[149]	batch variability[150]	reproduction of ECM[150]
glycosamino-glycan	chondroitin sulfate	coacervate gel formation[151]	needs chemical cross-linking[152]	chondroitinase proteolysis[153]
	hyaluronan	ionotropic gel formation[154]	needs chemical modification[155]	cell adhesion and hyaluronidase proteolysis[156]
polysaccharide	agarose	gel formation below 36 °C[157]	nonbiocompatibility[158]	ether functionality[159]
	alginate	ionotropic gel formation[160]	L-guluronic content nonbiocompatibility[161]	hydrolysis chelation degradability[160]
	k-carrageegan	ionotropic gel formation[161]	potential immunogenicity[162]	hydrolysis degradation[163]
	chitosan	chemical gel formation[160]	high viscosity[164]	antibacterial/antifungal[26]
	dextran	ionotropic gel formation[165]	potential immunogenicity[166]	aqueous two-phase microsphere formation[167]
	gellan gum	ionotropic gel formation[168]	poor cell adhesion[169]	transparent[170]
	methylcellulose	methylation-dependent physical gel formation[171]	nondegradable[171]	natural biocompatibility[171]
polyethers	poly(ethylene glycol)	covalent cross-linking[172]	poor cell adhesion[172]	soft and hard tissue compatible[173]
polyesters	poly(caprolactone)	covalent cross-linking[174]	slower degradation from hydrophobicity[174]	FDA approval for several devices[174]
	poly(glycolic acid)	covalent cross-linking[175]	potential immunogenecity[176]	bioabsorable polymer[175]
	poly(glycerol sebacate)	covalent cross-linking[177]	potential acidic biodegradation byproducts[178]	recovery from mechanical deformation[179]
	poly(lactic acid)	covalent cross-linking[180]	potential immunogenecity[181]	finely controlled degradation[180]
	poly(propylene fumarate)	covalent cross-linking[182]	immunogenicity[183]	finely controlled tensile strength[184]
polymer (other)	poly(2-hydroxyethyl methacrylate)	covalent cross-linking[185]	nondegradable[186]	transparent[187]
	poly(vinyl alcohol)	covalent cross-linking[188]	poor cell-adhesion[189]	biocompatibility[188]
	poly(acrylamide)	covalent cross-linking[190]	neurotoxic monomer, nondegradable[191]	finely tunable stiffness[190]
poloxamer	Pluronic F127	self-assembly[192]	needs copolymer[192]	reverse thermal gelation[193]

medical device is a federally regulated product which must be tested through clinical trials before it can be used.[124] In the European Union, products in which physical cells are combined with a natural or synthetic scaffold are classified as advanced therapy medicinal products (ATMP). These products are currently regulated under the legislation for medicinal products or medical devices (Regulation (EC) No 1394/2007), which has recently been established as a common regulation for all member states.[125−127]

For most medical products, the raw materials and end-product regulations as described above are well-established. As a manufacturing process; however, AM techniques do not align well with current regulatory standards, which rely on the manufacturing process to be standardized, validated and continuously monitored.[123,128] Most AM technologies involve an individualized design and manufacturing process for every part produced, rather than a standard common process for all parts. The current regulatory frameworks do not account for these differences. These individualized processes pose considerable challenges in assessing product quality assurance and testing.[129,130] This customizable nature will require a strategy to ensure quality control in every step of the process: production of raw materials, design control of the 3D printed model, validation of the manufacturing process along with its governing software, and finally end product testing.

1.16. CONSIDERATIONS FOR FORMULATING BIOINKS

Ink formulations for initial applications of 3D printing did not require biocompatible properties. Now, the goal of printing human tissues has necessitated the development of cytocompatible inks formulated from biomaterials. Moreover, bioprinters need to be able to transform biomaterial-based inks into biologically functional 3D structures. Designing printable biomaterials can be challenging due to the complex set of requirements that a bioprinted construct must fulfill. Ideally, constructs will be derived from biocompatible components with mechanically biomimetic features and programmable degradation in order to facilitate resorption throughout biological remodeling processes. The bioink must also be capable of being fully cured using processing which is safe for biological materials, due to the sensitivity of cells and biomolecules. The bioink should also be optimized to minimize stress induced damage to biological material from the printing process. Some printing mechanisms involve localized heating or pressure-induced extrusion. In these cases, cellular inclusions may benefit from bioink formulations that exhibit low thermal conductivity or shear-thinning properties. As a minimal requirement, biomaterials must allow for cell attachment, proliferation and function, as well as the remodeling process after printing. Cells should ultimately be able to degrade and replace the printed materials with natural ECM at a desired rate.

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A variety of ECM components can be added to bioinks to ensure proper extracellular biomimicry. The most popular formulation strategy has been to use naturally derived polymers as the base component, including collagen, gelatin, fibrin, hyaluronic acid, chitosan, and silk in rapid scaffold prototyping.[55,56,131−133] These polymers possess specific biochemistry which mimic properties of extracellular matrix, thus manipulation of these polymers, and the biomimetic properties that they confer, allows control over cell attachment and subsequent migration on and through a printed scaffold. This not only affects the proliferation of cells within the scaffold, but also the rate of integration with host tissue. The natural biochemical moieties of these materials reduce the need for further functionalization with surface ligands.

Additional degradable polymers such as alginate, agarose, agar, and PLA have been used in bioplotting and printing applications as well.[88,100,134] Synthetic polymers do not innately mimic ECM; however, they facilitate consistent processing and printing and are compatible with many functionalization strategies. Synthetic molecules such as PEG and others in Table 2 have been functionalized and used in several tissue engineering applications.

Ultimately, if a 3D bioprint is to differentiate into new functional tissue, cells must proliferate and replace the printed materials with new ECM. Resident cells drive this remodeling process by secreting proteases to initiate biomaterial degradation, or in the case of hydrolytic polymers, remodel the matrices during the loss of biomaterial. As a scaffold degrades, it can further impact the remodeling process depending on whether any toxic byproducts are produced during degradation. Toxic products can interfere with cellular physiology directly or by producing an unfavorable environment such as by affecting pH. The degradation of the chemical, physical, and mechanical properties of a scaffold should also be optimized. Physical geometry must be maintained in physiological conditions as designed to ensure adequate cell functions via mass transfer and related needs.

The strategies for bioink printing can be divided into two directions: bioink prints as functional scaffolds or scaffold-free printing strategies, which only use cells as the printing material. In both scenarios, viable cell-laden bioprints of relevant size require suitable nutrient transport through vasculature mimics or pore architecture. If the material is not sufficiently robust or exhibits excessive swelling, the intricate pore and vascular structure can be lost, preventing nutrients from supplying the cells in the constructs. The printed materials must also physically support the intended structure, thus mechanical properties must be sufficient for the designated tissue or location.

Bioinks also must often be stored in a reservoir before being transferred onto the printing surface. The properties of bioink blends vary, although most are stored as liquids. This is unlike the solid plastic filaments common to other 3D printing applications. The initial viscosities of bioinks also vary and must be taken into account when formulating compatibility with a bioprinter. Converting bioinks from a printable fluid state into the solid state within the short amount of time allotted for printing requires various types of cross-linking, and gel transition methods. Initial viscosity affects the print speed, the ability to build 3D structures, and in some cases dictates the cross-linking requirements. Structures printed with higher viscosity bioinks are initially more stable and may require less cross-linking to be resilient to culture conditions. These inks may also facilitate more rapid generation of scaffold height in a layer-by-layer approach compared to low viscosity prints. However, sufficient bioink cross-linking must occur within the timespan of a single print layer before subsequent layers can be deposited. A major challenge is exploiting cross-linking mechanisms in order achieve structural integrity without applying cytotoxic processing.

As seen in Table 2, many natural and synthetic polymers can be physically or chemically cross-linked into a gel state for printable hydrogels, which are versatile for tissue engineering. For example, alginate can be cross-linked ionotropically using $CaCl_2$ solution. Alginate and other hydrogels display low polymer density, high water content, and a variety of gelation mechanisms that make them good candidates for 3D printing. Thermoplastic hydrogels can also be used where temperature control can transform molten biomaterials such as agarose, gelatin, and PLGA, which later solidify on the printing substrate.[89,194] Agarose-based hydrogels are formed after chilling the molten form from 60 to 36 °C, while other materials such as gelatin can be printed at cooler temperatures and then dissolve away in culture conditions (e.g., 37 °C).

Alternative methods have been developed in order to circumvent some of the drawbacks of traditional thermoplastic and cross-linking strategies. Photopolymers can be extruded in a continuous bead or deposited as discrete droplets through a series of inkjet heads which are mounted in-line with a UV curing lamp. End groups of many biomaterials can be modified with photoinitiator molecules such as acrylate or methacrylate to allow for photo-cross-linking capability. The most extensively used biocompatible photocurable materials are poly(ethylene glycol) diacrylate (PEGDA),[195] naturally derived gelatin methacrylate (GelMA)[196] and hyaluronic acid.[197] Photopolymerization can be processed under biologically compatible environments with no temperature dependence and no stringent post-processing conditions. However, photoinitiators and photosensitizers are often needed to ensure fast cross-linking, which limits the amount of materials that can be used as well as the biocompatibility of the products.[198]

1.17. BIOPRINTING BOTTLENECKS AND FUTURE NEEDS

Over the past two decades, there has been a tremendous amount of process in manufacturing living 3D constructs and there is currently an abundance of literature reports that demonstrate various levels of cell viability after bioprinting. However, cell survival may not give an accurate representation of bioprinting success, as functionality of the final tissue product should be considered the key outcome. Cells must perform their essential intended functions in the printed tissue construct, and maintain this function for a physiologically relevant length of time. At present, most reports limit studies of cell viability of function to days or a few weeks at best, yet most of these systems must function for much longer.

For more than a decade, material development has been a bottleneck for rapid scaffold prototyping. UV light, chemical cross-linking, and high temperatures negatively impact most biologically active additives,[7] thus printing strategies which utilize cellularized matrix gels and cell-pastes while avoiding deleterious curing mechanisms have been developed. However, the majority of these prints lack initial mechanical strength and are vulnerable to a wide range of external conditions resulting in melting, dissolution, or warping of the printed structures.[199] Higher cell concentrations may also inhibit some of the

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hydrogel cross-linking mechanisms.[132] Thus, there seems to be a balance between maintaining and differentiating cells at biologically relevant densities in bioprinted constructs and producing robust biopolymer constructs that can withstand mechanical and chemical stimulation.

This compromise between structural strength and biocompatible processing severely limits applications such as biomedical implants and modern composite scaffolds with structural components and microfluidic vasculature. These factors, structure/strength of the construct vs biological compatibility, seem at odds in terms of materials formulations for bioprinting. A strategy enabling the simultaneous printing or codeposition of bioactive components (molecules or cells) and biodegradable structural components, which either utilizes nondeleterious postprocessing; or does not require postprocessing, would overcome this problem. Further, codeposition of two or more streams, each optimized either for materials for biological functions, could also be used to overcome current limitations, although complicating the engineering and overall printing process.

The great hope of 3D bioprinting for producing reproducible biological constructs lies in two realms: producing structures for studies into implantation and regenerative medicine, and providing automated assays for high throughput *in vitro* drug and toxicity studies in lab grown tissues. To reach these goals, researchers must consider the complex interplay between developmental biology of proliferating and differentiating cells into new tissues and organs as well as material and printing issues that enable safe and inert formation of these constructs in a method which allows for repeatable and robust bioprints with long-term viability. Although these demands are lofty, the explosion of new literature and technologies involved with 3D bioprinting and AM presents an exciting field for researchers hoping to propel these processes into the mainstream of fields from basic science to clinical medicine.

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Notes

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