Guiding Lights: Tissue Bioprinting Using Photoactivated Materials

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Guiding Lights: Tissue Bioprinting Using Photo-Activated Materials

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Abstract. Photo-activated materials have found widespread use in biological and medical applications and are playing an increasingly important role in the nascent field of three-dimensional (3D) bioprinting. Light can be used as a trigger to drive the formation or the degradation of chemical bonds, leading to unprecedented spatiotemporal control over a material's chemical, physical, and biological properties. With resolution and construct size ranging from nanometres to centimeters, light-mediated biofabrication allows multicellular and multimaterial approaches. It promises to be a powerful tool to mimic the complex multiscale organization of living tissues including skin, bone, cartilage, muscle, vessels, heart, and liver, among others, with increasing organotypic functionality. With this review, we comprehensively discuss photochemical reactions, photo-activated materials, and their use in state-of-the-art deposition-based (extrusion and droplet) and vat polymerization-based (one- and two-photon) bioprinting. By offering an up-to-date view on these techniques, we identify emerging trends, focusing on both the chemistry and instrument aspects, thereby allowing the readers to select the best-suited approach. Starting with photochemical reactions and photo-activated materials, we then discuss principles, applications, and limitations of each technique. With a critical eye to the most recent achievements, the reader is guided through this exciting, emerging field with special emphasis on cell-laden hydrogel constructs.

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1. INTRODUCTION

Life on earth has evolved diverse mechanisms to harness the power of sunlight. Accordingly, the natural world is replete with examples of how living organisms interact with light to store energy, catalyze reactions, detect the external environment, and actuate. In addition to these natural examples, the discovery of light-emitting and light-sensing proteins¹⁻⁴ has revolutionized biotechnology, resulting in disease treatment (photodynamic therapy)⁵⁻⁷ and control of cellular processes at an unprecedented level of spatial and temporal resolution. The conformational changes of membrane receptors, in particular the channelrhodopsins, have established the field of optogenetics,⁸⁻¹⁰ and allow spatiotemporal control of neuronal activity¹⁰ but also control of gene expression of multiple cell types¹¹ and migration.¹² Recent biotechnological developments make possible light-induced editing of the genome by CRISPR-Cas9 photocaging.¹³ By putting photolabile cages on molecules such as peptides, drugs, second messengers, mRNA, ATP, one can render a whole host of cellular processes light-activatable at the subcellular level.

The power of light is increasingly used not only to control the cells behavior, but also to more intelligently design their 3D biomimetic extracellular environment. Through the connection of photo-activated biomaterials with bioprinting methods, cellular scaffolds are being printed that have complexity not previously obtained. Examples include: 1) printing/guiding at the resolution of micrometer-sized biological structures such as capillaries¹⁴⁻¹⁵ and neurites¹⁶ 2) printing multimaterial and multicellular constructs¹⁷⁻¹⁹ 3) controlled patterning of biological cues in the matrix²⁰⁻²² and 4) changes in mechanical properties at subcellular spatial resolution.^{14, 23-24} Promisingly, there is an increasing number of examples where bioprinted constructs have shown enhanced functionality compared to conventional homogeneous tissue-engineered environments.²⁵ The hope for the field is that these tissues could one day be used as living replacements to regenerate injured or diseased tissues and organs and as predictive high-content screening platforms for drugs.

Living tissues and organs display a hierarchical organization, and it has been postulated that this organization can be recapitulated by the length scales of various chemical/material reactions and biofabrication techniques.²⁶ Not all of the biological length scales (from atomic, molecular, macromolecular, cellular, cellular functional unit, tissue, organ, to an organism) can be replicated by current bioprinting techniques, however, certain length scales are possible (Figure 1, Table 1). For example, the extrusion bioprinting is an excellent method for printing tubular structures of several hundred microns in diameter, as this is the dimension of the bioink strand, which is extruded through the printer's nozzle.²⁷ Vat polymerization (VP) bioprinting, on the other hand, is a method in which a structure is solidified from a vat of photo-sensitive resin through controlled exposure to light. The method

can be performed at very high resolution, suitable even for manipulating properties of the extracellular environment at sub-cellular resolution.^{22, 28-29} In Table 1, we summarize the basic features of four bioprinting methods (extrusion bioprinting, droplet-based bioprinting, and projection/scanning and two-photon VP) that have profited most from the power and utility of photo-activated materials. General features of these methods, including feature size, maximum structure size, cost, and applicability for multimaterial printing or high throughput (HT) applications are shown.

Figure 1. Bioprinting modalities can be divided into deposition- and vat polymerization-based techniques.

Table 1. Comparison of deposition bioprinting techniques (green) and vat polymerization bioprinting techniques (blue).

	DEPOSITION		VAT POLYMERIZATION	
	Extrusion printing	Droplet printing	Projection VP	Two-photon stereolithography
Max. structure size	cm	cm	cm	mm
Average time to print 1 cm³ block	20 min	N.A.	1 hr	Days
Typical feature size	100-600 μm	50-500 μm	25 - 150 μm	100 nm – 1 μm
Multimaterial	***	****	*	*
High throughput	**	****	**	*
Typical viscosity of ink/resin	10,000 Pa·s	<15 mPa·s ^a <200 mPa·s ^b	10 – 5000 mPa·s ^c <90,000 mPa·s ^d	Viscosity-independent
Cost of device (\$)	30-250k	5k	30k-50k	90k – 500k

^aInkjet printing ^bMicro-valve printing ^cConventional VP ^dVolumetric VP

To select the right printing method for the desired application, it can be useful to understand the hierarchical organization of tissues and organs. Tissues are typically defined as an aggregation of cells with similar structure and function. These cell groups can be categorized as falling under one of four types: connective, epithelial, muscle, or nerve.³⁰ Higher-level tissues, however, can also be composite in nature, being made up of cell groups from more than one category. For example, muscle tissue can contain epithelial type cells in its vessels and connective tissue in the peri-, epi- and endomysia as well as innervation.³¹ In another example, vascular tissue and lymphatic tissue are composed of a single layer of endothelium (a specialized type of epithelial cells) together with smooth muscle and adventitia (a type of connective tissue).³² Tissues can vary greatly in their cellular density, ranging from ~9000 cells/mm³ in tissues like cartilage³³ to ~150'000 cells/mm³ in solid epithelial organ tissues such as liver.³⁴ Organs can also be typed based on different criteria. For example, they can be ordered according to geometrical complexity, ranging from flat, tubular, hollow to solid organs.³⁵ Flat organs, such as cartilage³³ and cornea,³⁶ are avascular and contain just one or a few cell types. Most organs contain all four types of tissues. Figure 2 shows the important anatomical features of different types of organs that have been of interest in the biofabrication field.

Skin. Skin is a complex flat organ consisting of epidermis (epithelium), which is supported by an underlying dermis (connective tissue)³⁷ (Figure 2). As an epithelial tissue, epidermis provides key barrier, secretion, and protection functions. This tissue is primarily cellular with the packing density of cells around 35 cells/100 μm^2 .³⁸ An epithelial cell is polarized, with its basal domain/membrane facing the extracellular matrix (ECM) of the basement membrane, the apical side of the cell facing the lumen, and tight-junction-mediated contacts to neighboring cells laterally. Biofabrication of skin analogs has been considered relatively straightforward due to its 2D-like flat structure. A skin-mimetic construct with two characteristic layers of epidermis and dermis has been successfully biofabricated by an extrusion bioprinter (Figure 2 - Skin example).³⁹ However, the *in vivo* functionality of skin is also supported by glands, sensory neurons, immune cells, and hair follicles, which have complex microarchitectures and functions that are traditionally hard to mimic and still remain challenging. In particular, hair follicles have been the subject of tissue engineering and biofabrication approaches.⁴⁰

Blood vessels. Blood vessels, tubular organs, have a branching structure (Figure 2), where large arteries with a diameter of several millimetres branch into narrower arterioles (diameter 5-100 μm) and then to capillaries (diameter 5-10 μm).³² All structures of the macro/microcirculation are lined by a single layer of endothelial cells. All vessels except capillaries are enveloped by a circular layer of smooth muscle cells (tunica media) and an outer layer of innervated connective tissue (tunica externa).³² The arterioles play a particularly important role in vasodilation and regulation of blood pressure. This general layered organization is also seen in more complex hollow organs like bladder, stomach and esophagus, where the barrier, secretion, transport and sensing properties of the epithelium play an important role in the function of the organ. One approach to printing narrow blood vessels with μm resolution is to make use of two-photon stereolithography (2P-SL).¹⁵ Branched 3D microvasculature geometries were printed in native collagen. The endothelial layer was formed by HUVECs seeding after washing the uncrosslinked collagen from the vessel lumen (Figure 2 – Artery example).

Bone. Compact or cortical bone is a mineralized connective tissue with outstanding toughness due to its arrangement of collagen and hydroxyapatite mineral nanocrystals.⁴¹ The composition and ultrastructural architecture of the 3D ECM not only lend the mechanical strength⁴²⁻⁴³ but also provide the embedded cells with signaling and biophysical cues.⁴⁴ The tissue is organized into functional units called osteons (Figure 2). Osteons arise during the remodeling of the diaphysis of long bones.⁴⁵ Bone resorbing-osteoclast cells tunnel their way into bone, bringing blood vessels with them. Bone forming-osteoblasts then deposit layers of lamellar bone (osteoid) along the surface of the channels, incrementally narrowing the central channel, leaving only the small blood vessel to nourish the osteon. Osteoblasts, which are entrapped in the normal mineralized lamella, become osteocytes, which themselves are connected by a network of cytoplasmic synapses, filling channels called canaliculi.⁴⁶ Osteon-like concentric microgrooved patterns were created by combining photolithography with melt-casting (Figure 2 – Bone example). Microgrooved polycaprolactone surface patterns induce the corresponding cellular morphology and arrangement. Also, the surface exhibited suppressing of osteoclastogenic differentiation of RAW264.7 cells and modulated the secretion of osteoclastogenic cytokines by mesenchymal stem cells (MSCs). Osteoclast formation and activity created pathological conditions to delay the process of bone repair.⁴⁷

Liver. Solid organs such as the liver and the heart have also been of interest to the biofabrication field. In the liver, the parenchymal tissue is organized into hexagonal-shaped lobules, the functional repeating unit of the liver.⁴⁸ Each lobule is a mini-filtration system in which blood from the hepatic artery and portal vein enters the lobule at its outer spokes and passes by the hepatocytes before exiting through the central vessel. The hepatocytes are polarized based on exposure to gradients in nutrients, oxygen, and morphogens.⁴⁹ Lobule-mimetic hexagonal structures have been successfully biofabricated using projection VP with multimaterials and multiple cell types (Figure 2), where enhanced hepatic cellular functions were confirmed compared to 2D or single-cell type culture.⁵⁰ Still, most biofabricated structures so far retain very simplified features compared to the native tissue in terms of layer numbers, cell types, and the presence of perfusable vessels. Furthermore, with the current VP technique, it is difficult to achieve HT biofabrication of such microarchitectures requiring high resolution, which should be addressed if one aims to replace a whole organ or large piece of tissue with bioprinted one.

Heart. The heart, composed primarily of cardiac muscle, is a highly cellular contractile tissue, again with hierarchical organization.⁵¹ The cardiac muscle or myocardium is embedded between the epi- and endocardium layers. The myocardium is composed of single cells, the myocytes, which are around approximately 100 μm in length and are joined together at their long ends by intercalated discs, structures that assure tight connectivity and synchronized contractility of the tissue. The cytosol of myocytes is filled with myofilaments, themselves composed of sarcomeres, the basic contractile

unit of muscle. In biofabrication of this organ, scaffolds to help achieve alignment myotubes are critical. One approach has been to use bioprinting to extrude hydrogel strands, which then guide the seeded cells to form oriented, contractile cardiac tissue (Figure 2 – Heart example).¹⁹

In addition to microarchitectures, solid organs have, by necessity, developed a complex arborization of blood vessels, lymphatics and neural “stromata”. Metabolically active cells can be no more than a few hundred microns away from the nearest capillaries (the size of most “functional units”) due to the diffusion distance of oxygen.⁵² This organic structure of solid organs is challenging to achieve by any engineering method, and thorough knowledge of the complex and interdependent pathways and crosstalk involved in organ morphogenesis is important. In addition to the significant challenge of engineering tissues of this spatial complexity, epithelial solid organs have a very high density, with multiple cell types, making tissue engineering very challenging from a metabolic demand perspective. We believe that photo-activated biomaterials and bioprinters of increasing sophistication will enable the engineering of tissues and organs that mimic their native counterparts to the extent that they can be used in clinical settings.

Figure 2. Hierarchical organization of flat (skin), tubular (artery), and solid (bone, liver, heart) organs of the body and relevant examples from the biofabrication literature. Skin: adapted from with permission ref 39. Copyright 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. Artery: adapted with permission from ref 15. Copyright 2016 WILEY-VCH Verlag. Bone: adapted with permission from ref 47. Copyright 2019 Elsevier Ltd. Liver: adapted with permission from ref 50. Heart: adapted with permission from ref 19. Copyright 2016 Elsevier Ltd.

The goal of this review is to provide an overview of the light-based biofabrication toolbox, which consists of photochemistries, photo-activated polymers, and bioprinting technologies (Figure 1). In the photochemistry section, we restrict ourselves primarily to those reactions, which can be performed in water, are relatively fast, and occur under physiological conditions, allowing for cell encapsulation without significant cytotoxicity. For the polymers, we concentrate on hydrogels, which are three-dimensionally crosslinked hydrophilic polymer networks that can absorb large amounts of water without dissolution and provide an excellent mimic of native extracellular environments of cells. For the bioprinting methods, we focus on the most common deposition-based bioprinting methods (extrusion and droplet printing) and vat polymerization-based bioprinting methods (one-photon and two-photon) (Figure 1). We discuss which photochemistry, polymer, and technology combination is appropriate to achieve a desired print quality and resolution, and which combinations are compatible with the specific biological demands of the embedded cells. Finally, we adopt quite a broad definition of the word “printing”, namely as an automated process in which cells, materials, and biological cues are drawn onto a 3D canvas according to a pre-designed (computer) model. In this analogy, the palette consists of photo-activated polymers, cells, and biological molecules, and we refer to these materials as **bioinks** when used in deposition-based bioprinting, and **bioresins** for vat polymerization-based bioprinting. The “paintbrushes” are the printers, which can draw 3D structures using photo-activated formation of chemical bonds and erase material from the drawing by means of photo-activated cleavage of bonds.

2. PHOTO-ACTIVATED MATERIAL TOOLBOX

From a biomaterial chemistry perspective, photochemical reactions can either lead to the formation of new bonds (photocrosslinking) or the cleavage of existing bonds (photodegradation). The constructive and destructive nature of photo-activatable compounds allows for the biofabrication of biomaterials with predetermined microstructure, to modulate hydrogel stiffness⁵³, to degrade hydrogel to obtain high precision patterns in mechanical properties⁵⁴, or to specifically immobilize/remove biochemical cues.⁵⁵ By doing so, hydrogels able to mimic the native ECM can be achieved. Light-induced biofabrication of tissue models is also dependent on the appropriate selection of hydrogel precursors, photoinitiators (PIs), and light dosage. The following section will survey various photochemical processes and materials and their applicability in 3D bioprinting technologies for cell-laden hydrogels.

2.1 Light-Induced Crosslinking

Using light in 3D bioprinting techniques is already well established, perhaps most widely in preparing crosslinked hydrogels containing viable cells.⁵⁶⁻⁵⁷ There is a broad range of photo-activated groups that, upon light stimulus, either form molecules with unpaired valence electrons called radicals, or undergo an internal electronic activation, allowing them to react. Crosslinking based on radical polymerizations has found widespread usage in 3D bioprinting, as radicals are, contrary to ionic species, stable in aqueous physiological conditions and tolerant to hydrophilic compounds. Other possible strategies for light-induced crosslinking are cycloaddition reactions such as Diels-Alder reactions or 1,3-dipolar cycloaddition. Depending on the polymerization mechanism, crosslinking can proceed either through a chain-growth (Figure 3A) or step-growth process (Figure 3B).⁵⁸

visible radiation. NVP was reported to significantly reduce the oxygen inhibition time, increase the rate of polymerization and the final monomer conversion for the synthesis of hydrogels.⁶⁷⁻⁶⁸ Recently Aguirre-Soto et al. proposed that the reduction in oxygen inhibition is due to the complexation between NVP and Eosin Y, which enhances the rate of photoinduced electron transfer to the coinitiator triethanolamine, while two oxygen molecules are consumed in this event.⁶⁷

Another approach takes advantage of thiol reactivity to reduce oxygen inhibition. While peroxy radicals incorporated into polymer chains are not reactive toward monomer addition and propagation, they are reactive toward hydrogen abstraction from a thiol functional group.⁶⁹⁻⁷⁰ The generated thiyl radical can then undergo addition reactions with monomers to form thioether bonds and carbon-centered radicals that are able to propagate further. This approach applied to hydrogel formation uses multifunctional thiols in conjunction with multifunctional (meth)acrylates. Crosslinking occurs by a mixed-mode polymerization mechanism, where competition exists between the chain-growth homopolymerization of the (meth)acrylate and the step-growth mechanism of a thiol-ene polymerization.^{62, 71}

Secondly, radical photocrosslinking is defined by reaction diffusion-controlled kinetics. The mobility of macroradical chain ends is strongly limited, so that the only way for radical sites to move is through the propagation reaction of unreacted double bonds, so-called reaction diffusion.⁷² Thus, termination can occur only by propagation reactions. The photopolymerization of multifunctional vinyl monomers leads to a considerable amount of unreacted double bonds. In the context of 3D bioprinting, the complete functional group conversion and oxygenated conditions are critical for the viability of living cells in the crosslinked polymer network. Moreover, radical species' reactivity in the crosslinked polymer network is locally driven by diffusion (trapped carbon radicals) and results in high microstructural heterogeneity. It was observed that radical photopolymerization of multifunctional monomers produces a heterogeneous network containing highly crosslinked regions (microgel domains), as well as less densely crosslinked regions.⁷³ Due to the inhomogeneous network architecture, which is created during the radical curing process, the resulting hydrogel materials show shrinkage stress that could lead to deformation or mechanical failure of the construct.⁷⁴

Another drawback is that the formation of the highly crosslinked hydrogel can lead to volume relaxation of the polymer toward their equilibrium specific volume. The process of volume relaxation can occur at a longer than or similar time scale to polymerization kinetics and is accompanied by volume shrinkage and stress development. Volume shrinkage behavior is significant for applications such as 3D bioprinting where shape- and volume-fidelity of the printed construct are important.⁷⁵⁻⁷⁶

2.1.2 Photoinitiated Step-Growth Polymerization

Thiol-ene Reactions: Highly efficient reactions of thiols with reactive carbon-carbon double bonds (enes) have demonstrated tremendous applicability in hydrogel network formation from photocrosslinkable materials.⁷⁷⁻⁷⁸ Thiols (Figure 4 (1)) react with alkenes (2) by radical-mediated or base-catalyzed reactions. The radical-mediated thiol-ene reaction requires a PI to generate a radical initiator species (3) that abstracts the hydrogen radical from the thiol to form a thiyl radical (4). Then, the thiyl radicals attack the double bond of the alkene, generating a carbon-centered radical (5), which then abstracts the hydrogen radical from another thiol to regenerate the thiyl species, thus repeating the cycle. Thiol-ene hydrogel networks are formed rapidly and quantitatively under ambient conditions. Moreover, they produce polymer networks that are highly uniform with reduced shrinkage and mechanical stress.⁷⁹ In contrast to conventional radical chain-growth polymerization, thiol-ene step-growth polymerization is insensitive to oxygen inhibition. All these properties make thiol-ene reactions suited for hydrogel formation in the presence of cells and biological materials.⁸⁰ A potential drawback for crosslinking is that the thiol-ene reaction cannot be considered strictly bioorthogonal. Specifically, the presence of a thiol-containing natural amino acid, cysteine (abundance 1.7%)⁸¹ may be able to generate thiyl radicals, which can compete during crosslinking. However, several cell types have been successfully encapsulated into thiol-ene hydrogels, including human MSCs⁸², fibroblasts⁸³⁻⁸⁴, fibrosarcoma⁸³, and pancreatic beta-cells⁸⁵.

Thiol-ene hydrogels are prepared by covalent crosslinking of macromers containing thiols and alkenes. Reactive groups are introduced on macromers by a reaction with functional groups commonly found on natural and synthetic polymers, including amines, carboxylic acids, aldehydes, and reduced disulfides. The choice of the alkene species also influences the polymerization mechanism. Norbornene, vinyl sulfones, vinyl ethers, and maleimides moieties have been increasingly utilized due to the incapability of their carbon-centered radical to homopolymerize through the chain-growth mechanism (Figure 4, gray shading).⁸⁶⁻⁸⁷ In such systems, the network formation proceeds only by step-growth mechanisms (Figure

4). Although a judicious selection of thiol-ene reactants and conditions can minimize side reactions, thiol-functionalized macromers can react with each other to form disulfides.⁸⁸

Recently, Chu et al. identified that the presence of encapsulated chondrocytes alters the formation of PEG hydrogels by chain-growth radical polymerization and thiol-ene crosslinking.⁸⁹ It is hypothesized that chondrocytes interact and deplete propagating radicals and thiol crosslinker before hydrogel formation. Hence, hydrogel immediately surrounding the chondrocyte resulted more loosely crosslinked than the bulk hydrogel, leading to the development of a stiffness gradient around the cell.

Figure 4. Schematic of step-growth and chain-growth modes of photoinitiated thiol-ene reactions. The gray shading of the chain-growth mechanism denotes the competitive nature of thiol-ene reactions, which occur in a predominantly stepwise mechanism.

Thiol-yne Reactions: Reactions between thiols and alkynes are conceptually similar to thiol-ene reactions. The main difference is that alkyne is difunctional and can react with two thiol molecules to form a dithioether.⁹⁰

Thiol-Michael addition: The thiol-Michael addition is one of the most widely used click reactions in polymer science. The light-mediated approach was introduced by Xi et al., where the base, a primary amine, was caged using a photolabile group 2-(2-nitrophenyl)propyloxycarbonyl (NPPOC). Upon irradiation with 320-390 nm, the amine is released and exhibits catalytic activity. Although thiol-yne and thiol-Michael addition have similar advantages as thiol-ene reactions, both have yet to be realized in 3D biofabrication settings.⁹¹

Although photoinitiated radical reactions are the most widely employed crosslinking mechanism of hydrogel fabrication technologies, crosslinking can also be achieved with reagents that have innate photochemical activity, without the need for a PI. Although yet to be realized in 3D biofabrication technologies, these reactions represent fast crosslinking strategies. Among these are photo-strain-promoted azide-alkyne cycloadditions, tetrazole-alkene, and tetrazine-alkene cycloadditions.⁹²

Photo-Strain-Promoted Azide-Alkyne Cycloadditions (photo-SPAAC): Cycloadditions comprise a broad and versatile class of reactions in which unsaturated molecules combine to form a cyclic adduct. They have been successfully applied to prepare injectable and cell encapsulating hydrogels.⁹³ Photo-SPAAC relies on photochemical decarbonylation of cyclopropanones to produce cyclooctynes (Figure 5A). Cyclopropanone-caged dibenzocyclooctynes are hydrolytically stable⁹⁴ and do not react with organic azides. Upon irradiation using 350 nm wavelength light, cyclopropanones release carbon monoxide to form cyclooctyne, which undergoes strain-promoted cycloaddition with azides. The photouncaging of cyclopropanone is characterized by high quantum efficiency in aqueous physiological conditions. Hence, the photo-SPAAC has been applied for labeling proteins.⁹⁵ It was also demonstrated that a SPAAC click reaction could be triggered by two- and three-photon excitation of cyclopropanone-caged dibenzocyclooctyne using infrared (IR) lasers of wavelength 690 nm and 1050 nm, respectively.⁹⁶

Nitrile Imine-Mediated Tetrazole-Ene Cycloaddition (NITEC): NITEC is a prominent example of orthogonal ligation chemistry (Figure 5B). Tetrazoles can be photo-activated, generating a highly reactive nitrile imine intermediate, which undergoes 1,3-dipolar cycloaddition reactions with various electron-deficient olefins (alkenes) to form a stable pyrazoline-based covalent linkage.⁹⁷

Tetrazole to imine conversion can be triggered with low-power UV lamps, LED lights, or laser beams. Photo-induced NITEC ligations have been applied to selectively label alkene-containing proteins *in vitro*⁹⁸⁻⁹⁹ and *in vivo*¹⁰⁰⁻¹⁰¹ without apparent toxicity. NITEC was used as catalyst-free crosslinking chemistry to prepare hydrogels from tetrazole- and methacrylate-functionalized 4-arm PEG.¹⁰² The fast

reaction rate and irreversible nature of the click reaction allowed hydrogel formation within minutes. Elegant work by Yu et al. designed water-soluble naphthalene-based tetrazoles that could be activated by 2P excitation at 700 nm.¹⁰³ Two-photon-triggered NITEC reactions were employed to perform fluorescence imaging of microtubule dynamics in living Chinese hamster ovary cells that were pretreated with fumarate-modified docetaxel.

Tetrazine Ligation: First introduced in 2008, the reaction of s-tetrazines with alkene derivatives, referred to as tetrazine ligation, has emerged as one of the most potent chemistries in the bioorthogonal toolbox (Figure 5C).¹⁰⁴⁻¹⁰⁵ The reaction proceeds rapidly at room temperature in aqueous and organic solvents, produces high yields of product, and is chemoselective and bioorthogonal. In order to achieve spatial control, it was possible to use light and photoredox catalysts to generate reactive tetrazine from an unreactive dihydrotetrazine (dHTz) precursor.¹⁰⁶ Truong et al. reported hydrogel formation using tetrazine and norbornene functionalized 4-arm PEG. Tetrazine groups were generated *in situ* by photo-oxidation of dHTz in physiological conditions and irradiation with red light (625 nm, 10 mW/cm²) in the presence of methylene blue as a photosensitizer. Upon oxidation, gel formation was reported to occur within minutes. Table 2 compares the main features of chemistries used for hydrogel formation.

Photodimerization of Anthracene: While the materials containing cinnamate and coumarines dimerize upon UV irradiation by $[2\pi+2\pi]$ cycloaddition, anthracene photodimerization proceeds by a $[4\pi+4\pi]$ cycloaddition mechanism (Figure 5D). Compared to the formation of a cyclobutane ring by $[2\pi+2\pi]$ cycloaddition, anthracene undergoes photocycloaddition to give cyclooctane-type dimers. Consequently, the dimer of anthracene is less strained and more stable. Typically, irradiation by 365 nm light is used to induce the photocycloaddition of unsubstituted anthracene. The reaction does not require a catalyst and does not generate any by-product or radicals that might damage cells. The presence of substituent on anthracene was used to enhance the photosensitivity under visible-light irradiation. Placing an electron-rich substituent group on the anthracene (Figure 5D) significantly red-shifted the light absorption compared to unsubstituted anthracene. This concept has been applied by Truong et al., where visible light irradiation (400–500 nm) drove a cycloaddition of anthracene groups covalently attached to 4-arm PEG chains, which enabled hydrogel formation.¹⁰⁷

Figure 5. General photo-induced click chemistry reactions A) Photo-Strain-Promoted Azide-Alkyne Cycloadditions (photo-SPAAC), B) Tetrazole-Ene Cycloaddition (NITEC), C) Tetrazine Ligation, and D) Photodimerization of Anthracene.

2.1.3 Photomediated Redox Crosslinking

Photoinduced-Copper-Catalyzed Azide-Alkyne Cycloadditions: Photo-CuAAC are both orthogonal and efficient, and considerable effort has been made to render CuAAC chemistry light sensitive. Photo-CuAAC involves the *in situ* generation of Cu(I) via the photomediated process. PIs are introduced to the system to evoke the reduction of Cu(II) to Cu(I), which is needed to catalyze azide-alkyne cycloaddition (Figure 6A).¹⁰⁸

Tyramine Oxidation: While the tyramine crosslinked hydrogels are generally formed through a reaction with hydrogen peroxide and horseradish peroxidase (HRP), hydrogel production using light was reported as well. The photochemical process is usually initiated using photosensitizers such as riboflavin, water-soluble metal complex tris(2,2'-bipyridyl)-ruthenium(II) chloride hexahydrate [Ru(II)(bpy)₃]Cl₂ or Eosin-Y (Figure 6B). Loebel et al. studied single and two-photon crosslinking of tyramine-substituted hyaluronate using laser light at 488 nm and 710 nm, respectively.¹⁰⁹ Photorheometry showed gelling properties in minutes, and using 2P-SL, predefined micropatterns were produced.

Figure 6. Photomediated Redox Crosslinking A) Photoinduced-Copper-Catalyzed Azide-Alkyne Cycloadditions (photo-CuAAC) B) Tyramine Oxidation.

Table 2. Advantages and disadvantages of the chemical crosslinking strategies outlined in Section 2.1

Crosslinking Strategies	Advantages	Disadvantages
Radical Polymerization	<ul style="list-style-type: none"> • Ease of synthesis and robustness • Diverse array of functional monomers • High tolerance of polar functional groups compared to ionic polymerizations 	<ul style="list-style-type: none"> • Oxygen inhibition • Toxicity of unreacted groups • Radicals damaging to biological materials • Heterogeneous hydrogel networks
Thiol Conjugations	<ul style="list-style-type: none"> • Rapid crosslinking • Oxygen tolerance • Tunable reaction kinetics • Low natural abundance of cysteine reduces cross-reactivity with biomolecules 	<ul style="list-style-type: none"> • Potential for thiol exchange • Maleimides undergo hydrolysis • Oxidation to disulfides over time • Side reaction with amines • Not bioorthogonal, potential reaction with cysteine

	<ul style="list-style-type: none"> • Homogeneous hydrogel networks 	
photo-SPAAC	<ul style="list-style-type: none"> • Highly efficient photouncaging in water • No added catalyst needed • Gelation time of bicyclononyne-azide <1 min⁹³ 	<ul style="list-style-type: none"> • Laborious synthesis of alkynes • Cyclooctynes undergo side reaction with thiols
NITEC	<ul style="list-style-type: none"> • High selectivity • No added catalyst needed • Gelation time of tetrazole-acrylate groups in minutes¹⁰² 	<ul style="list-style-type: none"> • Thermodynamic equilibrium between open azide form and preferred tetrazole form.
Tetrazine Ligation	<ul style="list-style-type: none"> • High selectivity and bioorthogonality • Gelation time of tetrazine-<i>trans</i>-cyclooctene groups <2 s¹¹⁰ 	<ul style="list-style-type: none"> • Tetrazine instability
Photodimerization of Anthracene	<ul style="list-style-type: none"> • Bioorthogonal • Single type of functional group for crosslinking • No added catalyst needed • Gelation time in minutes¹⁰⁷ 	<ul style="list-style-type: none"> • Synthesis of functional anthracene is multiple step process
photo-CuAAC	<ul style="list-style-type: none"> • Bioorthogonal • Dark polymerization allows for shorter light exposure 	<ul style="list-style-type: none"> • Copper catalyst cytotoxicity
Tyramine Oxidation	<ul style="list-style-type: none"> • Single type of functional group for crosslinking 	<ul style="list-style-type: none"> • Not bioorthogonal, potentially reacts with tyramine or tyrosine

2.2 Water-Soluble Photoinitiators (PIs)

2.2.1 Single-Photon Initiators (1PIs)

PI is a critical component to start the photocuring because most monomers alone do not produce initiating species with a sufficient quantum yield upon light exposure. Interest in PIs began after the progress of industrial photopolymerization in the late 1970s.¹¹¹ The vast majority of industrial processes utilize oil-soluble PIs and their stable emulsions when aqueous dispersibility is required. However, the development of novel materials for biological and medical applications generated a strong demand for truly water-soluble PIs.¹¹² This section aims to review progress in water-soluble PIs and presents criteria for evaluating and selecting PIs for use in biofabrication.

The critical requirements for PIs for hydrogel biofabrication in the presence of living cells are 1) aqueous solubility, 2) low cytotoxicity, 3) high efficiency in generating radicals by visible light and low-intensity light sources, and 4) excellent reactivity toward monomers. In conformity with the first law of photochemistry, high efficiency in generating free radicals can be achieved only when the absorption spectrum of a PI largely overlaps with the irradiation profile of the utilized light source. Table 3 summarizes the physico-chemical properties of several water-soluble PIs used in 3D bioprinting. 1PIs are classified by the radical-generating mechanism into *Norrish Type I* and *Type II* photolysis (Figure 7A, B).¹¹³⁻¹¹⁵ Upon irradiation, *Type I* PIs undergo a homolytic bond cleavage to form two radicals that initiate the polymerization (Figure 7A). *Type II* PIs undergo a bimolecular reaction in which the excited triplet state of a PI reacts with an auxiliary molecule (hydrogen donor), thereby producing an initiating radical (see an example of Eosin Y initiation) (Figure 7B).

One of the first and still the most commonly used water-soluble 1PI is the α -hydroxyketone 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (I2959, Table 3). However, its major drawback is limited molar absorptivity at wavelength 365 nm ($\epsilon \approx 5 \text{ M}^{-1} \text{ cm}^{-1}$)¹¹⁶, resulting in the need for high concentrations to fabricate hydrogels. Although water-solubility of I2959 (5 g/L)¹¹⁶ is adequate for many applications, the structural analog 2-hydroxy-1-[3-(hydroxymethyl)phenyl]-2-methyl-1-propanone (APi-180®, Table 3) offers much higher solubility in water but has an absorption spectrum peaking at 329 nm. Increasing use by the bioengineering field of new monochromatic light sources (LED, laser diodes) emitting in the 365–405 nm range created a strong demand for water-soluble PIs absorbing in a similar

range. Monoacylphosphine oxides (MAPOs), originally used exclusively for organic formulations, provide sufficient photosensitivity at wavelengths in the 380–450 nm range. Their water-soluble analogs were conveniently prepared by treatment of the oil-soluble PI ethyl phosphinate with lithium or sodium iodide providing the water-soluble phenyl(2,4,6-trimethylbenzoyl)phosphinate salts (LAP) and Na-TPO. Both offer an excellent water solubility, especially LAP (47 g/L), which considerably exceeds that of I2959.¹¹⁶ Furthermore, LAP absorbs at 365 nm with a molecular extinction coefficient, ϵ , of $218 \text{ M}^{-1} \text{ cm}^{-1}$, while the ϵ of I2959 is only $5 \text{ M}^{-1} \text{ cm}^{-1}$, which implies that LAP can be used at considerably lower concentrations than I2959 with reduced cytotoxic effects.¹¹⁶ Therefore, owing to favorable cytotoxicity and visible light absorption, LAP has become the first choice in state-of-the-art water-soluble PIs for 3D biofabrication.¹¹¹ Another class of phosphinate-type PIs is bisacylphosphine oxides (BAPO), which have absorption spectra that are even more red-shifted than those of MAPO PIs. Especially for UV-LED light sources with peak emissions at 385, 395, and 405 nm, BAPO-type PIs provide suitable absorption properties. However, the synthesis of BAPO-type PIs is more demanding than for MAPO compounds. Corresponding, water-soluble alkali metal salts are accessible in a two-step procedure.¹¹⁶ The BAPO derivative can be esterified with polyethylene glycol (PEG) to provide a liquid, non-ionic water-soluble PI.¹¹¹

Another class of PIs is azo compounds. Their excitation results in a photofragmentation of the weakest C – N bond and release of a nitrogen molecule. Photochemically-induced elimination of nitrogen from aliphatic azo compounds is accompanied by the formation of alkyl radicals, which initiate radical polymerization (Figure 7C). Commercially available water-soluble PI 2,2'-Azobis[2-methyl-N-(2-hydroxyethyl)propionamide] (also known as VA-086) was used in 3D bioprinting of Gel-MA with 365 nm light source.¹¹⁷

Recently, a growing interest in the use of Ru^{II} complexes for metal-catalyzed radical polymerizations has given rise to the design of an efficient ruthenium PI that can be activated under visible light. Irradiation of the water-soluble metal complex tris(2,2'-bipyridyl)-ruthenium(II) chloride hexahydrate [Ru(II)(bpy)₃]²⁺ together with sodium persulfate leads to the triplet charge transfer state of Ru. In the presence of sulfate anions, the Ru-excited state deactivates by electron transfer reactions, leading to Ru^{III} species and SO₄ radicals, which eventually leads to crosslinking reactions.¹¹⁸⁻¹¹⁹

A variety of PI systems containing dyes (e.g., Eosin Y, Riboflavin) have been described for 3D bioprinting. In these systems, it has been proposed that radicals are formed in the interaction of the excited dye in the presence of electron donors such as tertiary amines.¹²⁰⁻¹²¹ Eosin Y is excited by green light to its singlet state, from which it undergoes intersystem crossing (ISC) to its triplet state. Then it is reduced by triethanolamine (TEA), and subsequent proton transfer generates two radicals, which initiates the polymerization. *N*-Vinylpyrrolidone (NVP) has been employed as a comonomer in the eosin-mediated synthesis of hydrogels to aid in reducing oxygen inhibition and enhancing the rate of radical polymerization and the final conversion.⁶⁷ The riboflavin-TEA photoinitiating system has significant absorbance in the 300–500 nm range and biocompatibility is significantly higher than the conventional I2959. However, it was found to be less efficient for 2P-polymerization.¹²¹

Various initiating strategies have been developed in order to accelerate the printing speed. In a recent study,¹²² a hybrid semi-conductor/gold nanoparticle (CdS-Au) PI was developed for rapid 3D printing. This new type of PI generates radicals in the presence of dissolved oxygen through photocatalysis, and the generated reactive oxygen radicals could induce photopolymerization of water-soluble acrylamide monomers. The CdS-Au had a very high molar extinction coefficient ($\sim 10^7 \text{ M}^{-1} \text{ cm}^{-1}$ at 385 nm), which is 11 orders of magnitude higher than that of I2959 ($0.0003 \text{ M}^{-1} \text{ cm}^{-1}$ at 385 nm) and therefore led to faster and more complete photopolymerization than the I2959 system. Still, biocompatibility and cytotoxicity of the PI have to be studied.

Figure 7. Examples of photoinitiating mechanisms Type I and Type II photocission, photofragmentation, and two-photon activation.

Table 3. Chemical structures of common water-soluble PIs used in 3D biofabrication techniques.

PI	Chemical Structure	Water solubility (g/L)	λ_{max} (nm)	ϵ_{max} ($\text{M}^{-1} \text{cm}^{-1}$)	Ref.
I2959		5	328 365	296 5	116
APi-180		74	329	97	116
LAP		47	365 380 405	218 191 30	116
Na-TPO		29	380	250	116
BAPO-OLi		54	383	197	116
BAPO-ONa		60	383	256	116
PEG-BAPO		1.38	390	450	111
VA-086		45	375	30	123

Ru(bpy) ₃ /SPS		10	450	14600	118
Eosin Y		1	515 516	69800 86500	124-125
Riboflavin		0.06-1.2	374 449	10300 12100	126-127

2.2.2 Two-Photon Initiators (2PIs)

In recent decades, two-photon stereolithography (2P-SL) has garnered a great deal of attention due to the possibility of fabricating 3D structures of complex chemical and physical hierarchy reminiscent of natural materials.¹²⁸ 2P-SL takes advantage of the non-linear nature of two-photon absorption (2PA), which confines photochemical crosslinking of the material to small laser focal volume and allows structures to be fabricated with unprecedented spatiotemporal resolution. In contrast to single-photon absorption, 2PIs absorb two photons simultaneously, which allows the use of low energy near-infrared (NIR) light. The advantages of NIR light are improved depth penetration in tissue and less harm to living cells compared to UV light.¹²⁹

Several factors affect the design and application of PIs for 2P-polymerization. The first requirement is a large 2PA cross-section of the initiator. The 2PA absorption cross-section (σ^{2PA}) is measured in Göppert-Mayer units (GM, 1 GM = 10^{-50} cm⁴ s/photon) and quantifies a probability of 2PA at a wavelength of interest.¹³⁰ From the excited state, the PI can either decay through ISC to a long-lived triplet state (T_1) from which radical active species can be generated (Figure 8) or decay through a fluorescence emission to the ground singlet state (S_0) (see Figure 8). Therefore, only a portion of the absorbed energy is used to trigger photochemical reactions with the generation of free radicals. Fluorescence represents the most relevant competing process, but also other deactivation pathways such as cis-trans isomerization can take place, reducing the efficiency of the PI.¹³¹ The σ^{2PA} can be measured by direct methods (i.e. nonlinear transmission and z-scan¹³²) or indirect methods (i.e. 2P excited fluorescence).¹³¹

Figure 8. Jablonski diagram of PI radical generation and competing fluorescence decay process (green). After the simultaneous absorption of two photons (red arrows), the excited PI decay through an intersystem crossing to the triplet state (T_1). From this state, PIs can generate radical species which trigger photochemistry reactions.

In principle, molecules with a π -conjugated system typically exhibit 2PA activity. The molecular structure design of 2PI to improve the 2PA properties has been focused on the enhancement of the intramolecular charge transfer by the extending π -conjugated system and using strong electron-donating and electron-accepting groups. These can be classified into symmetric ($A-\pi-A$, $D-\pi-D$) and nonsymmetric ($D-\pi-A$) molecules, in which D stands for an electron donor, A stands for an acceptor, and they are linked by a π -conjugate bridge (Figure 7D).¹³¹

New, highly efficient, and water-soluble 2PIs developed by Liska's group have met the increasing demand for 2P-polymerization of 3D hydrogels (Table 4). These include the benzylidene cycloketone-based 2PIs named G2CK, E2CK, and P2CK, which have carboxy groups to ensure water solubility.¹³³ Recently, a supramolecular water-soluble initiator WI was prepared through host-guest interactions, where host molecules 2-hydroxypropyl- β -cyclodextrins encompassed hydrophobic PI 2,7-bis(2-(4-pentanoxy-phenyl)-vinyl)anthraquinone. The resulting supramolecular 2P initiator WI has a $\sigma^{2PA} \sim 200$ GM at the wavelength of 780 nm.¹³⁴

One of the main problems with using 2P-SL in the presence of cells is the diffusion of the 2PI from the ECM into the cell's cytosol, where they exhibit considerable cyto- and phototoxicity. Liska and colleagues synthesized macromolecular 2PI HAPI, where the MGABA initiator precursor was linked to a hyaluronan backbone. It was demonstrated that the macromolecular nature of HAPI hinders the cellular translocation and exhibits superior biocompatibility compared to reference PI E2CK. Moreover, 3D hydrogel structures containing living cells were produced by the 2P-polymerization of Gel-MA at high scanning speeds of 100 mm/s.¹³⁵

Three water-soluble benzylidene cyclanone dyes with cyclobutanone (T_1), cyclopentanone (T_2), and cyclohexanone (T_3) moieties were designed and synthesized, and their photophysical properties were investigated in water.¹³⁶⁻¹³⁷ Benzylidene cyclanone dyes were modified with four sodium carboxylate groups to achieve water solubility. The maximum σ^{2PA} of T_1 - T_3 in water was determined as 567 GM, 808 GM and 231 GM at 820 nm and 3D nanopatterns based on 2P-polymerization of bovine serum albumin (BSA), water-soluble acrylic ester monomer (SR610) and hyaluronic acid derivative (HA-GM) were successfully biofabricated using these 2PIs. The distinct disadvantage of the benzylidene cyclanone 2PI is their severe cytotoxicity at concentrations used for 2P-polymerization.

Systematic investigations of the relationship between molecular structure and 2PA properties in various solvents were performed for chromophores based on the distyrylbenzene framework.¹³⁸ One successful example of rational 2PI design is a distyrylbenzene chromophore with subsequent quaternization using trimethylamine, which affords the water-soluble molecule 1,4-bis(4-(N,N-bis(6-(N,N,N-trimethylammonium) hexyl)amino)-styryl)-2,5-dimethoxybenzene tetraiodide (WSPI).¹³⁹ In an aqueous medium, WSPI showed $\sigma^{2PA} \sim 360$ GM at the wavelength of 730 nm. This initiator was used for the first time for 2P-polymerization in the formulation containing up to 80% of water and a living organism (*C. elegans*) as a real-time biocompatibility sensor. Large σ^{2PA} enabled the production of 3D microstructured scaffold from PEG-diacrylates (PEG-DA) with a fast scanning speed of 10 mm/s under laser power of 140 mW.

Recently, Tromayer et al. developed the first photocleavable water-soluble azoinitiator for 2P-polymerization, transcending the limitations of charge transfer π -conjugated PI.¹⁴⁰ Novel tetrapotassium

4,4'-(1,2-ethenediyl)bis[2-(3-sulfophenyl)diazenesulfonate] (DAS) showed lower σ^{2PA} of around 40 GM at a wavelength of 800 nm compared to the established 2PI P2CK. Although DAS was used at double the concentration compared to P2CK due to lower σ^{2PA} , it supports to date the highest scanning speed up to 1 m/s, and the threshold power of 2P-polymerization was 45-100 mW.

Table 4. Chemical structures of typical water-soluble photoinitiators used in 2P-microfabrication.

2PI	Chemical Structure	λ_{abs} nm	ϵ_{max} $\text{M}^{-1} \text{cm}^{-1}$	λ_{2P} nm	σ^{2PA} GM ^a	Ref.
G2CK		-	-	800	163	133
E2CK		471	3.5	800 830	201 480	133, 141
P2CK		506	5.5	800	176	133
WI		290	-	750	282	134
HAPI		466	3.5	840	400	141
T1		522	2.87	820	567	136
T2		513	5.5	820	808 604 ^b , 1032 ^c	136- 137
T3		478	4.94	820	231	136

WSPI		423	4.9	730 800	360 120	138- 139
DAS		370	2.0	800	40	140

^a2P absorption cross section values in water, 1 GM = 10⁻⁵⁰ cm⁴ s/photon. ^bnonlinear transmission (NLT) method. ^cTwo-photon-excited fluorescence method.

2.3 Photodegradation and Photouncaging Reactions

Using light offers many unique means of reaction control, such as selective irradiation wavelength, timing, and spatial restriction of the light exposure to control the photochemical reaction. It is particularly useful for precise conjugation of bioactive molecules (e.g., growth factors (GFs), signaling proteins, and morphogens) within the hydrogels.¹⁴² Immobilization of bioactive molecules (patterning) allows for modulation of cell fate determination, cell phenotype, or cell-material interactions. Furthermore, photo-activated materials can enhance cellular systems guidance into 3D microenvironments found in physiological tissues.^{22, 143}

Photouncaging refers to the photolysis of the caged compound (Figure 9A). Specifically, photoremovable protecting groups (PPGs) have been applied to on-demand activate substrates within the hydrogel for reactions with proteins^{55, 144} or to liberate functional chemical groups on polymer chains, such as thiols, enabling their further click-reaction with a target (bio)molecule.^{20, 145-147} Therefore, this approach has found widespread application to photopattern biomolecules. The selection criterion for the PPG to be used depends on the application. Generally, an ideal PPG used in a cell-laden biomaterial should have: 1) strong light absorption at wavelengths above 365 nm to avoid cell photodamage, 2) stability and solubility in physiological environments, 3) transparent photochemical byproducts to avoid competitive absorption, 4) low cytotoxicity, and 5) photoreaction with high quantum yield or efficiency for release.¹⁴⁸

One of the methods to fabricate photodegradable hydrogels is to incorporate a PPG into the crosslinks within the hydrogel network. Upon light irradiation, photolabile linkers are cleaved, and hydrogel degrades (Figure 9B). Consequently, the reduction in the number of crosslinks in the polymer network results in softening (e.g., lower stiffness, porosity) of the hydrogel or its complete dissolution. The preparation of hydrogels incorporating photodegradable groups is achieved by the conjugation of photolabile molecules to a polymer chain-end and subsequent polymer crosslinking. Also, a crosslinking reaction could occur by a photopolymerization, such as using acrylates¹⁴⁹⁻¹⁵³ or using click chemistry^{14, 28, 154-155}. This section will focus on the chemistry of photosensitive functional groups and trends for their use in light-responsive soft matter.

Figure 9. Schematic illustration of photomediated uncaging and degradation of photoactive hydrogels. Examples of (A) photouncaging and (B) photodegradation reactions.

2.3.1 Nitroaryl Groups

***ortho*-Nitrobenzyl (oNB):** Among the known PPGs, oNB derivatives (Figure 9A1) have emerged as the most commonly employed in biological applications. The mechanism of photolysis of oNB has been investigated by time-resolved spectroscopy.¹⁵⁶⁻¹⁵⁷ After light absorption, the excited singlet state of oNB is followed by an ISC to its triplet state. Then, the primary photochemical process is an intramolecular hydrogen transfer from the *o*-alkyl substituent to the nitro group, which is followed by the formation of the *aci*-nitro tautomer. Subsequently, the *aci*-nitro intermediate rearranges to the nitroso derivatives (*o*-nitrosobenzaldehyde or nitroso ketone) and release of the original uncaged compound occurs (Figure 10). Various substituents at the benzylic position of oNB affect the spectral properties of the chromophore. The most suitable strategy to obtain oNB derivatives with a redshifted absorption band is to introduce either an electron-withdrawing group at the para position or electron-donating group at the meta position. In this way, the absorption maximum of oNB can be shifted from 345 nm to 420 nm.¹⁵⁸⁻¹⁵⁹

oNB can also be cleaved by 2PA.¹⁶⁰⁻¹⁶¹ 2PA cross-section of oNBs protecting groups have been reported in the range of 0.015 – 0.065 GM at 750 nm, which is below the limit of 0.1 GM for efficient biological utilization.¹⁴⁸ Only recently, Lunzer et al. demonstrated a strategy to enhance the two-photon induced photocleavage reaction in oNB-based hydrogels.¹⁵³ In their report, the formed hydrogel was treated with a water-soluble two-photon active chromophore, cyclic benzylidene ketone-based molecule, which acted as a photosensitizer, i.e., efficiently absorbing two photons and then transferring the energy to oNB linkers. This modular system was proved to be biocompatible in the presence of encapsulated cells.

Figure 10. Reaction mechanism of the uncaging process of oNB caged carboxylic acid bearing compound.

Nitrodibenzofuran (NDBF): To increase the absorbance at a longer wavelength, oNB aromatic core was extended to NDBF. Irradiation of NDBF (Figure 9A2) with 365 nm light or 2P excitation at 800 nm resulted in efficient deprotection. NDBF was shown to be efficient for thiol caging, circumventing the undesired isomerization of coumarin caged thiol to the dead-end product upon photolysis.¹⁶²

2-(2-Nitrophenyl)propoxycarbonyl (NPPOC): Deprotection efficiency was considerably improved by placing substituents on the aromatic ring of the oNB group. NPPOC (Figure 9A3), oNB homolog, was involved as a photocage of the alkoxyamine-modified substrate. The resulting NPPOC-photocaged alkoxyamine was subsequently reacted with aldehyde functionality via photo-mediated oxime ligation.¹⁶³ Using this approach, Farahani et al. created a cell-laden hydrogel with a spatially photopatterned protein.¹⁶⁴ To this end, the protein functionalized with photocaged alkoxyamines was introduced to the hydrogel network containing benzaldehyde-end groups. Upon UV light irradiation, the uncaged alkoxyamines formed stable oxime linkages with aldehyde moieties.

2.3.2 Coumarin

A general mechanism of the coumarin group photorelease is summarized in Figure 11. After initial light absorption, relaxation takes place to the lowest excited singlet state. Heterolytic C-X bond cleavage occurs through the intermediate, tight ion pair of coumarinylmethyl cation and leaving group conjugate base. The coumarinylmethyl cation reacts with the solvent to generate a stable coumarinylmethyl product. The liberated leaving group is unstable and undergoes slow decarboxylation.¹⁴⁸

From a biological perspective, coumarin (Figure 9A4) PPG is suitable for cell-laden hydrogels because the photodegradation produces nontoxic byproducts.¹⁶⁵ Furthermore, coumarin PPG exhibits larger σ^{2PA} than oNB, providing efficient multiphoton cleavage of coumarin-based hydrogels.^{148, 159, 166} Similarly to oNB groups, there is a growing interest and demand for alternative photolabile molecules with a red-shifted absorption spectrum allowing for visible light-triggered photocleavage.¹⁶⁷⁻¹⁶⁸ For example, Truong et al. recently obtained a photodegradable PEG hydrogel with photocleavable benzoquinone linkers.¹⁶⁹ The synthesis of the hydrogel was achieved via thio-bromo click reaction between star-PEG end-functionalized with bromoquinone and thiol groups. The photocleavage was induced by irradiation with blue light (400-500 nm) and consisted of two subsequent processes, photoreduction of quinone and thermal trimethyl lock ring closure. The complete gel dissolution was obtained after 10 minutes of irradiation.

Figure 11. Mechanism of photorelease of coumarin-caged compounds.

2.4 Common Photosensitive Bioinks and Bioresins

In the field of biofabrication, definitions of “bioinks” and “biomaterial inks” for deposition bioprinting were recently proposed. Bioinks are defined as “a formulation of cells suitable for processing by an automated biofabrication technology that may also contain biologically active components and biomaterials”.¹⁷⁰ For VP methodologies, we propose the use of the term “bioresins” to refer to the precursor solution containing cells from which objects are polymerized. Materials used for the formulation of bioinks are hydrogel precursors (monomers, macromers, oligomers) that can be (photo)crosslinked to form a hydrogel. Hydrogels have attractive features for use in tissue engineering and regenerative medicine, such as biocompatibility and biodegradability, and importantly, many of them are able to mimic the native ECM milieu. By doing so, cell attachment, spreading, growth, infiltration, differentiation, and signaling can be controlled, allowing hydrogels to mature into functional tissue analogs. Hydrogel biomaterials, including gelatin, hyaluronic acid, alginate, PEG, and their use as photocrosslinkable bioinks will be discussed in this section.

Gelatin

Gelatin is produced by the denaturation of collagen, which is a major component of natural ECM. It is highly biocompatible, contains Arg-Gly-Asp (RGD) cell-binding motifs, is enzymatically degradable by metalloproteases, and is inexpensive and generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). Therefore, gelatin has become the most common material used in bioprinting. However, gelatin has an upper critical solution temperature (UCST) of around 30°C,¹⁷¹ meaning it is water soluble above this temperature and forms hydrogel only at lower temperatures than its UCST. Gelatin is therefore not stable at physiological or cell culture conditions but can be chemically modified and crosslinked to form a stable hydrogel.

Gelatin contains several modifiable functional groups (hydroxyl, amino and carboxylic groups) from parent collagen amino acids and ornithine, which is formed after collagen denaturation.¹⁷² Crosslinking occurs by (photo)polymerization of reactive functional groups, typically methacryloyl, which are immobilized onto the gelatin backbone. Gelatin-methacryloyl (Gel-MA) can be reproducibly prepared by the reaction of gelatin with methacrylic anhydride (MAA) (Figure 12A). Gel-MA contains both methacrylate and methacrylamide groups because hydroxyl groups as well as amino groups are reactive toward MAA. Highly substituted Gel-MA after long reaction times contains higher amounts of methacrylates compared to Gel-MA with a lower degree of functionalization (DF).¹⁷³⁻¹⁷⁵ Abbreviation Gel-MA is sometimes incorrectly referred to as gelatin methacrylate, which is misleading as amino groups are more reactive toward MAA, and the majority of methacryloyl groups are thus methacrylamides. Methacrylamide groups on Gel-MA are hydrolytically more stable than methacrylate groups. It was shown that gelatin methacrylamide could be completely decoupled from Gel-MA through selective hydrolysis of methacrylate groups.¹⁷⁶ Hydrogels of purely gelatin methacrylamide were more compliant than those of Gel-MA. Another approach to enhance the mechanical properties of gelatin is to modify both amino and carboxylic groups sequentially. Van Hoorick et al. developed gelatin where firstly, primary amines were converted to methacrylamides, and subsequently, carboxylic groups were activated, and additional methacrylates were introduced using amino ethoxy methacrylate (AEMA) (Figure 12B).¹⁷⁷ As a result, faster crosslinking kinetics, lower swelling ratios, and higher stiffness have been achieved, which expands the possibilities of gelatin for 2P polymerization. Activated carboxylic groups of Gel-MA may also be used as a handle to covalently introduce biological functionalities. In this respect, Gel-MA was functionalized with the neurotransmitter dopamine to obtain a crosslinkable functional gelatin derivative suitable to print scaffolds enhancing neuronal differentiation of neural stem cells.¹⁷⁸

Gelatin derivatives for step-growth-based crosslinking approaches have also been reported.¹⁷⁹ The most common step-growth crosslinking chemistry applied for gelatin derivatives is thiol-ene photoinitiated click chemistry. Thiol-ene photocrosslinkable gelatin contains an alkene functionality such as norbornene (Figure 12C, D), vinyl ester (Figure 12E), vinyl sulfone, allyl ether (Figure 12F), or acrylate. Conversely, gelatin may be functionalized with thiols, which are crosslinked using an alkene crosslinker (PEG methacrylate (PEG-MA)¹⁸⁰, Gelatin-norbornene (Gel-NB)¹⁷⁹). Allyl groups were introduced onto gelatin by the reaction of allyl glycidyl ether (AGE) with primary amines.¹⁸¹

Figure 12. Overview of preparation methods of different photo-crosslinkable gelatins: A) Gelatin methacryloyl (Gel-MA). B) Gelatin methacryloyl amino ethoxy methacrylate (Gel-MA-AEMA).¹⁷⁷ C) Gel-norbornene (Gel-NB) synthesized via reaction with carbic anhydride.¹⁷⁹ D) Gel-NB synthesized via reaction with 5-norbornene-2-carboxylic acid. E) Gel-vinyl ester (Gel-VE).⁵¹⁵ F) Gelatin-allyl glycidyl ether (Gel-AGE).¹⁸¹

Hyaluronic Acid

Hyaluronic acid (HA) is a naturally derived, non-sulfated glycosaminoglycan comprised of repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid. HA is an unbranched, anionic polysaccharide having a large molecular weight ($\sim 10^4$ – 10^7 Da). It has widespread clinical use in orthopaedics as a treatment for osteoarthritic joint pain, in ophthalmology to treat dry eye disease and in plastic surgery as a dermal filler.¹⁸² HA is a dominant component of the ECM of many tissues and has a role in regulating cell growth, migration, and differentiation.¹⁸³ Moreover, HA has inherent biological functions, such as binding affinity to the cell surface receptor CD44 and degradability by mammalian enzymes.¹⁸⁴ Due to its biological features, hydrogels built from HA are being used to engineer many tissues including cartilage, heart, blood vessels, nerves, and skin.^{185–187} Naturally, HA has become one of the most prominent biomaterials used in 3D biofabrication. It contains several functional groups (primary and secondary hydroxyl, or carboxylic groups) in the backbone, which can be used to introduce photoreactive moieties (e.g., methacrylates, vinyl esters, norbornenes) (Figure 13). The most common chemical modifications consist of the methacrylation of primary hydroxyl groups through a reaction with MAA^{188–189} (Figure 13A) or glycidyl methacrylate^{190–191} (Figure 13B). Many other HA-based bioinks (i.e., HA-VE, HA-NB)^{192–194} with various cell types have been reported to date.¹⁹⁵

Figure 13. Overview of preparation methods of different photo-crosslinkable HA. A) HA methacrylate (HA-MA) prepared using MAA,¹⁸⁸⁻¹⁸⁹ B) HA methacrylate (HA-MA) prepared using glycidyl methacrylate,¹⁹⁰⁻¹⁹¹ C) HA-vinyl ester (HA-VE). D) HA-norbornene (HA-NB).¹⁹²⁻¹⁹⁴

Polyethylene glycol (PEG)

PEG is the most widely used synthetic hydrophilic polymer in biomedical applications including surface modification,¹⁹⁶ drug delivery,¹⁹⁷ tissue engineering,¹⁹⁸⁻¹⁹⁹ and 3D biofabrication.²⁰⁰ Compared to natural polymers, PEG can be synthesized with targeted molecular weight and architecture, such as linear (Figure 14A) or branched structures (Figure 14B), and with defined chain end groups. Although PEG-based hydrogels do not exhibit intrinsic biological activity due to the antifouling nature of PEG chains,²⁰¹ they can be tailored to specific applications through incorporation of RGD peptides for cell adhesion or MMP-cleavable peptides for proteolytic degradation.²⁰² Among the PEG-based bioinks, PEG-diacrylate (PEG-DA) and dimethacrylate (PEG-DMA),^{25, 203-205} PEG-divinyl ester (2-PEG-DVE)²⁰⁶, PEG-dithiol (2-PEG-SH)²⁰⁷, PEG-norbornene (2-PEG-NB),^{84, 207} PEG-vinyl sulfone (2-PEG-VS)²⁰⁸⁻²⁰⁹ have been utilized for light-induced 3D biofabrication methods.

Figure 14. Examples of photocrosslinkable PEG (A) linear derivatives, PEG-diacrylate (PEG-DA) and dimethacrylate (PEG-DMA),^{25,203-205} PEG-dithiol (2-PEG-SH),²⁰⁷ PEG-divinyl ester (2-PEG-DVE)²⁰⁶ and (B) branched derivatives, PEG-norbornene (2-PEG-NB),^{84,207} PEG-vinyl sulfone (2-PEG-VS).²⁰⁸⁻²⁰⁹

Alginate

Alginate is a water-soluble, negatively charged polysaccharide biopolymer isolated from brown seaweed. It has a linear structure and consists of two types of monosaccharide residues as a repeating unit, namely 1,4-linked β -D mannuronic acid (M) and α -L-guluronic acid (G). Typically, a hydrogel state

with alginate is generated through ionic crosslinks between the carboxylic acid moieties and divalent cations (such as calcium, strontium, or barium). However, ionic gelation is reversible, and when ions are released, the gel may dissolve. Thus, alginate was adapted for photopolymerization. The principle groups for alginate derivatization are the two hydroxyl groups and one carbonyl group present per monosaccharide residue. Similar to other polysaccharides, photopolymerizable methacrylate derivative (Alg-MA, Figure 15A) can be synthesized by the reaction of secondary hydroxyl groups with MAA.²¹⁰ Another strategy is to functionalize the carboxylic group with aminoethyl methacrylates (Figure 15B).²¹¹ Alginate can also be functionalized with norbornene, a reactive substrate for the radical thiol-one reaction (Alg-NB, Figure 15C).²¹² Alginate functionalized with phenyl groups (Alg-Ph, Figure 15D) for oxidative photocrosslinking was prepared via carbodiimide-mediated condensation of the carboxyl groups of alginate and primary amino groups of tyramine.²¹³⁻²¹⁴

Figure 15. Overview of preparation methods of different photo-crosslinkable alginates. A-B) Alginate methacrylate (Alg-MA),²¹⁰ C) Alginate-norbornene (Alg-NB),²¹² D) Alginate-phenyl (Alg-Ph).²¹³⁻²¹⁴

Collagen

Collagen is a main structural protein in the ECM and is thus naturally an attractive scaffold material for tissue engineering. Collagen is composed of triple helices, which self-assemble into fibrillar hierarchical structures providing mechanical strength, visco-elastic properties, bioactive adhesion sites, and biodegradability.²¹⁵ It has been used as a bioink material in 3D printing and crosslinked mainly using temperature or pH change.²¹⁶⁻²¹⁷ However, thermal crosslinking is a lengthy process, and therefore, combining collagen with other polymers reduces the time for gelation and allows the use of collagen in 3D printing.²⁰³ Collagen has also been chemically modified by photocrosslinkable (meth)acryloyl groups (Col-MA, Figure 16).²¹⁸⁻²²¹ Functionalization is mostly performed using amino groups of lysine and hydroxylysine residues, which react with activated esters, MAA, or glycidyl acrylate (Figure 16). However, reaction conditions often require careful optimization to avoid undesirable gelation during modification. Functionalization of collagen may cause loss of the fibrillar structure and the concomitant reduction of desirable features such as bioactivity and biodegradability.²²²

Figure 16. Schematic of photocrosslinkable collagen methacryloyl (Col-MA) synthesis.²¹⁸⁻²²⁰

Poly(Vinyl Alcohol) (PVA)

PVA is a hydrophilic polymer synthesized by hydrolysis of poly(vinyl acetate). It is widely used in the pharmaceutical industry because of its biocompatibility, and many PVA-based applications are FDA approved. Recently, Qin et al. introduced novel cell-responsive PVA hydrogels made by 2P polymerization.²⁹ To manufacture a hydrogel microstructure, PVA was functionalized with norbornene (PVA-NB) (Figure 17A) for the subsequent thiol-ene reaction. This system allowed the rapid photochemical construction of 3D cell-instructive hydrogel by NIR light at high resolution. Anseth and co-workers have developed photopolymerizable PVA acrylate macromonomers (PVA-A) (Figure 17B) with varying degrees of reactive acrylate groups.²²³ Pendant hydroxyl groups of PVA were reacted with acrylate-terminated ester anhydride. Another approach employs MAA to introduce methacrylate groups on the PVA backbone (Figure 17C).²²⁴

Figure 17. Schematic of photocrosslinkable PVA. A) PVA-norbornene,²⁹ B) PVA-acrylate,²²³ C) PVA-methacrylate.²²⁴

2.5 Biocompatibility of Photoactive Systems

During 3D biofabrication, cells are directly exposed to several factors, including the hydrogel precursors, PIs, radicals, light at different wavelengths and intensity, and the crosslinked hydrogel network itself. Although light irradiation might have various effects on a living organism, it is now widely used in the presence of cells for a range of biomedical applications.^{6, 225} It is generally accepted that visible (400-700 nm) and infrared (700-1000 nm) wavelengths are not detrimental to living cells, although prolonged irradiation might generate heat.²²⁶ One of the targets of photodamage is cellular DNA. UV-B (280-315 nm) wavelength might be absorbed by cellular DNA and induce deleterious effects, such as the reduction in cell growth and survival or change in protein expression.²²⁷ It has been found that long-wave UV-A (315-400 nm) wavelengths are less efficient in causing direct DNA damage. Recently, Ruskowitz et al. demonstrated that low doses of 365 nm UV light needed to induce thiol-ene gelation did not cause deleterious effects to hMSCs. Neither proliferation rates nor protein expression were affected. Moreover, apoptosis was not induced after exposure to varying intensity of 365 nm light. In contrast, exposure to lower wavelength UV light (254 nm) decreased proliferation, initiated pro-apoptotic pathways, and changed protein production.²²⁸

The ability of living cells to survive 3D biofabrication is dependent not only on irradiation wavelength, time, and intensity but also on hydrogel formation chemistry.²²⁹ Free radicals generated during photopolymerization are known to oxidize cellular lipid bilayer or cause oxidative stress to cellular DNA.^{85, 230-231} Recently, Wong et al. investigated firstly only the effects of low-dose, near-UV light (300–425 nm) on hMSC function and found no significant change in global gene expression after multiple exposures totaling 25 min at 3.5 mW/cm².²²⁹ In contrast, cells exposed to radical photopolymerization exhibit significant differences in gene expression, which include genes related to the DNA damage. They found gene expression changes due to the presence of free radicals, not to long-wave UV exposure. In another study, exposure to only 365 nm irradiation without radical species for less than 10 minutes at 10 mW/cm² was considered cytocompatible.²³² These results are motivating the field to use and further explore non-radical-based crosslinking chemistries for hydrogel formation in the presence of living cells.

As discussed in previous sections, natural or synthetic polymers are functionalized to introduce photopolymerizable groups to induce their photocrosslinking. However, radical-based crosslinking shows the occurrence of numerous (up to 25%) unreacted residual monomer groups²³³⁻²³⁴ (acrylates or methacrylates), which become entrapped within the hydrogel structure and upon hydrolysis exhibit potential cytotoxic effects. Various acrylic monomers show a cytotoxic effect on the cell viability of, i.e., HeLa S3 cells with a range of maximal inhibitory concentration (IC_{50}) values from 0.01 to 3.70 mmol/L.²³⁵ In contrast, methacrylates are considerably less cytotoxic than acrylates with similar substituents. Widely used as a synthetic hydrogel precursor, PEG-DMA showed a higher IC_{50} value (10.28 mmol/L) in HeLa S3 cells than IC_{50} of acrylic monomers. Although methacrylate-based systems are the most prevalent photopolymerizable moiety, alternative vinyl esters can also undergo photopolymerization and exhibit low cytotoxicity. Compared to methacrylate and especially acrylate monomers, the corresponding vinyl esters conclusively demonstrate considerably lower cytotoxicity and ability to support growth of MC3T3-E1 osteoblastic cells.²³⁶ Further, upon hydrolysis of vinyl ester moieties, the major degradation product is biocompatible PVA and unpolymerized vinyl ester groups hydrolyze to acetaldehyde, which can be metabolized *in vivo* into acetic acid.

Bryant et al. investigated the cytocompatibility of several PIs using the fibroblast cell line NIH/3T3 and encapsulated chondrocytes.²³⁷ Of the initiators investigated, I2959 was found to cause the lowest level of toxicity on NIH/3T3 fibroblast cell line at concentrations < 0.5% (w/w), light intensities 6 mW/cm² at 365 nm, and polymerization times up to 10 minutes. Also, it was demonstrated that chondrocytes survive the photoinitiation process of PVA hydrogel formation using conditions 0.05% (w/w) I2959, 8 mW/cm² at 365 nm light, and a 10-minute exposure time. Another study evaluated the cellular toxicity of I2959 on six different mammalian cell populations - human fetal osteoblasts, bovine chondrocytes, rabbit corneal epithelial cells, human MSCs, goat MSCs, and human embryonic germ cells.²³⁸⁻²³⁹ Different cell types have diverse susceptibility to radical-induced toxicity of identical PI concentration and light exposures. A range of I2959 concentrations (0.03 – 0.1% (w/v)) was well tolerated by all cell types studied.

To increase aqueous solubility, other initiators with improved cytocompatibility, such as MAPO and BAPO salts, have become state-of-the-art. The water-soluble initiator LAP has very low cytotoxicity (LC_{50} 3.1 mmol/L) with fibroblasts encapsulated in a gelatin hydrogel. It was shown that cell survival for fibroblasts encapsulated in LAP-initiated PEG-DA hydrogels was 95% using the concentration of LAP 0.22 mM (0.0065% w/v).²³¹ Also, BAPO initiators show very low cytotoxicity, hence making phosphinate PIs an ideal choice for biofabrication.^{116, 231}

3. 3D DEPOSITION BIOPRINTING

Bioprinting methodology and biomaterial development has exploded, and this review concentrates largely on advances published in recent years. Traditionally, the bioprinting space has been dominated by “deposition” approaches in which material is placed on a printbed with 3D automated control. Two common examples of this type of biofabrication are extrusion bioprinting and droplet-based bioprinting (Figure 1). Both of these have profited highly from photo-activated materials, but some of the major advantages with other types of crosslinking are also included for completeness. The reader is referred to excellent recent reviews on these topics.²⁴⁰⁻²⁴⁴

3.1 Extrusion Bioprinting

3.1.1 Principles of Extrusion Bioprinting

Extrusion bioprinting, also called robotic printing, direct ink writing (DIW), or contact bioprinting, is currently the most widely used 3D bioprinting method. Its popularity is due to its ability to rapidly produce centimeter-sized, cell-laden constructs, as well as to the commercial availability of low cost bioprinters and an increasing array of bioinks available for purchase from multiple vendors. The method is based on the flow of a continuous strand of biopolymer material out of a nozzle whose exit diameter is commonly in the range of ~100-600 μ m. When printing at an average speed of ~10 mm/s, centimeter-sized constructs are achievable in less than an hour. During extrusion printing, the polymeric strands are stacked on top of each other in a layer-by-layer fashion to construct the 3D element. There is generally continuous contact between the material flowing out of the nozzle and the material on the printbed, except when the nozzle is retracted for repositioning, during which the flow out of the nozzle is momentarily interrupted. Flow is initiated by pressure in most bioprinters, though syringe pumps have been used. A progressing cavity pump can be used for increased accuracy of bioink flow and precision of the final printed object.²⁴⁵ Extrusion bioprinting is analogous to the popular fused deposition modeling (FDM) printing, where the thermoplastic filament is heated to the melting temperature, T_m , of the polymer

and strands of melted polymer are extruded out of the hot end and stacked in a layer-by-layer fashion. In both cases, a computer-aided design (CAD) digital model of format .stl or .amf is sliced or converted to the machine language G-code. G-code is the most widely used numerical programming language to achieve automated control of machine tools and 3D printers.

Commercial extrusion bioprinters are configured so that either the build plate or the printhead/printing cartridge provides the movement in x, y, z or a combination thereof (Figure 18). Although the basic function of all extrusion printers is similar, certain printers can be upgraded to allow hybrid biofabrication (i.e. extrusion combined with melt electrowriting). Printing into a support bath might profit from a build plate which is stationary. Likewise, methods where the amount of deposited material is continuously monitored could benefit from a stationary stage. On the other hand, in the case of a heavy printhead with a significant amount of inertia, accurate printing might be better achieved by moving the buildplate and keeping the printhead fixed.

Figure 18. Various configurations of extrusion bioprinters showing movement of the build plate and printhead/bioink cartridge in x (red), y (blue) and z (green) directions. A) Cartesian Printers B) Delta Printers

Rheological Properties for Printability: The accuracy and reliability of extrusion bioprinting is largely dependent on the ability of the bioink to flow on demand, whereas the mechanical integrity of the print is dependent on the degree of layer-layer adhesion. Layers of uncrosslinked hydrogel fuse easily, but the layers will tend to flow and reduce print fidelity, whereas crosslinking each layer sequentially might lead to better shape retention, but less interlayer adhesion. In FDM, stop-start-stop of the flow is guaranteed by the rapid and precise melting and solidification of the material. Integrity of the printed product results from a certain amount of molecular entanglement between the layers, which is achieved as the hot layer slightly melts the cold layer below.²⁴⁶ Reproducing this level of control over the flow properties in bioprinting is much more difficult to achieve as hydrated polymers crosslink slowly, allowing gravity and surface tension to distort the printed object. This rheological challenge of extrusion bioprinting has been referred to as a ‘race against instabilities’.²⁴⁷

To obtain a more in-depth understanding of the flow and viscoelastic behaviour of bioinks and acellular biomaterial inks, standard rheological tests are carried out as a general method for evaluating printability. First, polymeric solutions have either Newtonian or non-Newtonian properties, where the latter is further categorized into shear thickening (increasing viscosity with increasing shear rate) and shear thinning (decreasing viscosity with increasing shear rate), and their respective time-dependent versions are known as rheopecty and thixotropy. Shear thinning is a feature of most bioinks which reduces shear stress applied to cells within the bioink. Secondly, shear recovery, which determines the return of a material to the elastic state, is often measured. Thirdly, the yield/flow point specifies when a solid starts flowing or the transition point from a viscoelastic solid to a viscoelastic fluid. The reader is referred to recent papers on rheological printability for more information.^{244, 248-250}

Material Strategies for Extrusion Bioprinting: The three most common crosslinking strategies used in extrusion bioprinting are 1) light-induced crosslinking of photoactivatable polymers (chain-growth and step-growth polymerization), 2) ionic crosslinking, often using alginate, and 3) temperature-based crosslinking through use of thermosensitive polymers like gelatin. Methacryloyl derivatives of gelatin,²⁵¹

collagen,²⁵² elastin,²⁵³ chitosan,²⁵⁴ chondroitin sulfate,²⁵⁵ and dECM²⁵⁶ can be used as extrusion bioinks whose printed structure can be crosslinked by light.

Although less common than bioinks based on photo-initiated chain-growth radical polymerization, the increasing use of bioinks based on step-growth thiol-ene click chemistry is a noteworthy trend in extrusion bioprinting. Thiol-ene clickable bioinks made from poly(glycidol),²⁵⁷ gelatin,¹⁸¹ and alginate²¹² have recently been published. Yeh et al. formed step-growth hydrogels using a photo thiol-ene reaction between norbornene-functionalized poly (glycerol sebacate) and pentaerythritol tetrakis (3-mercaptopropionate), which yielded 3D-printable structures and impressive deformational behavior.²⁵⁸ Other semi-synthetic bioinks based on thiol-ene reactions include photocurable PEG-DA and thiolated fibrinogen fragments²⁵ and methacryloyl collagen with thiolated HA.²⁰³ A bioink containing equimolar thiol/ene ratio of Gel-NB and thiolated gelatin was printed with adipose-derived stem cells (ADSCs). When benchmarked against Gel-MA, the thiol-ene bioink better promoted adipogenic differentiation, likely due to the more homogeneous polymer network and/or the large and bioactive thiol crosslinker.²⁵⁹ Finally, in a comparison of 10% w/v Gel-NB and 10% w/v Gel-MA, and LAP and I2959 as PI, the more rapid crosslinking with the combination of LAP (at 365 nm) and Gel-NB allow printing of tall (20 mm) structures.²⁶⁰

Rendering a biopolymer light-sensitive through methacrylation or other means results in a loss of viscoelasticity,^{27, 261-263} which has serious consequences for its ability to be bioprinted. In certain types of bioprinting discussed later in this review, i.e., droplet and vat polymerization, the loss of viscosity is auspicious, as low-viscosity materials are beneficial. However, for extrusion printing, special methods are needed to avoid the post-printing flow of the bioink (Figure 19).

PROBLEM	SOLUTION

LOW VISCOSITY	EMBEDDED PRINTING	UV CROSSLINKED
SOLUTION

NANOPARTICLES / NANOFIBRILS	SELF-HEALING	JAMMED / ENTANGLED

Figure 19. Common strategies to achieve good extrusion printability

Embedded Printing: The search for ideal bioinks is akin to finding a “sweet spot” that combines excellent printing fidelity with a biological environment that robustly supports the embedded cells.

Several strategies have been devised in recent years to achieve this. Extrusion of bioinks or biomaterial inks into a shear thinning fluid bed is one method to overcome the need for self-supporting bioinks (Figure 19). The method, called embedded printing, uses a support material which is solid at low shear stress, but flows under conditions of high shear stress (i.e., in the vicinity of the moving nozzle). For example, 0.2% (w/v) Carbopol ETD 2020 is a non-thixotropic granular material that undergoes local shear thinning fluidization near the nozzle without perturbing adjacent regions and undergoes rapid recovery.²⁴⁷ This allows previously “unprintable” structures to be made (Figure 20A). For bioprinting applications, the support bath is often made of a slurry of gelatin particles. Photo-activated HA-MA with 0.1% (w/v) PI I2959 could be printed into the support, UV crosslinked, after which the support could be melted away.²⁶⁴ A 20 μm resolution of this technique has recently been achieved by replacing the slurry particles with highly uniform gelatin microparticles made by coacervation (Figure 20B).²⁶⁵ Interestingly, the gelatin microbeads of the support material were incorporated into the printed structure giving it natural porosity once the gelatin had been eluted at 37°C.²⁶⁶ Additional biomaterials that have been used for embedded printing include slurries of agarose²⁶⁷ and gellan²⁶⁸, but most interesting, with respect to the topic of this review, is the approach using a support material made of photo-activated alginate (oxidized and methacrylated).²⁶⁹ Bioinks composed of high-density solutions of MSCs could be printed into an alginate support bed, and the alginate was then UV crosslinked into a porous “mold” that could support the development of the cellular structure²⁶⁹ (Figure 20C). In a reverse configuration, the support bath can be filled with cell aggregates from induced pluripotent stem cell (iPSC)-derived cells in which sacrificial pluronic channels were printed. This approach allowed the biofabrication of “vascularized” tissues with cell densities approaching that of solid epithelial organs (Figure 20D).²⁷⁰

Figure 20. Examples of embedded printing. A) Photocrosslinkable polyvinyl alcohol is “written” in a granular bed consisting of Carbopol polymer (left), and in water after removal of the support (right). B) Highly concentrated collagen bioink is extruded into a bath of coaservated gelatin microparticles according to a human heart scaled to neonatal size. C) Scaffold-free pure cellular bioink is printed into a bath of oxidized, methacrylated alginate slurry with a 27-gauge needle (Scale bar: 600 μm). D) Pluronic sacrificial inks are printed into a bath of cell aggregates, leaving channels in high cell density constructs after removal of Pluronic (Scale bars: 50 μm (top row) and 500 μm (middle and bottom rows)). A and D: Adapted with permission from ref 247 and ref 270, respectively. Copyright 2015 The Authors and Copyright 2019 The Authors, respectively. Some rights reserved; exclusive licensee American Association for the Advancement of Science. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC) <http://creativecommons.org/licenses/by-nc/4.0/>. B: Adapted with permission from ref 265. Copyright 2019 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. C: Adapted with permission from ref 269. Copyright 2019 The Royal Society of Chemistry.

Rapid UV Crosslinking: Other methods to increase printability capitalized on rapid crosslinking to prevent flow and improve print fidelity. By exposing each layer to UV light (Figure 19) (or spraying CaCl_2 on alginate prints²⁷¹ or H_2O_2 on tyramine-modified prints²¹³), one can stabilize each layer before printing the next²⁷²⁻²⁷³ and therefore print taller structures without sagging.²⁷⁴ In another approach, ‘pre-crosslinking’ by light exposure of Gel-MA and HA-MA bioinks was used to improve their rheological properties and printability.^{272, 275} Also, exposure of HA-MA or HA-NB to UV light as it is ejected from a transparent glass capillary tube could increase the resolution of the print, as well as protect cells from shear stress.^{194, 273} By optimizing the UV intensity and the length of the capillary, good printing resolution was obtained for low viscous precursors of Gel-MA (5% w/v), HA-MA (2.5% w/v), PEG-DA (5% w/v) and HA-NB (2% w/v).²⁷³ Here, it was possible to print multimaterial cell-laden filaments using a core-shell

nozzle and alternating the on-off status of the core and sheath flow. Finally, the polymer content of Gel-MA bioinks could be reduced even further through co-extrusion with alginate with coaxial nozzles (see section on Hardware Advances and Figure 22). Recently, double-network stiff hydrogel components from PEG-DA and alginate were printed alongside cell-laden softer printed strands to give soft tissue grafts more stability.²⁷⁶

For completeness, soft and fluidic biomaterial precursors can be co-fabricated with a much stiffer material, often a thermoplastic-like poly-caprolactone (PCL), which then relaxes the need for self-supporting, perfectly printable inks. PCL filaments have been printed alongside those of ECM particle-containing inks,²⁷⁷ whereas de Ruijter has taken advantage of a technique known as melt electro-writing (MEW) for reinforcement.²⁷⁸ Advances in the MEW scaffolding have achieved impressive deformational and structural properties, and their stability often circumvents the need for bioprinting at all as the hydrogel is often injected or cast into the scaffold.²⁷⁹⁻²⁸¹

Nanoparticle/Nanofibril Inks: Another common strategy for printing low concentrations of photo-activated materials like Gel-MA is through the addition of nanoparticles and nanofibers, which afford the material a higher yield stress and shear thinning properties (Figure 19). Recently, as little as 1% Gel-MA was printed with excellent resolution using the addition of (2,2,6,6-tetramethylpiperidin-1-yl)oxodanyl (TEMPO)-treated nanocellulose.²⁸² The resolution of these scaffolds was improved by dripping 45 mM CaCl_2 onto the constructs during printing, which was thought to interact with the carboxylic groups of the cellulose nanofibrils. Viscosity-enhancing components that have been added to bioinks include laponite,²⁸³ graphene oxide,²⁸⁴ and carbon nanotubes.²⁸⁵⁻²⁸⁶ In fact, the addition of nanoparticles is of significant importance for the field, as it is an effective method for both contributing to shear thinning and for bringing additional functionality into the bioink. For instance, carbon nanotubes and metal nanowires can render materials conductive, which could benefit electrically active tissues like cardiac and neural tissue.²⁸⁷⁻²⁸⁸ Nanosilicates have affinity binding to GFs, drugs, and other morphogens important in maintaining the viability of the cells and their ion release can support the osteogenic phenotype.²⁸⁹ Cidonio et al. showed that both osteogenesis and angiogenesis were enhanced in laponite-Gel-MA bioinks compared to Gel-MA alone, due to interactions between the GFs with the laponite.²⁸³ In addition to the aforementioned nanoparticles with intrinsic shear thinning properties, recent studies demonstrated the use of non-shear thinning solid silica nanoparticles (ca. 40 nm in diameter) as a rheology enhancer via induction of electrostatic or dynamic covalent interactions between nanoparticles and polymer matrix.²⁹⁰⁻²⁹¹ The rheological properties of such nanocomposite bioinks can be manipulated to a great extent by controlling the size, concentration of nanoparticles, and more importantly the type of interactions between nanoparticles and polymers.²⁹⁰⁻²⁹¹ The function of these nanoparticles was to serve as a mechanical reinforcement and mechanism to dissipate mechanical energy.²⁴⁰

Self-healing Bioinks: In an effort to reproduce the flow properties necessary for bioprinting, it is suggested that materials crosslinked with reversible bonds could rapidly self-heal and be a promising strategy for producing extrusion bioprinting materials (Figure 19).²⁴⁰ These bioinks can be of the supramolecular type, based on non-covalent interactions (i.e., electrostatic, hydrophobic, hydrogen and guest-host interactions) or can take advantage of reversible/exchangeable covalent bonds (i.e., boronate ester, imine, hydrazone).²⁹²⁻²⁹³ Although these reactions do not involve light, photo-activated materials have indeed been critical to enhancing properties of the final construct. As an example, Highley et al. utilized guest-host interactions in a bioink composed of hyaluronan modified by adamantine and cyclodextrin for both the extruded ink as well as for support bed.²⁹⁴ The G-H interactions provided initial stability, but for long-term stability, a secondary network based on HA-MA was crosslinked with UV light. In a second approach, which did not require post-print crosslinking, Kim et al.²⁹⁵ combined oxidized HA (OHA), a soluble glycol chitosan (GC) and adipic acid dihydrazide (ADH) for a self-healing bioink based on a competitive formation of imine bond (aldehyde of OHA and primary amine of GC) and acylhydrazone bonds between OHA and ADH. OHA and HA directly modified with ADH could also produce a self-healing and printing hydrogel, which could be additionally stiffened and patterned with HA functionalized with norbornene plus a tetrathiol and PI.²⁹⁶ Taking advantage of non-covalent interactions between hydroxydopamine and proteins, Shin et al. showed that modification of ECM components with gallol moieties, an aromatic ring containing three hydroxyl groups, could form hydrophobic interactions that enhanced the short-term shear-thinning properties of the material, printing fidelity, and adhesion to tissue surfaces.²⁹⁷

Granular Bioinks: Typical bulk hydrogels have pore sizes in the range of 10-100 nm, and in addition to limited transport of oxygen, nutrients and waste products of these materials, the embedded cells are dependent on protease- or hydrolytic-dependent cleavage of the polymer network to gain space for proliferation, migration, and ECM deposition. The introduction of interconnected

macroporosity/supermacroporosity (macroporosity is defined here as pore sizes between 50 nm - 10 μm and supermacroporosity between 10 - 500 μm) has been shown to support formation of neurons, establishment of capillary beds, and proliferation and formation of cellular networks.²⁹⁸⁻³⁰⁰ Structures printed from macroporous bioinks would be able to benefit from the superior cell infiltration, spreading or migration which have been observed in structured hydrogel environments.³⁰¹ The temporal control of polymerization afforded by photo-activated materials can play an important role. Macroporous bioinks were first reported based on aqueous phase separation that occurs between two immiscible aqueous phases of Gel-MA and poly(ethylene oxide)(PEO), where cells were loaded in the Gel-MA phase.³⁰² Once the phase-separated bioink was stabilized by photocrosslinking, the PEO was subsequently removed, leaving porous structures which highly promoted cell spreading. Perhaps an even more robust method to create macroporous materials uses granular microgels. These materials have excellent mass transport properties due to the interconnected void space between the microgels (see excellent reviews^{301, 303-304}). For example, the Khademhosseini group used microfluidic-assisted Gel-MA beads, which were annealed together with additional UV light exposure to form a high content Gel-MA material with excellent viability of the seeded human umbilical vein endothelial cells (HUVECs).³⁰⁵ In a similar manner, the Segura group generated PEG microgels using microfluidics and then annealed the spheres together with UV (as well as activated Factor XIII (FXIIIa) or click chemistry).³⁰⁶ Cells within these so-called Microporous Annealed Particle (MAP) gels demonstrated excellent cell spreading, proliferation, and network formation compared to smaller-pore bulk gels, and the material showed improved *in vivo* wound healing in mice.²⁹⁸ The same group has also used tetrazine-norbornene click reactions to tune the interaction strength between microgels and by extension the stiffness of the microgel scaffold.³⁰⁷ Using colloidal interactions to form structured hydrogels, Nair et al. found that the vasculogenesis could be tuned based on the structure of the porous void fraction.³⁰⁰ They were able to demonstrate the formation of a network of dense and interconnected endothelial cell (EC)-lined capillaries within the colloidal macrostructured gels. Recently, Caldwell et al. showed the importance of porosity on cell behavior.³⁰⁸ They compared MSC-seeded macroporous scaffolds made from large (~190 μm), medium (~110 μm) and small (~14 μm) spherical microgels and found that the large spheres significantly induced the cytokine secretion.³⁰⁸

Auspiciously, “soft jammed” granular microgel materials exhibit shear thinning and/or self-healing properties that make them particularly suitable for extrusion bioprinting (Figure 19). Being composed of already crosslinked materials, granular bioinks also have an inherent resistance to flow. For example, Highley et al. used a microfluidic-based approach to make jammed bioinks also containing cells, though the stability of these structures was weak and required the presence of a support gel.³⁰³ Xin et al. used electrospraying to create PEG microbeads that were printable and could be annealed using thiol-ene click chemistry.²⁰⁷ Composite bioinks containing microgels suspended in a continuous medium has also been reported. Song et al. used microgels from 10% gelatin to achieve shear thinning bioinks and suspended these microgels in a 3% gelatin solution that could later be stabilized by transglutaminase.³⁰⁹ Methods to produce microgels have also been an area of active research with “in-air” production of microgels achievable with faster rates and without the oil phase.³¹⁰

Recently, methods to produce uniform elongated, high-aspect-ratio microgels have also been produced with greater potential to influence cell alignment and migration compared to spherical microgels.³¹¹⁻³¹² Kessel et al. showed that elongated gel microstrands were also applicable to extrusion bioprinting (Figure 19).³¹³ High-aspect ratio HA-MA microstrands entangled, producing a printed construct with greater stability compared to spherical granular gels. The microstrand constructs did not need to be secondarily crosslinked and the properties of the printed bioink could be adjusted by the time of exposure of the bulk gel to UV light. An added advantage is the alignment of the microstrands along the printing direction which contributed to the formation of anisotropic structures and alignment of cells.³¹³ The mechanism of shear thinning in the nozzle of extrusion printers using entangled inks is illustrated in Figure 21, along with our current understanding of how molecules, nanoparticles, and microgels align and/or deform during flow. In each of these examples, stabilization of the structures by photo-activated materials can be advantageous.

Figure 21. Theoretical mechanisms of shear thinning in the nozzle of extrusion bioprinters

Hardware Advances for Extrusion Bioprinting: One of the powerful aspects of extrusion bioprinting is the biological relevance of the dimension of the strands which are deposited. These strands can be deposited in patterns reminiscent of the vascular branching³¹⁴, and channels can be formed within hydrogels when the deposited material is a sacrificial bioink.³¹⁵⁻³¹⁷ Other approaches to directly bioprint vascular structures have taken advantage of coaxial needles to print hollow structures from low concentrations of photo-activated materials. For instance, by placing low viscosity Gel-MA (~ 0.08 Pa·s), cells, and alginate in the core of the nozzle and CaCl_2 in the sheath component, it was possible to reduce the Gel-MA concentration to 4.5%²⁷⁵ (Figure 22A). Liu et al. were able to further reduce the Gel-MA concentration to 2.5% by polymerizing a core of Gel-MA, CaCl_2 and cells within a sheath of alginate.³¹⁸ Interestingly, two-material “Janus” type strands can also be made where the inner core of the co-axial extruders is fed via a y-junction microfluidic head (Figure 22B). Costantini et al. also printed coaxial Janus strands of PEG-fibrinogen, alginate and I2959, where one half of the core contained C2C12 myogenic cells and the other half BALB/3T3 fibroblasts.²⁵ Finally, triple coaxial nozzles can be used to print tubular structures where CaCl_2 is placed in both the outer sheath and inner core³¹⁹ (Figure 22C) or when the inner core contains CaCl_2 in a fugitive Pluronic ink which diffuses to crosslink two outer alginate-containing sheath layers (Figure 22D).^{18, 320} Some disadvantages of the coaxial vessels are their possible collapse and poor adhesion to adjacent strands. Recently, it has been suggested that more uniform endothelialisation of channels can be achieved by adding endothelial cells to sacrificial core gelatin strands that are printed alongside photo-activated sheath materials. As the gelatin liquefies, the released cells are shown to evenly cover the inner surface of channels.³¹⁷

Figure 22. Coaxial nozzles can be used to print low concentrations of photo-activated materials by adding alginate, which rapidly crosslinks in the presence of CaCl_2 . A) A solid strand of bioink can be rapidly crosslinked with CaCl_2 in the sheath.¹⁹ B) A Janus arrangement allow printing of two bioinks within a single strand.²⁵ C) Hollow structures can be printed using triple coaxial nozzles.^{318,319} D) Two-layered hollow structures can be printed with triple coaxial nozzles (25G/18G/15G), where the core material is CaCl_2 -containing Pluronic.^{18,320}

Recent hardware advances have also improved resolution of the method, one area in which extrusion bioprinting does not compare favorably to other techniques. For example, Kenics mixing elements were placed prior to the exit of the nozzle, creating complex “chaotic” patterns of multimaterials in the printed strands (Figure 23A).³²¹ Although most commercial extrusion bioprinters have multiple printheads allowing two or more bioinks to be used, switching between printheads is often cumbersome, slow and disrupts the print flow. The introduction of microfluidic printheads was a natural evolution to rapidly switch the flow of bioinks in accordance with the order and volume of each material needed for the

multimaterial print.³²²⁻³²⁴ Capillaries from up to seven different reservoirs have been merged into a single printhead, where the actuation of valves and pressure allows continuous flow during the switching.³²³ A similar approach switched the flow between small nozzles that were bundled into one large nozzle.³²⁵ Finally, in order to further increase the size and complexity of printed structures, the concept of parallel printing, also termed voxelated printing, has recently been published (Figure 23B).³²⁶ Hardware advances are making possible the printing of new types of bioinks which need to be mixed just prior to exiting the nozzle or where particles like cells can be concentrated at the tip.³²² Finally, the incorporation of “lab-on-a-tip”-type diagnostics could be used to expand the functionality of extrusion bioprinting.

Figure 23. Examples of A) chaotic printing with a Kenics mixture in the nozzle (Scale bar: 1 mm), and B) parallel “voxelated” printing of gelatin using 8 printheads and very rapid switching of the flow (Scale bar top row: 8 mm, Scale bar bottom row: 2 mm). A: Adapted with permission from ref 321. B: Adapted with permission from ref 325. Copyright 2019, Springer Nature.

3.1.2 Applications

Extrusion bioprinting has been applied to almost every cell and tissue type. However, the great majority of applications are at an early stage and there is no bioprinted product currently on the market. Due to the large body of current literature, we have restricted ourselves to just a few illustrative examples of situations where bioprinted constructs have made significant advances in mimicking the properties of native tissues. Given the resolution of extrusion bioprinting, the method has been optimal for the production of tubular structures and channels, and in particular, the study of the interactions between channels. For example, Song et al.⁵⁴ made channels using a sacrificial G-H bioink that was printed into a support bath. The support could be stabilized postprinting by a UV-triggered thiol-ene reaction. After endothelialisation of the channels, another channel releasing angiogenic factors was used to create signaling gradients, which induced sprouting of capillary structures into the support bed medium (Figure 24A).⁵⁴ With this system, one could study the effect of shape and curvature on the process of vascular sprouting. In a second example, adjacent PEO-pluronic sacrificial channels were printed into a bed of gelatin-fibrin, and after removal of the sacrificial material, proximal tubular ECs were perfused into one channel and glomerular microvascular ECs into the adjacent one (Figure 24B).³²⁷ The authors observed perfusion of albumin and glucose out of the proximal tubule into the vascular lumen, indicating that complex transport processes reminiscent of reabsorption process in kidneys could be modeled.

Extrusion bioprinting has also been used to study the importance of spatial interaction distance between cell types. For example, Maiullari et al. combined HUVECs and iPSC-derived cardiomyocytes³²⁸ to recreate some of the complex inhomogeneities of cardiac tissue (Figure 24C). They found that the maturation of cells in bioprinted materials was superior to that of bulk gels, largely due to the printed macroporosity and improved mass transport. Additionally, they found staining of alpha-sarcomeric actin, which is indicative of aligned cardiomyocytes, was promoted in the printed samples and much reduced in bulk, non-printed gels. In particular, the greatest *in vivo* alignment of the cardiomyocytes was found when the two cell types were printed within the same strand (called Janus geometry) using a microfluidic head, and the effect was diminished when the two cell types were printed in alternating layers (Figure 24C). In another study, anisotropic functional myocardium was extrusion bioprinted with an alginate/Gel-MA bioink containing 1×10^7 HUVECs/mL and I2959 in the core of a nozzle with CaCl_2 flowing in the

sheath of the coaxial extruder. The aspect ratio of the grid ranged from 2x2, 2x3, 2x4 to 2x5, and maximum cell alignment was found in the 2x5 configuration (Figure 24D).¹⁹

Figure 24. Examples of extrusion bioprinting applications. A) Vascular sprouting was influenced by the curvature of the channel. B) Proximal tubule epithelial cells (green channel) were printed in close proximity of glomerular microvascular endothelial cells (red channel) as an *in vitro* model of renal reabsorption. C) Cardiomyocytes and HUVECs were printed within a single strand (Janus) or in alternating layers (Scale bar: 50 μm). D) Contractile endothelialized myocardium was printed using an alginate/Gel-MA bioink which was later seeded with cardiomyocytes. Long-term 28-day contractility was achieved when a 2x5 aspect ratio grid was used. A: Adapted with permission from ref 54. Copyright 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. B: Adapted with permission from ref 326. C: Adapted from ref 328 (Distributed under the Creative Commons Attribution License). D: Adapted with permission from ref 19. Copyright 2016 Elsevier Ltd.

Another strength of extrusion bioprinting is the ability to precisely position two or more cell types to better mimic their *in vivo* distribution. While these examples do not use light-activated materials, they could be adaptable to the materials described in this review. Derr et al. used extrusion bioprinting to manufacture skin equivalents (SEs) consisting of connective tissue dermis and epithelial epidermis.³²⁹ The bioink for the dermis was composed of fibrinogen, collagen type I, gelatin and elastin combined with human dermal fibroblasts. Using the microvalve of the bioprinter, laminin/entactin was jetted onto the dermal surface, followed by extrusion of the neonatal dermal epithelial keratinocytes, which formed a complete monolayer after 14 days. The SEs showed good functionality with intact “outside in” and “inside out” barrier function,³²⁹ as confirmed by cadherin staining of the epidermal cells and collagen VII staining of the basement membrane (Figure 25A). In another recent example, the Cho group, who has pioneered the use of soluble ECM extracts to improve biological functionality of bioprinting materials, used a triple coaxial nozzle to position ECs on the inner surface of their biofabricated vessels and smooth muscle cells on the exterior (Figure 25B). The biofabricated grafts were patent in an *in vivo* rat model and stable endothelialisation and maturation of the smooth muscle layer were observed.¹⁸

Figure 25. Applications of multicellular extrusion bioprinting. A) Bioprinted skin substitutes have barrier function with staining for basement membrane protein type VII collagen and Cadherin-1 (CHD1). B) Triple coaxial extrusion bioprinted vascular graft with single layer of endothelial cells facing the lumen and an exterior layer of smooth muscle cells. Left: Adapted with permission from ref 329. Copyright 2019 Mary Ann Liebert, Inc. Right: Adapted with permission from ref 18. Copyright 2019 AIP Publishing LLC.

Finally, a unique feature of extrusion bioprinting is its ability to align proteins and molecules along the printing direction, thus providing cells within the printed structure with important topological/guidance cues. In these cases, the shear stress, which is often thought to be a detrimental factor, can be beneficial in increasing alignment of the structures. Kim et al. used a 5% collagen bioink which, when printed at a speed of 10 mm/s and using a 100 micron nozzle diameter, allowed sufficient alignment of the collagen to orient human ADSCs, H9C2 cardiac muscle cells and MC3/3-E1 bone cells.²¹⁷ Interestingly, the application of 50 mM glycine and 150 mM KCl was critical to support the fibril formation of the collagen (Figure 26A). In a related application, collagenous bioinks derived from decellularized corneal ECM were extruded with 20G, 25G and 30G nozzles, which increased the alignment of the collagen fibrils compared to a non-printed control. The embedded keratocytes showed increasing cellular alignment as seen by f-actin staining, but also increased expression of α -smooth actin at the highest gauge needle, where shear stress is highest (Figure 26B).³³⁰ This increased expression is a sign of myofibroblast differentiation, a process associated with reduced corneal transparency. Fibrin can also align during extrusion as demonstrated by a study using a bioink containing fibrinogen, HA, and factor XIII. Strands were printed into a bath containing thrombin and PVA, which could stabilize the fibrin in an aligned orientation. Embedded primary Schwann cells could then align along the fibrin microstrands (Figure 26C).³³¹

Figure 26. Examples of cellular alignment according to the print direction. A) Human adipose-derived stem cells align along fibrillated collagens. B) In a corneal model, keratocytes were printed in bioink consisting of decellularized corneal extracellular matrix. Using nozzles of decreasing diameter, there was increasing alignment of the collagen fibers compared to non-printed (NP) control. The smallest 30G nozzle however causes upregulation of α -smooth muscle actin (Scale bar: 50 μ m). C) Primary Schwann cells aligned according to bioprinted fibrin-HA strands (Scale bar: 100 μ m). A: Adapted with permission from ref 217. Copyright 2019 Elsevier B.V. B: Adapted with permission from ref 330. Copyright 2019 IOP Publishing Ltd. C: Adapted with permission from ref 331. Copyright 2016 Elsevier B.V.

3.1.3 Limitations and Outlook

Despite the substantial output of research using extrusion techniques and advances to produce good phenotypic models, extrusion bioprinting suffers from certain disadvantages. Looking toward the

translation of bioprinted products to the clinics, it is important to bear in mind certain potential limitations of the method. Firstly, extrusion has inherently limited resolution, and printing of cell-laden bioinks with good viability requires the use of nozzle diameters of several hundred microns. In reality, the diameter of the strand can be even larger due to exudate swelling. The printed strands are therefore an order of magnitude larger than the dimension of an average cell, making control of cellular and subcellular environment of individual cells not possible. Another potential limitation related to extrusion through the small nozzle size is the possible deleterious effects of shear stresses and extensional strains on cells as they are mixed into viscous bioinks and during their entry into and out of the nozzle. Shear stress can reduce cell viability, but it can also alter proliferation capacity and phenotypic expression of the cells,^{330, 332} where simple live/dead assays may give an incomplete picture of the state of the cells after printing.

Perhaps a second restriction of the technique relates to the difficulty in controlling the flow of bioinks, which makes reproducibility and accuracy problematic to achieve. The strand must hold its shape until it is crosslinked, but it must also coalesce with the under- and overlying strands so that the part has good integrity. The rheological window, which allows these seemingly contradictory behaviors, is quite narrow. Multiple factors can affect the rheological properties of the bioink, including the mixing process, the DF, molecular weight of the polymer, and the addition of cells. To complicate the situation further, many bioinks may relax or have thixotropic or time-dependent shear thinning behavior, meaning the print process could change within a cartridge of bioink or from cartridge to cartridge.³³³ In practice, these viscosity variations may be compensated by manual tuning of the printing process, particularly the printing pressure and speed. Given that there is a range of printing parameters, the amount of material that is actually deposited on the build plate, and hence the number of cells in the cellular product, can vary substantially.

Print fidelity of layer-by-layer hydrogel objects is more difficult, as hydrogels often undergo unpredictable dimensional changes. As has been observed in the deposition of strands by FDM, the profile of the strand is circular as it exits the nozzle, but as the strand is deposited, it makes a 90° turn. Once deposited, its cross-sectional dimension is more ellipsoid (wider in width than height). The sagging of the z direction is difficult to predict and increases with the number of layers. Therefore, the z error (programmed line thickness minus the actual line thickness) will result in changes in the distance between the nozzle tip and printed layer, which in extreme cases causes the nozzle to trail through the ink or the strand to break up. Again, manual adjustments to the printer are often necessary, when the number of printed layers exceeds around ten.

In order to improve print fidelity and keep the strand from flowing, a rapid cessation of flow post-printing is often achieved by exposure of UV light after each layer, which allows much taller structures to be printed (Figure 27).²⁷⁴ Layer-by-layer crosslinking, however, can affect the layer-layer adhesion and/or cause a gradient in mechanical stiffness. Since the UV light can penetrate hundreds of microns into the printed construct depending on the optical properties of the material, the initial layers are exposed to more UV light than the upper layers. Post-printing of the entire construct has advantages in terms of construct integrity, but a disadvantage is that the z-error increases with increasing weight of the overlying layers.

Figure 27. Light-mediated printing of tall structures. PEG-DA was printed without crosslinking (a) and with crosslinking between layers (b)-(e), the latter of which allow the printing of tall structures but diminished the layer-layer adhesion strength. Adapted with permission from ref 274. Copyright 2017 Elsevier B.V.

Currently, bioprinting of tens or hundreds of identical products necessary for *in vitro* screening and for toxicology and safety studies for regulatory purposes is challenging. The fidelity of the printed construct compared to the original .stl file needs to be assessed prior to submission to regulatory authorities.³³⁴ To assess print fidelity, algorithms have been developed, but improvements on both the processing of bioinks and the hardware to detect and correct printing errors are required.

Despite these limitations, extrusion bioprinting has unique advantages compared to its competitors. It is the only biofabrication technique which can create truly anisotropic properties³¹³, as the flow of the material can be used to effectively align fibrils, microgels, and by implication, cells. To benefit from this phenomenon, non-planar slicing software³³⁵ and curvilinear printing³³⁶ could be harnessed to exploit the natural anisotropy of tissues or organs, replacing arbitrary layer-by-layer slicing. Extrusion bioprinting explicitly benefits from high-yield stress, viscous materials, thus the functionality of extrusion bioinks can be augmented through the addition of multifunctional nanoparticles and high cell densities in ways not possible for other techniques. Extrusion bioprinting excels in multimaterial applications and many of the most exciting advances have come on the hardware side (parallel printing³²⁶, chaotic printing³²¹, Janus printing²⁵). Finally, for clinical-sized grafts, extrusion bioprinting outperforms all other techniques and is the method of choice for replicating tubular structures like small caliber vessels and nerves found in the human body.

3.2 Droplet-Based Bioprinting

3.2.1 Principles of Droplet-Based Bioprinting

Droplet-based bioprinting originated from inkjet printing, which had been widely used for 2D printing of inks onto papers for decades. A conventional inkjet printer prints text or graphics by jetting ink droplets from an orifice, depositing them on a paper followed by a drying process. In 1994, the first inkjet-based 3D printer was brought to the market by Solidscape,³³⁷ where heated liquid wax droplets were deposited on a substrate and rapidly solidified by cooling to build a 3D construct in a layer-by-layer fashion. Later, starch- and plaster-based ink materials came on to the market, and finally in 2000, a 3D inkjet printer called Quadra (Objet Geomerics Ltd., currently a branch of Stratasys Ltd.), utilizing photo-activated materials as inks, became commercially available. The 3D inkjet printing technique, in combination with photo-activated inks and light-mediated solidification, is also known as multijet or polyjet 3D printing, which is widely used for general rapid prototyping as well as manufacturing 3D parts.³³⁸ Since then, inkjet-based 3D printers have expanded their applications to other fields, including the deposition of living cells to build 3D cellular constructs. As the field grows, diverse droplet-based bioprinting methods have been developed, such as micro-valve and acoustic-droplet-ejection bioprinting.³³⁹ Since inkjet and micro-valve bioprinting have been widely adopted for directly building cell-laden constructs (Figure 28), these two techniques and their use in combination with photo-activated bioinks are discussed in this section. A comprehensive overview of general droplet-based bioprinting can be found in a recent review paper.³³⁹

Figure 28. Mechanisms of generation of droplets in drop-on-demand bioprinting. Left: In inkjet bioprinting, ejection of an ink drop is induced by pressure generated through either formation of vapour bubbles upon heating (Thermal) or displacement of a piezoelectric actuator (Piezoelectric). Right: In micro-valve bioprinting, a droplet is ejected when the solenoid coil induces magnetic fields which pull the plunger upwards and open the orifice. Since the ink in a cartridge is under pneumatic pressure at all times, when the orifice is opened, droplets can be ejected.

Hardware

There are two different modes of droplet-based printing: continuous jetting printing (CJP) and drop-on-demand (DOD) printing.³⁴⁰ In CJP, an ink is continuously ejected and spontaneously breaks into droplets due to Rayleigh-Plateau instability, which describes the tendency of a continuous liquid stream to minimize the surface area of the stream by forming droplets.³⁴¹ The generated droplets are electrically conductive so that electric or magnetic fields can position them at the intended position. Droplets not used for printing are collected and recirculated. Conversely, in DOD printing, individual droplets are jetted on demand. In bioprinting applications, the DOD mode is preferred over CJP because it avoids ink recirculation, something which can raise the risk of contamination as well as waste valuable bioink material, and the DOD printing does not require electrical conductivity of the bioink. DOD printing typically involves the following three steps: 1) generation of droplets, 2) deposition of droplets on a substrate, and 3) layer-by-layer solidification.

In inkjet printing, droplet generation can be induced by thermal, piezoelectric or electrostatic mechanisms, with thermal and piezoelectric-based bioprinters being the most widely adopted^{243, 342} (Figure 28). In thermal bioprinting, the jetting of a droplet is powered by pressure generated through rapid formation and collapse of an air bubble during rapid heating and cooling of a resistor. In piezoelectric bioprinting, the displacement of a piezoelectric actuator generates pressure to eject the fluid. Thermal bioprinters are relatively cheap compared to piezoelectric ones. However, the local temperature of the ink can increase up to 300 °C for a few microseconds, which could limit material types to the ones resistant to thermal degradation.³⁴³ In contrast, piezoelectric bioprinters are relatively expensive but compatible with a wider range of materials without any of the risks caused by high temperature compared with thermal bioprinters. For bioprinting cell-laden constructs, both bioprinters have been extensively utilized. The typical volume of droplets generated by the inkjet printers ranges from 1 to 100 pL, with a typical diameter of 10-100 µm. The rate of droplet generation is up to 30 kHz.^{339, 342}

In addition to inkjet printing, micro-valve bioprinting is another DOD printing method widely adopted for biofabrication. A micro-valve bioprinter generates ink droplets on demand by controlling the movement of a plunger by a solenoid coil (Figure 28). When a voltage pulse is applied, the solenoid coil induces magnetic fields, which pulls the plunger upwards and opens the orifice. The bioink in a cartridge is under pneumatic pressure at all times, and thus when the orifice is opened and the pressure is high enough to overcome the viscosity and surface tension of the bioink, droplets are ejected. In this way, droplets can be generated at a few kHz, and the typical range of orifice diameters is 100–300 µm, which

generates larger droplets (100-600 μm) compared to the thermal or piezoelectric inkjet bioprinter. However, it is more tolerant to viscous bioinks (1-200 mPa·s) compared to inkjet bioprinters.^{339, 344}

Bioink considerations for droplet-based printing

In addition to hardware, physical properties of a bioink should meet certain criteria to facilitate droplet-based bioprinting. In particular, surface tension, viscosity, and density are important parameters. A detailed physical description about printing fluids is suggested in the study by Jang et al.³⁴⁵ Briefly, one can predict printability of a fluid using the following equation proposed by Fromm:

$$Z = \frac{1}{Oh} = \frac{\sqrt{\gamma\rho l}}{\eta} = \frac{\sqrt{We}}{Re} \quad (1)$$

where Oh = Ohnesorge number, γ = surface tension of a fluid, ρ = density of a fluid, l = characteristic dimension (the radius of the printer orifice), η = viscosity of a fluid, We = Weber number, Re = Reynolds number.

For inkjet printing, a study based on numerical modeling suggested that a material with a Z value (the reciprocal of Ohnesorge number) between 1 and 10 is printable.³⁴⁶ Later, a slightly different range of Z values ($4 < Z < 14$) was suggested, which was determined experimentally using a piezoelectric inkjet printer.^{345, 347} For a fluid with a Z value smaller than 1, too-high shear stress by high viscous fluid results in too much viscous dissipation to form a droplet, whereas a fluid with a Z value larger than 10 would form a main drop accompanied with small satellite droplets. For micro-valve printing, this range could differ from inkjet printing. Choi et al. reported that fluid with a Z value between 0.23 and 84.77 was able to form stable droplets and be printed using a custom-made micro-valve printer.³⁴⁸ Ejection of a fluid with a low Z value (<1) was enabled by increasing the pneumatic pressure up to 200 kPa to overcome viscosity dissipation, and a backflow stopper helped with preventing liquid backflow and forcing the liquid to move forward. Printing a fluid with a high Z value (>14) was achieved by optimizing printing pressure and droplet velocity to avoid the formation of long filament-shaped droplets and satellite droplets. They claimed that the optimization could be done within a wider range of pressure and velocity compared to inkjet printing, because the micro-valve printer had a lower minimum velocity requirement (0.63 m/s) than an inkjet printer (1.4 m/s). However, Schulkes reported that a fluid with the Ohnesorge number (Oh) larger than 1 (i.e., $Z < 1$) forms a stable filament rather than a droplet, which implies that stable droplet-based printing with an ink having a low Z value close to this limit would be challenging.³⁴⁹

For a bioink droplet, viscosity is often the most important parameter in determining printability, because most bioinks are based on biocompatible aqueous solutions; so there are no significant differences in surface tension (20-70 mJ/m²) or density ($\sim 1000 \text{ kg/m}^3$) between bioinks, but viscosity can vary greatly. The typical viscosity of bioinks for a DOD inkjet bioprinter is $<15 \text{ mPa}\cdot\text{s}$ ³⁴² and for a micro-valve bioprinter $<200 \text{ mPa}\cdot\text{s}$. In addition to the physical properties of a bioink, the travel speed of the droplet should be optimized, where the speed needs to be high enough to overcome the surface tension at the orifice and at the same time low enough to prevent the droplet from splashing on a substrate.³⁵⁰

Figure 29. Photographs of printed strands with respect to delay and drop spacing (left) and schematic representation of printed strand behavior at an intermediate temperature (right). Uniform lines (III, in the middle of the scheme) can be obtained with optimized drop spacing and delay (see white area). Adapted from ref 351.

In addition to the successful ejection of a droplet from the orifice, interactions between droplets and the receiving substrate are important for quality printing. The shape of a droplet is affected by substrate properties such as hydrophobicity and roughness. For printing continuous strands, the spacing between droplets should be also adjusted to form a uniform line. The conditions for printing stable droplets or

strands using an inkjet printer have been intensely investigated both theoretically and experimentally.³⁵¹⁻³⁵⁴ Soltman et al. demonstrated that the shape of a printed line depends mainly on spacing between droplets, lengths of delay (time between ejection of drops, 1/frequency), and temperature for a given droplet volume.³⁵¹ In this study, an aqueous ink of a conductive polymer, poly(3,4-ethylenedioxythiophene)/poly(styrenesulfonate)(PEDOT/PSS, 1.3 w/w%) was deposited onto a glass slide coated with poly(4-vinylphenol). Depending on the drop spacing and delay, the deposited droplets along a line exhibited different behaviors (Figure 29). When the spacing between droplets was greater than the droplet diameter, no coalescence occurred and isolated droplets were formed. As the distance decreased, the droplets coalesced to form a uniform line, yet a further decrease in spacing led to bulging along the line. The delay and temperature are important parameters that affect evaporation of liquid in a drop and thus drop volumes. For example, a shorter delay will lead to less evaporation than a longer delay, yielding larger drops, which require more spacing to form a uniform line than smaller drops. Indeed, for a given drop spacing, a shorter delay resulted in bulging, whereas a prolonged delay led to formation of a uniform line. The width of a printed line is affected by the surface contact angle of the deposited ink drop as well as the size of a drop. A hydrophilic substrate will induce a higher degree of droplet-spreading than a hydrophobic one, which results in larger widths of printed lines. This tendency was experimentally demonstrated by Smith et al.³⁵³ using a silver-containing ink, where 358 μm and 82 μm of line widths were obtained on a glass (contact angle 5.9 °) and a Teflon (contact angle 58 °) substrate, respectively. Overall, material properties, drop coalescence, delay, drop volume and drop-substrate interactions determine resolution of DOD printing.

Figure 30. General procedure of droplet-based printing (top) and different methods of crosslinking (bottom).

Crosslinking methods

After the deposition of droplets, crosslinking immediately follows (Figure 30, top). In addition to photocrosslinking, various crosslinking chemistries have been adopted for droplet-based bioprinting including ionic³⁵⁵⁻³⁵⁶ (e.g. Ca^{2+}), chemical³⁵⁷ (e.g., protamine, poly-L-lysine), enzymatic³⁵⁸⁻³⁵⁹ (e.g., horseradish peroxidase (HRP), transglutaminase), and thermal crosslinking³⁶⁰⁻³⁶¹. For each type of crosslinking chemistry, several different methods of droplet printing have been demonstrated (Figure 30, bottom).

For ionic, enzymatic, or chemical crosslinking, solidification can be induced by sequential deposition of bioink followed by another type of ink containing crosslinking agents. For example, iPSCs were successfully printed by sequentially depositing an ink composed of alginate and a bioink comprised of cells and CaCl_2 crosslinking solution.³⁵⁶ Despite the fragility of iPSCs, the viability of printed cells showed no significant difference from non-printed cells (>80%), implying high cytocompatibility of the printing method. In another system, a 3D cell-laden construct was printed by sequential printing of two inks, one composed of phenolic hydroxyl (Ph)-modified polymer and HRP and the other of Ph-modified polymer and hydrogen peroxide.³⁵⁸ Crosslinking between Ph moieties on gelatin or alginate was induced when

the second ink, containing hydrogen peroxide, was printed on top of the layer printed using the first ink, with HRP.

A jetting-spraying method is an approach where each deposited layer was spray-coated with crosslinkers, which is suitable for ionic or chemical crosslinking. Yoon et al. demonstrated that the method is compatible with various crosslinking agents, including CaCl_2 , thrombin, protamine, poly-L-lysine and chitosan oligosaccharide.³⁵⁷ In addition, a centimeter-scale cell-laden hydrogel was biofabricated with high printing fidelity at high printing speed (75 mm/s), which was attributed to the fast crosslinking made possible by the sprayed crosslinker mist. Similar to extrusion printing, printing in a bath has also been used for droplet-based printing. One common system is to print an alginate-based bioink into a bath filled with CaCl_2 solution. For instance, cell-laden vessel-like structures were generated by printing 1% alginate solution into a bath filled with a CaCl_2 solution.³⁶² The solution provided a buoyant force as well as fast crosslinking so that vascular structures with overhangs were achieved. Since droplet-based printers are only compatible with inks having low viscosity, some studies have utilized approaches where a bioink composed of cells and low viscosity crosslinker is printed in a bath filled with uncrosslinked hydrogel precursor solution. In the study by Cui et al., a bioink comprised of thrombin/ Ca^{2+} and cells was printed into a fibrinogen biopaper to form fibrin channels.³⁶³ The printed endothelial cells (ECs) were aligned inside the fibrin channel and proliferated to form confluent layers in 21 days. In another approach, stabilization of ejected droplets was induced by self-assembly of lipid monolayers on the surface of droplets.³⁶⁴ Droplets of an aqueous ink were printed in a bath filled with oil, where lipid molecules are self-assembled on droplet surfaces, and the deposited droplets were further stabilized by lipid-lipid interactions between droplets to form stable bilayers. The droplets in resulting printed constructs were highly cohesive, providing the constructs with structural integrity. In the follow-up study, the method enabled biofabrication of cellular constructs where multiple cell types were patterned with the minimum feature size of ca. 200 μm .³⁶⁵

A few thermoresponsive polymers have been explored as a bioink or biomaterial ink for droplet-based printing. Blaeser et al. printed thin-walled cylindrical structures by ejecting agarose ink kept at 39°C into a bath containing fluorocarbon support liquid at 20°C.³⁶¹ In another study, a poly(*N*-isopropylacrylamide-co-2-(*N,N*-dimethylamino)-ethyl acrylate) bioink was printed onto a substrate at 37°C to generate arrays of aggregates of ECs, which eventually were fused to each other.³⁶⁶ Thermal crosslinking requires high concentrations of polymers for fast crosslinking, which hinders thermoresponsive polymers from being used as a sole bioink for droplet-based bioprinting. Instead, the thermal crosslinking is often used in combination with other crosslinking methods to obtain additional mechanical stability after printing. For instance, a bioink comprised of CaCl_2 and cells was printed into a mixture of alginate and collagen.³⁵⁵ Ionic crosslinking of alginate provided fast crosslinking during printing to maintain the printed structure, and the printed construct was further mechanically stabilized by inducing thermal crosslinking of collagen in the incubator at 37°C for 3-5 h.

Lastly, light-induced crosslinking has been utilized to solidify cell-laden constructs. In most studies in droplet bioprinting, photocrosslinking was induced by continuous irradiation on the printing stage (Figure 30, bottom). One needs to be careful not to expose the cartridges to the light throughout the printing procedure, which would cause undesirable crosslinking and clogging of the orifice. The photocrosslinking method has several advantages over other crosslinking modes in droplet-based bioprinting. Compared to sequential printing or jetting-spraying methods, photocrosslinking does not require a separate step for applying crosslinker, which could reduce the printing time. In addition, a wide range of materials could be used as photo-activated bioinks through conjugation of photoactive moieties to polymers, whereas ionic or thermal crosslinking is only relevant to a limited range of materials (e.g., gelatin, alginate). Furthermore, one could obtain additional structural stability by irradiating a printed construct after printing. For this, photocrosslinking is often used in combination with other crosslinking chemistries. For example, in the study by Biase et al.,³⁶⁷ a mixture of pluronic-diacrylate, Eosin Y and TEA was printed onto a substrate at room temperature to induce thermal crosslinking during printing, which was followed by photocrosslinking by light irradiation after printing.

Photo-activated bioinks in droplet-based bioprinting

Most photo-activated bioinks developed for droplet-based printing are based on PEG-DMA or Gel-MA. The bioinks for droplet-based printing are limited by the requirement of low viscosity to avoid clogging. PEG-DMA, in general, has low viscosity and is easy to functionalize, which makes it attractive for droplet-based printing. In addition, compared to Gel-MA, mechanical properties of PEG-DMA gels are more tunable and suitable for printing mechanically robust constructs. For example, compressive moduli of

printed constructs using 10% and 20% (w/v) PEG-DMA inks were 37 kPa and 395 kPa, respectively.³⁶⁸ On the other hand, PEG itself does not provide any biological cues, which often leads to reduced viability or cellular functions of printed cells. To address the issue, efforts have been made to enhance biological properties of PEG-DMA-based bioinks. In the study by Gao et al.,³⁶⁹ acrylated RGD and MMP-sensitive peptides were added to PEG-DMA. This ink was used to bioprint hMSCs within a layer-by-layer 3D tissue using a thermal inkjet bioprinter. The bioprinted hMSCs were then cultured in either a chondrogenic or osteogenic environment. hMSCs printed using the ink with acrylated peptides exhibited better mechanical maturation and higher chondrogenic or osteogenic marker expression compared to those printed using the ink without peptides. The same research group also reported a bioink composed of PEG-DMA and hydroxyapatite particles, which promoted formation of bone-like tissue during 21 days of *in vitro* culture.³⁷⁰

Gel-MA-based bioinks are more biologically active compared to PEG-DMA, due to the presence of bioactive amino acid sequences such as RGD. However, Gel-MA is usually too viscous for inkjet or micro-valve printing, and the one with low DF even forms a gel at room temperature. Therefore, Gel-MA solution at low concentrations (<5 wt%³⁷¹⁻³⁷²) has been used as bioink to avoid any risks of clogging. Several approaches have been explored to improve properties of Gel-MA-based bioinks for droplet-based printing. In general, methacrylation interrupts physical crosslinking of gelatin resulting in decreased viscosity. Therefore, for droplet-based printing, gelatin with high DS is desirable due to its low viscosity. However, high DS also leads to a high degree of crosslinking of gelatin, and thus the formation of a stiff gel. To independently control the photocrosslinking and viscosity of Gel-MA, Hoch et al. synthesized methacrylated and acetylated gelatin.³⁷³ The viscosity of GM10 (Gel-MA synthesized by adding ten-fold excess of MAA to gelatin) is suitable for inkjet printing (~6.7 G' mPa·s at 37 °C for 15 wt% solution). However, the formed hydrogel retained a high storage modulus (~55 kPa for 15 wt% solution), indicating formation of a stiff gel. Acetylated- and methacrylated gelatin had viscosity as low as that of GM10 (~5.2 mPa·s at the same condition) but formed a soft gel (G' ~25 kPa for 15 wt%). Another approach to controlling the mechanical properties of Gel-MA-based bioinks is to add PEG-DMA.²⁰⁰ A bioink comprised of 10 wt% PEG-DMA and 1.5 wt% Gel-MA was successfully printed using a thermal inkjet bioprinter, and the compressive modulus of a bioprinted construct was ca. 40 kPa.

Compared to the other biofabrication techniques, droplet-based printing is highly compatible with multimaterial printing. Similar to extrusion-based printing, a droplet-based printer can have multiple printheads, each of which contains a cartridge filled with a bioink and single or multiple nozzles. In addition, droplet-based printing allows deposition of multimaterials in a single droplet. For example, arrays of droplets having varying ratios of PEG-DMA to Gel-MA have been printed using a micro-valve bioprinter, which was used to study cellular response to different ECM compositions.³⁷⁴⁻³⁷⁵ To generate such arrays, droplets of Gel-MA were deposited with increasing volume followed by deposition of PEG-DMA with decreasing volume, resulting in the generation of arrays of droplets with the same volume. The resultant dual-material droplets exhibited homogeneous opaqueness, even though the degree of mixing of two materials in a droplet was not further studied. The method of printing multimaterials without additional mixing is possible because of the low viscosity of bioinks used in droplet-based printing. However, to date, this approach has not been fully exploited to bioprint a 3D construct with multimaterial bioinks.

3.2.2 Applications

Droplet-based bioprinting allows for the generation of complex arrays/patterns of drops containing cells and/or biologics for HT screening as well as tissue engineering. In addition, several types of tissue analogs such as cartilage, bone and vessels have been biofabricated using this technique in combination with photo-activated bioinks.

HT screening and patterning: Generation of droplets with varying compositions can be achieved by depositing different types of bioinks on a spot of varying volume. For example, in the study by Ma et al.,³⁷⁴ an array of droplets with varying ratios of Gel-MA to PEG-DMA was bioprinted using a micro-valve bioprinter. Specifically, a Gel-MA bioink (5 wt%) containing human periodontal ligament stem cells was first deposited with decreasing droplet volume. The volume of a droplet was controlled by varying the duration of valve opening between 1 - 5 ms. Subsequently, a PEG bioink (10 wt%) containing the same cells was deposited on the same array of Gel-MA droplets to achieve Gel-MA:PEG-DMA ratios of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5. The bioprinted stem cells exhibited highest viability as well as greatest spreading area in the condition with the highest Gel-MA ratio (5:0), and as the ratio of Gel-MA in a drop

decreased, the viability and spreading area also decreased (Figure 31). In a follow-up study,³⁷⁵ the same screening platform was used to find the optimal bioink composition for supporting *in vitro* osteogenic differentiation of human periodontal ligament stem cells, where the bioink composed of Gel-MA and PEG-DMA at a ratio of 4:1 was chosen, considering both biological properties and mechanical stability. The selected bioink with the cells was injected to an alveolar bone defect in a Sprague Dawley rat and led to the better healing of the defect compared to the same hydrogel without cells or a saline solution. Even though both studies only utilized Gel-MA and PEG-DMA, the approach holds great promise for HT screening of various biologically active materials.

Figure 31. A) Bioprinted arrays of cell-laden hydrogel drops with varying ratios of Gel-MA and PEG-DMA for HT screening. B) Live/dead staining images of cells in printed gels with a Gel-MA (5 wt%)/PEG-DMA (10 wt%) at ratios of 5/0, 4/1, 3/2 (top, left to right), 2/3, 1/4, 0/5 (bottom, left to right). (Scale bars: 200 μm) Adapted with permission from ref 374. Copyright 2015 IOP Publishing Ltd.

In addition, droplet-based printing allows the generation of micropatterns of biomolecules or cells. This strategy becomes powerful when the patterns are printed onto functional substrates. For example, microarrays of transfected cells with a spot diameter of ca. 500 μm were generated by seeding cells on the plasmid micropatterns printed by a piezoelectric inkjet printer.³⁷⁶ To achieve local deposition of cells on the patterns, the glass substrate was first functionalized with PEG, followed by printing using an ink composed of a plasmid (encoding two different fluorescent proteins, Venus and mCherry) and ECM proteins. When cells were seeded on the printed micropatterns on the PEG-functionalized substrates, the cells selectively attached to the patterns. The generated arrays could be a platform for functional analyses of specific genes on cells based on cell phenotypes. In a recent study by Jia et al.,³⁷⁷ patterning of biomolecules such as fibronectin and GFs on biomimetic nanofibrous matrices was demonstrated. Droplets were printed onto six different electrospun fibrous matrices composed of PCL and collagen. The printing resolution was affected by the wettability of the matrices as well as fiber organization, where higher wettability led to a larger printing area and isotropic or anisotropic fiber orientations resulted in round or oval shapes of droplets, respectively. By optimizing the materials and printing conditions, arrays of spots with high resolution ($25 \pm 10 \mu\text{m}$) were printed on the matrices. Using the optimized conditions, transforming growth factor beta 1 (TGF- β 1) was printed on the PCL/collagen electrospun fibrous mat. The amount of TGF- β 1 deposited on each microspot was controlled by changing the number of printed layers of the TGF- β 1 solution (100 ng/mL), where the layer numbers were varied from 1 to 10. As a result, the expression of a myofibroblastic differentiation (α -smooth muscle actin, α -SMA) increased with the amounts (i.e., layers) of deposited TGF- β 1 on a microspot.

Figure 32. Droplet-based bioprinting of cartilage analogs. A) Left: Schematic of bioprinting onto an *ex vivo* osteochondral plug. Right: Distribution of cells (green) throughout a printed (left) or cast (right) scaffold. (Scale bars: 100 μ m) B) Biomimetic fibrocartilage analogs bioprinted by a micro-valve printer. The region stained as red contains TGF- β 1 and MSCs and the green region contains bone morphogenetic protein 2 (BMP-2) and MSCs. C) Expression of chondrogenic and osteogenic marker genes by the cells in printed constructs containing BMP-2 or TGF- β only (single phase) or both (multiphase) after 36 days of *in vitro* culture. A, B: Adapted with permission from ref 200. Copyright 2013 The Royal Society of Chemistry. B, C: Adapted from ref 371.

Tissue implants: Photo-activated bioinks in droplet-based printing have been mainly studied for printing cartilage and bone analogs. Cui et al. developed a PEG-DMA-based bioink containing human chondrocytes (at 5×10^6 cells/mL) and I2959 (at 0.05 %).³⁶⁸ The bioink was directly printed onto an *ex vivo* osteochondral plug with cartilage lesion (4 mm diameter, 2 mm depth) using a thermal inkjet bioprinter (Figure 32A). Due to the low viscosity of the ink, cells in a cast gel sedimented, resulting in inhomogeneous cell distribution, whereas bioprinting with simultaneous layer-by-layer photopolymerization led to the formation of a gel having homogeneously distributed cells. Bioprinted chondrocytes in a 10 % PEG-DMA bioink underwent *in vitro* maturation, where increased gene expression of collagen II and aggrecan was observed after 6 weeks of culture compared to the gels cultured for 2 weeks. Furthermore, the *in situ* printed gel was well integrated into the osteochondral plug, resulting in significantly increased interface failure stress after 6 weeks of *in vitro* culture. In the follow-up study, they demonstrated that addition of Gel-MA to the PEG-DMA bioink led to enhanced biological activities of printed cells.²⁰⁰ 1.5 w/v% of Gel-MA was added to a PEG-DMA bioink (10 w/v%) containing hMSCs (at 6×10^6 cells/mL), and a cylindrical construct (with 4 mm diameter, 4 mm height) was printed onto the osteochondral plug (Figure 32A, left). As confirmed in the previous study, cells were uniformly distributed throughout the printed construct, but casting of the same bioink resulted in sedimentation of cells (Figure 32A, right). With Gel-MA present in the bioink, printed cells showed enhanced expression of marker genes for chondrogenesis or osteogenesis when cultured in chondrogenic or osteogenic media, respectively. In another study, biomimetic patterning of growth factors was achieved using a micro-valve bioprinter.³⁷¹ 5% Gel-MA-based bioinks containing MSCs and growth factors were used to bioprint a fibrocartilage analog connecting tendon and bone (Figure 32B). To recapitulate the microenvironment of native tissue, two different bioinks containing either BMP-2 or TGF- β 1 were printed as an interlocking structure (Figure 32B). For comparison, two types of single-phase hydrogels (containing BMP-2 or TGF- β 1 without patterning) were prepared as well. As a result, the expression of marker genes for both osteogenesis and chondrogenesis was enhanced after 36 days of *in vitro* culture compared to single phase hydrogels (Figure 32C). In addition, genes related to tendon or muscle were also upregulated in the fibrocartilage model.

3.2.3 Limitations and Outlook

As addressed in the previous section, droplet-based bioprinting has several advantages over other bioprinting techniques, including high compatibility with multimaterial printing as well as ease of HT arrays for screening. In addition, it has higher resolution and greater control over the deposition pattern compared to extrusion-based printing. However, there are critical drawbacks which impede wide application of the technique for biofabrication.

First, the small range of possible viscosities greatly limits the types and concentrations of materials which can be used, which in turn limits the properties of printed constructs. Polymers with high molecular weight or high concentrations cannot be used for droplet-based printing, and thus it is difficult to print mechanically robust constructs.³⁷⁸ Furthermore, clogging can easily occur even with a bioink with low viscosity, if the duration of printing is long. For applications requiring a bioink with high viscosity, one could choose to use the micro-valve bioprinter, which is compatible with materials with higher viscosity rather than thermal or piezoelectric inkjet printers. Other types of droplet-based printers could be an option for printing high viscosity bioinks without losing resolution or with higher resolution than inkjet printers. For example, an electrohydrodynamic (EHD) jet bioprinter can print an ink with maximum viscosity of ca. 3000 mPa·s,³⁷⁹ due to the different mechanism of droplet generation in EHD jetting. An EHD jet bioprinter generates droplets using an electric field between the conductive build substrate and the orifice, which is typically metal-coated. The ink in a syringe forms a meniscus at the orifice by back pressure, and when high voltage is imposed, a droplet of the ink is pulled out of the orifice and ejected. This “pulling from outside” mechanism allows for printing with higher resolution (orifice diameter > 100 µm) with high-viscosity inks compared to the inkjet or micro-valve printing where pressure change in a syringe pushes an ink and ejects a drop. However, the use of EHD jet printers for printing cells has not yet been either intensely studied or optimized. Considering that most cell-laden constructs have been printed using inkjet or micro-valve bioprinters to date, use of other types of droplet-based printer, including EHD jet printers, could solve some of the issues in current droplet-based bioprinting.

In general, droplet-based printing is not suitable for printing with a bioink with high cell concentrations (typically $\sim 1 \times 10^6$ cells/mL), partly due to possible clogging and partly due to an increase in viscosity upon the addition of cells.³⁸⁰ In addition, the small diameters of the orifices of droplet printers (10-150 µm) are not compatible with printing large cell aggregates or microcarriers. The low viscosity of bioinks can also cause sedimentation of cells when printing large constructs, leading to an inhomogeneous distribution of cells. Additionally, since the technique is non-contact printing that involves the flight of droplets, the printing condition should be carefully adjusted to prevent droplets containing cells from drying or crosslinking during flight.

Other concerns about droplet-based bioprinting are possible damage to proteins and/or cells by high shear stress and/or heat. The viability of cells bioprinted using a thermal inkjet printer depends on the type of cells. For example, Chinese hamster ovary cells and primary motoneurons had viability higher than ~90%,³⁸¹ whereas adult human dermal microvascular endothelial cells (HMVECs) – primary cells known to be highly sensitive to manipulation – resulted in 75% of cells apoptotic at 24 hours post-printing.³⁸² In addition, in a later study, overexpression of angiogenic heat shock proteins by printed HMVECs was observed, which could be useful for capillary formation of printed tissue as the authors claimed. However, there also exist the risks of uncontrolled activation of other functional heat shock proteins.³⁸³⁻³⁸⁵ Recently, thermal inkjet printing was also associated with upregulation of oncogenic pathways, increased phosphorylation of kinases and large-scale alternation in gene expression of breast cancer cells.³⁸⁶

Piezoelectric inkjet printing can also lead to mechanical stress-induced cell death. In particular, printing at high power and/or high vibration frequencies can cause damage to the cell membranes. Saunders et al. reported that the viability of bioprinted fibroblasts decreased with the printing voltage, where 98% and 94% viability were observed for cells printed at 40 V and 80 V, respectively.³⁸⁷ Furthermore, compressive mechanical stress caused by high vibration frequencies during piezoelectric inkjet printing can lead to denaturation of proteins. In the study by Nishioka et al., only 40% of HRP remained active after printing at a compression rate of $5.48 \times 10^4 \mu\text{m}^3/\mu\text{s}$, and the amount of damaged enzyme was reduced with decreasing the compression rate.³⁸⁸ For micro-valve bioprinting, high shear stress-induced cell death has been reported.^{356, 389} Viability of printed endothelial cells was greatly affected by pneumatic pressure, where ~60% and ~85% live cells were found after printing at ~276 kPa (40 psi) and ~35 kPa (5 psi), respectively.³⁸⁹ In the same study, the diameter of nozzles in a range of 150 – 400 µm only slightly affected viability. In addition, a long nozzle length (24.4 mm) led to lower viability of

printed human embryonic stem cells (ca. 70%) compared to viability (ca. 90%) of the cells printed with a short nozzle length (8.9 mm). Therefore, in droplet-based bioprinting, the printing conditions and methods should be carefully chosen according to the purpose of bioprinting. More importantly, the impact of droplet-based printing process on various biological activities should be further investigated.

In most droplet-based bioprinting with light-based crosslinking, the light is irradiated on the entire stage throughout the whole printing process, which could lead to over-curing of layers deposited earlier as well as cellular damage of over-exposed cells. Therefore, the light penetration and curing depth along with light exposure to the cells in different layers during printing should be comprehensively studied for deposition-based printing, which will be important in particular for printing large cell-laden constructs.

Nevertheless, droplet-based bioprinting has distinct advantages over other bioprinting techniques when generating HT arrays, printing with multimaterials, and precisely positioning bioink drops at picoliter resolution. We envision that one could achieve droplet-based biofabrication of clinically relevant HT screening systems in addition to 3D tissue architectures by exploring new material/chemistry combinations and resolving the method's current limitations.

4. VAT POLYMERIZATION BIOPRINTING

Vat polymerization (VP) printing produces 3D objects by selective light irradiation of photo-activatable materials to induce localized curing of liquid materials called resins, which fill a vat. VP printing was first developed and patented by 3D Systems in 1987³³⁷, where the term “stereolithography (SL)” was first proposed as a method for fabricating solid objects through layer-by-layer printing of photocurable materials. The SL was demonstrated using a laser beam, which thereafter has been heavily used for the printing of acellular structures out of epoxy or acrylic monomer-containing resins. Since the 2000s, the development of materials, including biocompatible, water-soluble PIs and bioactive-photoactive materials such as Gel-MA, led to application of the technique for the bioprinting of cell-laden structures. In particular, the number of publications with a focus on printing cells with SL has increased sharply since 2010.

In addition, advanced light exposure techniques such as digital light processing (DLP) and two-photon SL have evolved, providing high printing speed and/or resolution. In recent bioprinting research, the new technologies have been favored over traditional laser-based SL (commonly known as SLA), and therefore this section mostly focuses on DLP (projection) VP and 2P-SL, including key working principles and, important bioprinting applications, as well as current limitations of these techniques.

4.1 Projection VP

Projection VP, also known as DLP VP, is a manufacturing technique based on spatially-controlled irradiation, where light is transmitted through a digital photomask. In traditional single photon laser-based VP, also called SLA, a focused laser is guided by a scanning mirror to selectively induce crosslinking in a photo-crosslinkable material. The use of a focused laser provides high resolution. However, the point-by-point scanning of each layer is time-consuming and may ultimately lead to a reduction in cell viability and increased cell sedimentation when printing tall structures. For these reasons, projection VP has been preferred over laser-based VP in recent bioprinting studies. Recent advances in hardware, software, and material development allowed the achievement of fast and high quality bioprinting using projection VP. In particular, resolution and printing speed have been dramatically improved in two state-of-the-art projection VP systems: continuous liquid interface printing (CLIP) and volumetric printing (Figure 33).

Figure 33. Laser SL and different types of projection VP. Volumetric VP: Adapted with permission from ref 395. Copyright 2019, The Authors, some rights reserved; exclusive license American Association for the Advancement of Science.

4.1.1 Principles of Projection VP

In this section, the traditional VP printing method is described first, followed by emerging printing methods, namely CLIP and volumetric printing.

Figure 34. Working principles and components of projection VP. A) The schematic of a DMD-based DLP device. A light reflected on an “on” state micromirror is transmitted to the projection lens and eventually to the plane of printing, whereas an “off” state micromirror transmits a light to the light absorber. B) Two different configurations of z-movement in layer-by-layer projection VP. In the Bottom Up configuration, the printing stage moves up and photocrosslinking occurs at the bottom of a vat, whereas in the Top Down configuration the stage moves down and photocrosslinking happens at the air-liquid interface.

Hardware

The essential components of projection VP printers are a light source, a DLP device, a projection lens, a vat and a z-movement motor and controller. LEDs and UV lamps are commonly used as light sources, occasionally in combination with filters, to selectively transmit specific wavelengths. From the source, UV light travels through a DLP device capable of directing the light. DLP devices can be classified as liquid crystal displays (LCDs) or as digital micromirror devices (DMDs). A DMD is an array of micromirrors made of highly reflective aluminium. Each mirror has an “on” and an “off” state characterized by its tilting angle, where the “on” state directs the light to the plane of printing and the “off” state transmits the light to a light-absorber (Figure 34A). A typical tilting angle is ± 12 degrees where conventionally $+12$ degrees determines the “on” state and -12 degrees the “off” state.³⁹⁰ Each mirror in a DMD array corresponds to a pixel. Compared to LCDs, DMDs allow higher UV transmittance efficiency, faster switching of patterns and smaller pixel size,³⁹¹ which makes it preferred over LCDs in bioprinting.

Figure 35. Working principles of projection VP. A) General process for layer-by-layer projection VP. B) Schematic of the working principle of O₂ inhibition-based CLIP. Dead zone is created by diffused oxygen from an O₂-permeable window, which inhibits radical-mediated polymerization, allowing the continuous movement of the build platform and generation of 3D objects. C) Schematic of volumetric printing. A bath filled with a resin is illuminated by a projection at multiple angles. Photocrosslinking occurs at a region where the cumulative dose of irradiation is above the threshold. B: Adapted from ref 393. C: Adapted with permission from ref 395. Copyright 2019, The Authors, some rights reserved; exclusive license American Association for the Advancement of Science.

When light is reflected on an “on” state micromirror, it passes through a projection lens and is focused on the plane of printing. Light reaching the build plane initiates the polymerization of photo-activated bioresins in the vat. Once a layer is crosslinked, the build platform moves up or down to allow the fresh resin to fill the space between the object being printed and the build plane, followed by re-positioning for printing a new layer with a defined layer height (Figure 35A). Typically, the layer height is in the range of 25 μm to 100 μm in projection VP bioprinting. There are two possible configurations of the stage movement in the z-direction. Depending on this configuration, bioprinting is performed using a bottom-up or a top-down approach (Figure 34B). In the bottom-up configuration, light is transmitted from the bottom of the vat, and the build platform moves up from the bottom of the vat after printing each layer. In the top-down configuration, crosslinking occurs at the top surface of the vat and the build plane moves down after printing each layer. In a top-down setting, the size of a printed construct is limited by the depth of a vat, and radical-based photocrosslinking at the surface of a vat can be greatly inhibited by oxygen from the air. For these reasons, most bioprinting applications use a bottom-up configuration.

For bioprinting purposes, many laboratories have developed customized printing equipment by installing a DLP device on a laser-based SL printer such as SLA 250 from 3D systems (Rock Hill, South Carolina, US) or assembling it from an LED light, a DMD chip, aligning optics, and a movable stage. A few commercial DLP printers have been used for printing with bioresins such as Perfactory® 3 Mini (EnvisionTec, Gladbeck, Germany), and desktop DLP 3D printer M-ONE (Makex, Zhejiang, China). Recently, several DLP bioprinters have also become available, including Lumen X™ (Cellink, Gothenburg, Sweden) and Cellbricks (Cellbricks, Berlin, Germany).

The main limitation of the traditional layer-by-layer method is associated with the slow speed of bioprinting in the vertical (z) direction. Because of this, most cell-laden constructs bioprinted by this method have relatively large x-y dimensions compared to heights. In VP bioprinting, printing speed can be calculated by considering the exposure/crosslinking time per layer and the time for separation, recoating, and repositioning of the stage after each layer. Although exposure times range between 3 – 40 seconds, actual printing time is largely influenced by the washing and recoating steps. Lim et al. reported a printing time of 1.5 hours to print a 5 mm×5 mm×5 mm cube (z-step size of 50 μm and a bioresin comprised of Gel-MA, PVA-MA, and Ru/SPS initiating system²²⁴). In this experiment, the irradiation time per layer was only 10 seconds but the time for printing a layer was 54 seconds. Similarly, in another study¹²⁰, the reported printing speed was 2 minutes/layer where a mixed PEG-DA and Gel-MA bioresin was used with the Eosin Y/TEA/ NVP initiating system.

A new technique called CLIP has recently been introduced to overcome the limitations of VP printing. In CLIP, radical-induced photocrosslinking is inhibited in a “dead zone” created by oxygen³⁹²⁻³⁹³ diffused from a gas permeable window (Figure 35B). In this dead zone, photoexcited photoinitiators or free radicals are quenched by oxygen or peroxides, and therefore solidification of a resin does not occur, which allows for the simultaneous movement of the build platform, photopolymerization and recoating. The thickness of the dead zone was adjusted by controlling the type of gas (dead zone thickness: oxygen > air > nitrogen) as well as the photon flux, where the higher photon flux resulted in the thicker dead zone. This method allows for continuous elevation of the printing platform without the necessity for multistep procedures as in the traditional approach (Figure 35), resulting in accelerated printing speed. Complex constructs of tens of centimeters in size were printed at 25-100 mm/hour with a vertical resolution (i.e., slicing thickness of a digital model) of several tens of μm . Furthermore, the continuous printing significantly reduces the staircasing effect and produces an object with much smoother surface compared to laser- or DLP-based VP.

Continuous printing can be achieved by choosing different types of inhibitors instead of oxygen. Beer et al.³⁹⁴ demonstrated another continuous VP printing method using two-color lights, one initiating photopolymerization and the other inhibiting the reaction. In this study, bis[2-(ochlorophenyl)-4,5-diphenylimidazole] (o-Cl-HABI) was utilized as a PI, which generates lophyl radicals upon UV irradiation to inhibit radical-mediated polymerization, thereby creating a dead zone adjacent to the illumination window. Camphorquinone was chosen as a second type PI to induce photocrosslinking by irradiation of visible light. However, the CLIP technique has not yet been demonstrated in the presence of cells.

The principle of inhibition allowed for printing genuinely layer-less 3D objects by a printing technique called volumetric or tomographic printing.³⁹⁵ This printing method is based on a computational reconstruction of projection images from cross-sectional scans of a 3D digital model. The computational reconstruction is the reverse process of computed tomography in medical imaging. Using this technique, projection images are generated using an established tomography algorithm at different angles from 0 to 360 degrees.³⁹⁶⁻³⁹⁷ A bath containing a photo-activated resin is illuminated by light through each projection at different angles (Figure 35C), and then photocrosslinking is induced at a region where the cumulative dose of irradiation is above a threshold. In principle, the level of this threshold can be adjusted by doping the resin with oxygen. Technically, light illuminates in a single direction through a dynamic photomask and the chamber filled with a resin is rotating (Figure 33). The printing principle allows for printing with a resin with high viscosity (up to 90,000 mPa·s) or pre-crosslinked solid resin. However, printing with a resin with low viscosity might lead to deteriorated printing resolution due to possible local flow disturbance of the resin in a rotating printing chamber. The simultaneous photocrosslinking in 3D significantly accelerated the printing process, where a centimeter-scale construct was produced within one minute. Even though it has not been demonstrated yet, the principle could be applied to non-radical based systems by utilizing other types of molecules instead of oxygen, which inhibits photopolymerization of the system to a certain extent. Furthermore, the method has been proven to be compatible with cell-laden bioresins.³⁹⁸ Centimeter-scale cell-laden constructs were bioprinted using a Gel-MA bioresin with the LAP initiating system. The bioprinted chondroprogenitor cells showed viability higher than 85% and increasing metabolic activity over one week. Considering the demonstrated printability and the cytocompatibility, the volumetric VP printing holds great promise to become a leading bioprinting technique in the near future.

Resolution

The lateral resolution of projection VP mainly depends on the DMD type and illuminating area. For a given number of micromirrors (i.e., pixels) of a DMD, illumination of a large area results in larger pixel sizes and reduced resolution (Figure 36). For example, for a DMD with 1080p (1920*1080p) when the desired width of an object is 100 mm, the lateral resolution will be $\sim 52 \mu\text{m}$ (100 mm divided by 1920).

Figure 36. Lateral resolution of projection VP. Each micromirror corresponds to a pixel, therefore for a given size of a DMD (typically 1920*1080), the lateral printing resolution decreases with increasing the projection area.

The vertical resolution depends on the light penetration depth, and thus is affected by type and concentration of photoinitiators, irradiation dose, photoactive moieties, and light-absorbing additives.³⁹⁹ The thickness of a polymerized layer (e) can be calculated as:

$$e = \frac{1}{\alpha c} \ln\left(\frac{t}{t_0}\right), \quad t_0 = \frac{T}{\alpha c F_0} \quad (2)$$

where α = Napierian coefficient of molar extinction of PI ($\text{L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$), c = concentration of PI, t_0 = threshold irradiation required to start the polymerization, t = irradiation time, T = irradiation threshold value (photons/ m^3), F_0 = light flux arriving on the surface (photons/ $\text{m}^3 \text{ s}$).

Equation 3 suggests that the vertical resolution of VP printing could be improved by reducing the irradiation time close to the crosslinking threshold, which will result in thinner layer heights. Secondly, the use of a PI with a high extinction coefficient at a high concentration will generate thin, crosslinked layers. When printing cells, both irradiation conditions and PI type/concentration cannot be widely varied because of cytocompatibility issues. Therefore, the third strategy, the addition of photoabsorbers, will be useful to efficiently control the penetration depth. Photoabsorbers in a bioresin will increase the threshold for photocrosslinking, and thus the light can be confined at the illuminated surface, leading to a small crosslinked layer thickness. The confined photoactivation will also prevent over-curing of neighboring pre-cured layers. In fact, several biocompatible photoabsorbers have been successfully demonstrated to effectively enhance bioprinting. Sakai et al.⁴⁰⁰ developed an alginate-based bioresin for projection VP bioprinting with a visible light projector. High printing fidelity was achieved by adding a visible light absorber, Acid Red, to the bioresin comprised of an alginate derivative and $[\text{Ru}(\text{bpy})_3]^{2+}$ /SPS initiating system (maximum absorption at $\sim 450 \text{ nm}$). Without the photoabsorber, the printed construct had a poorly defined structure, whereas the addition of 0.06 w/v% Acid Red to the resin significantly improved the printing quality (Figure 37A). An increased concentration of Acid Red (0.08 w/v%) led to decreased printing quality compared to the resin with 0.06 w/v%, which is attributed to inefficient photocrosslinking due to excess light absorption by Acid Red. Acid Red did not compromise high cell viability ($>90\%$) of the bioresin. For the same initiating system, Ponceau 4R, a biocompatible food dye, was used as another visible light absorber in combination with a bioresin composed of PVA-MA and Gel-MA to improve printing quality.²²⁴ Addition of Ponceau 4R (1 wt%) resulted in decreased curing depths, which eventually prevented over-curing during printing and thus significantly improved the quality of printing (Figure 37B). Other types of food dyes have recently been identified as efficient photoabsorbers for an LAP initiating system, which allowed for generation of complex vascular structures in monolithic hydrogels using a projection VP printer.⁴⁰¹ Among several tested candidates, tartrazine was chosen as a photoabsorber because of its light absorption range matching to the light of the printing system (405 nm) and its hydrophilicity which makes it easy to wash out the chemical after printing. Production of various entangled vascular networks in transparent hydrogels including PEG-DA and Gel-MA was demonstrated using a resin containing the food dye photoabsorber (Figure 37C). The lowest voxel resolution was $50 \mu\text{m}$. In addition, primary hMSCs were successfully bioprinted using the bioresin composed of PEG-DA and Gel-MA containing the photoabsorber, and the printed cells maintained high viability and osteogenic differentiation potentials.

Figure 37. 3D-printed hydrogels using photo-activated resins with/without photoabsorbers. A) Printed hydrogels using an alginate-based resin containing different amounts of Acid Red (0, 0.06 or 0.08 w/v%). B) Curing depth (C_d) of a resin composed of PVA-MA and Gel-MA with/without Ponceau 4R according to light intensity, and photographs of printed gels using each resin. C) 3D design of a complex vascular structure, and a photograph and microCT scan of the printed vascularized hydrogel (20 wt% PEG-DA) based on the 3D design (Scale bar: 3 mm). A: Adapted from ref 400. B: Adapted with permission from ref 224. Copyright 2018 IOP Publishing Ltd. C: Adapted with permission from ref 401. Copyright 2019 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science.

The presence of light-scattering components such as micro/nanoparticles can affect both lateral and vertical resolution.⁴⁰²⁻⁴⁰⁴ Scattering within a resin results in decreased lateral resolution, because scattered light can induce uncontrolled photopolymerization in the neighboring region. On the other hand, similar to the effect of light-absorbing additives, light-scattering in a resin leads to reduced penetration depth, resulting in enhanced vertical resolution. This trend has been well studied in a report by Sun et al. using the Monte Carlo ray tracing method for ceramic microparticle-containing resins irradiated with a laser at 364 nm.⁴⁰⁴ They showed that the curing depth of a resin increases with the particle sizes (in a range of 0.3 – 1 μm), whereas the curing radius decreases with the particle sizes, which was attributed to the stronger light scattering by the particles with a diameter (0.3 μm) close to the wavelength of the light (0.364 μm). In the same study, other particle properties, including refractive index and absorption coefficient, were also shown to affect the resolution.

In practice, the curing depth (C_d) can be determined from the following empirical equation⁴⁰⁵:

$$C_d = D_p \ln \frac{E}{E_c} \quad (3)$$

where D_p = light penetration depth, E = irradiation dose per area and E_c = threshold irradiation dose to start the photocrosslinking. The calculation of the curing depth is an important first step in determining optimal settings for the VP printing system.

Considerations for bioprinting cells in a vat

Figure 38. Microscopic photographs of cell-laden hydrogels with (left) and without (right) cell sedimentation (Scale bars: 1 mm). Adapted with permission from ref 406. Copyright 2010 The Royal Society of Chemistry.

As discussed in Section 2, materials and chemistries play an important role in the printing of cell-laden structures to keep the cells viable and functional. Since VP bioprinting is a nozzle-free process, cells are less likely to be damaged during the printing process compared to deposition-based bioprinting. However, cell sedimentation could be an issue when printing large constructs, which would result in an inhomogeneous distribution of cells throughout a construct. Indeed, Chan et al. observed cell sedimentation during printing using a bioresin composed of PEG-DA (700 Da, 20 w/v%) and fibroblasts (2×10^6 cells/mL)⁴⁰⁶ (Figure 38, left). The sedimentation occurred during printing in a vat with a top-down configuration, where the vat was filled with the bioresin at the start of the printing without further mixing during printing. The issue was resolved by printing using a bottom-up configuration where fresh bioresin is added after printing each layer (Figure 38, right). In some studies, buoyancy-modifying components were added to control the viscosity of the bioresin and prevent cell sedimentation. Lin et al. added Percoll, colloidal silica nanoparticles coated with polyvinylpyrrolidone, to a PEG-DA bioresin (4 kDa, 10 w/v%) to obtain a homogeneous distribution of human ADSCs in a printed gel.⁴⁰⁷ They suggested the optimal Percoll concentration to achieve the most homogeneous cell distribution was 37.5 v/v%. In another study, however, a much lower-concentration (6.5 v/v%) Percoll was used for the same purpose with a PEG-DA bioresin (1 kDa, 15 w/v%) containing HUVECs (2×10^6 cells/mL). In addition to Percoll, small amounts of HA-MA (0.5 w/v%) with high MW (700 kDa) were used to provide a PEG-DA bioresin with buoyant forces.⁴⁰⁸ Lim et al. confirmed that bioprinting using a bioresin composed of PVA-MA/Gel-MA (10 wt%/1 wt%) and hMSCs (5×10^6 cells/mL) produced a construct with homogeneous cell distribution.²²⁴ The result was attributed to the high viscosity of the bioresin (ca. 13 mPa·s) as well as gentle movement of the printing platform during the normal printing process.

Bioresins for projection VP

Several aspects should be considered when formulating a bioresin for projection VP. Firstly, for a successful re-coating process, the viscosity of a bioresin should not be too high. Hinczewski et al. reported that the highest viscosity to ensure sufficient layer recoating is 5 Pa·s.⁴⁰⁹ Simultaneously, the viscosity of the bioresin should prevent cell sedimentation. In a study by Kim et al.,⁴¹⁰ cells dispersed in 10% Gel-MA solution with a viscosity of 3 mPa·s eventually sedimented after 1 h without any mixing. However, if the same solution was slowly added in the vat after each layer, cells were homogeneously distributed throughout the printed construct. Similarly, Lim et al. reported that the gentle movement of the printing vat prevented cells from sedimenting in the bioresin (viscosity ~13 mPa·s).²²⁴ In volumetric printing, however, a bioresin with high viscosity (up to 90,000 mPa·s) or crosslinked hydrogels should be used, since printing is performed in a rotating chamber. A too-low viscosity of the bioresin would lead to fluctuations and perturbations that would prevent photocrosslinking at specific positions in 3D.

The choice of photocrosslinking chemistry also plays an important role in projection VP bioprinting. To date, Gel-MA or PEG derivatives in combination with a PI, I2959 or LAP, have been most intensely used for printing cell-laden constructs using projection VP. Recently, several visible light-initiating systems have been adopted for projection VP-based bioprinting primarily to improve biocompatibility. For example, Eosin Y initiating system has been used in combination with Gel-MA.⁴¹¹ The composition of the bioresin was 15 w/v% Gel-MA, 0.02 mM Eosin Y, 0.2 w/v% TEA, 74 nM NVP and fibroblasts (at 8×10^6 cells/mL). The bioresin was printed into a grid structure using a DLP printer with the wavelength range of 480-600 nm. However, the time for crosslinking one layer during printing was 2 min, which is relatively slow compared to other photo-initiating systems. The same research group also studied a bioresin utilizing the VA086 photo-initiating system with a light wavelength of 405 nm.⁴¹² A cell-laden structure was printed using a bioresin comprised of 10 w/v% Gel-MA, 1 w/v% VA-086, and fibroblasts with a laser-based VP printer at 3 mm/s. The viability of the bioprinted cells was >90 % at day 3, which is higher than usually reported for bioresins involving I2959 as a photoinitiator.

As for materials, PEG derivatives (PEG-DA, PEG-DMA) have been widely used in early studies. However, the recent trend in bioprinting has noticeably moved toward more biologically active materials such as Gel-MA. As described in the previous sections, PEG derivatives have general advantages in terms of tunable mechanical properties, ease of functionalization, and biocompatibility. However, they also have some issues that have been addressed in several studies. In the study by Raman et al.,⁴¹³ a bioresin based on PEG-DMA-1 kDa allowed for DLP printing of feature sizes down to 3 μ m, whereas the smallest feature size with a bioresin of PEG-DA-3.4 kDa was 30 μ m, which was attributed to a higher degree of swelling of the bioresin with higher molecular weight compared to the one with lower molecular weight. However, the viability of bioprinted fibroblasts in this study was higher for the PEG-DA-3.4 kDa bioresin (ca. 93 %) than that of the PEG-DMA-1 kDa (ca. 70 %). The study implies that the molecular weight of PEG should be carefully chosen in consideration of the desirable resolution and cell types for

each specific application. Another concern regarding the use of PEG-based bioresins is the lack of biological cues, which often leads to low viability of bioprinted cells. To improve this aspect, PEG has been modified with bioactive peptides⁴⁰⁶ or used in combination with biologically active molecules. For example, the addition of Gel-MA to a PEG-DA-based DLP resin led to improved viability of bioprinted cells from 60% for a PEG-DA-only resin to 85% for the resin composed of 50% PEG-DA and 50% Gel-MA.¹²⁰

Figure 39. Bioprinted hydrogels using functional resins and DLP bioprinters. A) Fluorescence images of three different types of bioprinted cells using standard or porous Gel-MA resin after 7 days of *in vitro* culture. F-actin and nuclei were stained as green and blue. B) Photographs of printed Sil-MA gels before and after compression and a printed sheet-shaped gel sutured to form a tubular structure. Tensile modulus of Sil-MA gels with different concentrations (10, 20, 30%) (Scale bars: 1 cm). C) Various 3D constructs printed using the Sil-MA resin (Scale bars: 1 cm). The vascular structure was perfused with water (blue color). D) Chemical structure of AHPG. E) Schematic of formulation of AHPG-based resins with controlled degrees of crosslinking. F) Structural stability of bioprinted microgels using an AHPG/Gel-MA bioink at days 1, 4, and 7 (Scale bars: 100 μ m). A: Adapted with permission from ref 302. Copyright 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. B,C: Adapted from ref 410. D-F: Adapted with permission from ref 417. Copyright 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

Various Gel-MA-based bioresins have been reported for DLP bioprinting.⁴¹⁴ In some studies, the biological activity of Gel-MA-based bioresins were further enhanced by the addition of functional

materials. For example, Yu et al. showed that the addition of dECM enhanced biological activities of bioprinted cells by providing direct bioactive signals present in ECM.⁴¹⁵ They prepared a bioresin composed of Gel-MA and heart or liver dECM, and confirmed that both bioprinted cells better expressed tissue-specific markers compared to the Gel-MA-only bioresin. Another approach to improve the biocompatibility of a bioresin is to control porosity to enhance the diffusion of oxygen, nutrients, and cellular waste. Using the same porous Gel-MA resin described in the extrusion bioprinting section, the macroporous gel with the ratio of 4:1 (Gel-MA:PEG, v/v) had smaller pore sizes (24.8 μm) than the one with the ratio of 1:1 (58.7 μm).³⁰² The Gel-MA bioresin with higher porosity led to enhanced viability, spreading and proliferation of bioprinted cells at day 7 compared to the one with lower porosity (Figure 39A).

Although the majority of recent studies in the literature use PEG or gelatin-based bioresins for VP bioprinting, other functional polymers could provide desirable properties for bioresins. For instance, HA-MA-based bioresins were utilized to bioprint ECs where bioactivity of HA supported the proliferation of ECs and vascularization.^{50, 416} More recently, glycidyl methacrylate-conjugated silk fibroin (Sil-MA) was synthesized and used in a DLP bioprinter.⁴¹⁰ The mechanical properties of the Sil-MA bioresin were tuned by controlling the concentration (10 - 30%), where 30% polymer concentration exhibited very high mechanical strength (910 kPa at break) due to the intrinsic tensile strength of silk fibroin (Figure 39B). In addition, the Sil-MA bioresin showed good printability, which enabled printing of various biological structures (trachea, heart, lung, vasculature) (Figure 39C).

A recent study synthesized an acrylated hyperbranched polyglycerol (AHPG) (Figure 39D) as a crosslinker for various photosensitive polymers including PEG monoacrylate, polyacrylamide (PAAm) and Gel-MA, providing tunable mechanical properties.⁴¹⁷ The degree of acrylation of AHPG was tunable over a wide range (10-70%) due to its hyperbranched nature (Figure 39E). The mechanical properties of gels were controlled using AHPG with different degrees of acrylation and molecular weight. For instance, 20% PEG monoacrylate gels had an elastic modulus ranging from ~5 kPa to ~60 kPa. The synthesized AHPGs in general retained low cytotoxicity (viability > 80%), and arrays of cell-laden microgels were successfully bioprinted using a DLP bioprinter and bioresin composed of Gel-MA and AHPG (Figure 39F).

Figure 40. Various approaches for multimaterial printing based on projection VP A) Bioprinting using two types of bioresins with sequential recoating of each layer, and fluorescence images of bioprinted constructs (Green: hepatic progenitors in Gel-MA, Red: supporting cells in Gel-MA/HA-MA) (Scale bars: 500 μm). B) Schematic of a microfluidic device with four inlets combined with a DLP device. C) Bioprinted, biomimetic hydrogels containing multiple cell

types. Muscle: a bioprinted multicellular pattern comprised of C2C12 cells (red), fibroblasts (blue) and HUVECs (green) after 48 h of *in vitro* culture. Tendon: a bioprinted structure composed of osteoblasts (blue), MSCs (red), and fibroblasts (green), and the inset shows the region of fibroblasts at high magnification where cells were stained with green (f-actin) and blue (nuclei) dyes (Scale bars: 500 μ m). D) Digital models for variable height micromixers (VHMs) with different numbers of repeating units and schematic of a VHM device combined with a DLP printer. A: Adapted with permission from ref 50. B,C: Adapted with permission from ref 17. Copyright 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. D: Adapted with permission from ref 418. Copyright 2016 The Royal Society of Chemistry.

In principle, printing multimaterials using a VP-based bioprinter is possible by using different bioresins for each layer. For example, Ma et al.⁵⁰ biofabricated a liver analog by depositing different bioresins containing hepatic progenitors (in Gel-MA) or supporting cells (HUVECs and ADSCs in the mixture of Gel-MA and HA-MA) in the same layer using a customized DLP bioprinter (Figure 40A). As a result, different types of cells were positioned in a liver lobule-like hexagonal structure, which led to enhanced *in vitro* maturation of the hepatic progenitor cells. However, this method is only applicable for printing thin constructs of a few layers, because the process is extremely labor-intensive as it required manual supply of bioresins to each layer and washing steps. For this reason, the printed liver analog in the study had only one layer. To print multimaterials more efficiently than the manual method, microfluidics-enhanced projection VP systems have been established by several groups.^{17, 418-419} Miri et al. developed an automated multimaterial biofabrication system based on four microfluidic inlets coupled to a printing chamber (Figure 40B).¹⁷ In this system, for printing a layer, a bioresin is supplied to the fluidic chip, followed by light irradiation and subsequent washing. The device enabled the biofabrication of a cell-laden skeletal muscle model and a tendon-to-bone insertion model, both composed of multimaterials and multiple cell types (Figure 40C). Liu et al. reported another type of microfluidic device combined with a printing chamber.⁴¹⁸ They developed a variable height micromixer (VHM), a microfluidic channel-based mixing device that can be combined with a DLP printer to print cell-laden hydrogels (Figure 40D). Three different types of VHMs with varying numbers of repeating units (1*1 units, 3*3 units, 9*9 units) were prepared and tested for mixing efficiency, where the VHM with 9*9 unit had highest complexity of geometry with highly varying local z-heights (Figure 40D). As a result, the VHM with more complex geometry (9*9 units) provided more efficient mixing at a low flow rate (320 μ L/min) compared to the VHM with simple geometry (1*1 units), which required a much higher flow rate (2.4 mL/min) to obtain sufficient mixing. Combined with a DLP printer, cell-laden hydrogels were biofabricated by supplying Gel-MA and the cell suspension into two separate inlets that were subsequently mixed in the VHM and photocrosslinked in the printing chamber. The VHM device enables possible *in situ* adjustment of the resin composition within a layer or during printing, allowing biofabrication of more complex architecture.

4.1.2 Applications

Recent progress in the biofabrication of cell-laden constructs using projection VP opens up possibilities for the generation of functional tissue analogs closely mimicking microscale biological and physical features of native tissue. Several studies have tried to bioprint specific types of tissue and assess biological aspects of them in depth, where bioprinting was used to 1) biofabricate macroscale cell-laden 3D hydrogels according to 3D models, 2) recapitulate microscale biological, mechanical and/or geometrical features of native tissue to form tissue analogs, or 3) generate ECM-mimicking micropatterns, providing cells with biophysical cues.

Figure 41. Bone and cartilage analogs bioprinted by volumetric printing. A) A bioprinted MSC-laden trabecular bone model (left) and a μ CT scan of the model (right) (Scale bar: 2 mm). B) Left: The bioprinted MSCs (pink) cultured in osteogenic medium for 7 days (Scale bar: 1 mm). Right: The bioprinted bone model containing MSCs (O-MSCs, pink) was seeded with endothelial colony-forming cells (ECFCs, green) and MSCs (P-MSCs, yellow) and cultured *in vitro* for 3 days. ECFCs were interconnected with each other and filled the pore space of the bioprinted construct (Scale bar = 500 μ m). C) A bioprinted meniscus analog (top). *In vitro* matrix deposition in the analog cultured for 28 days (bottom) (Scale bars = 2 mm). Adapted with permission from ref 398. Copyright 2019 The Authors. Published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

To date, several bioresins have been developed for bioprinting 3D-tissue analogs of cartilage or bone based on PEG derivatives,⁴²⁰ Gel-MA,^{398, 421-422} HA-MA,⁴²¹ PVA-MA²²⁴, which showed good printability as well as biocompatibility. In particular, volumetric printing showed great promise of printing large-scale structures within a reasonable time.³⁹⁸ A trabecular bone model was biofabricated using a bioresin composed of Gel-MA and MSCs based on a μ CT-scanned 3D model. A 3D trabecular architecture (8.5 $\times$ 9.3 mm) retaining interconnected porous structures was generated in 12.5 seconds with resolution of $144.69 \pm 13.55 \mu\text{m}$ (the smallest resolved feature size) (Figure 41A). The bioprinted MSCs were homogeneously distributed throughout the construct and successfully cultured in osteogenic medium for 7 days (Figure 41B, left). The porous structure of the printed model allowed the construct to be seeded with endothelial colony-forming cells and MSCs, generating a multi-cellular structure. After 3 days of co-culture of three different types of cells in the construct, the formation of early angiogenic sprouts by the endothelial colony-forming cells was observed, and the post-seeded MSCs were aligned along with the nascent sprouts (Figure 41B, right). The observations imply that the bioprinted MSCs were able to promote the angiogenic process of the neighboring cells by generating paracrine signals. In addition to the bone model, a centimeter scale chondroprogenitor-laden meniscus was printed in 28 seconds using a Gel-MA bioresin (Figure 41C). After 7 days of *in vitro* culture, the bioprinted cells showed increased metabolic activity compared to day 1, glycosaminoglycan (GAG) and collagen deposition as well as mechanical maturation. The compressive modulus of the bioprinted tissue was ca. 25 kPa at day 1 and reached ca. 267 kPa after 28 days of *in vitro* culture.

Figure 42. Bioprinted liver analog with mechanical heterogeneity. A) Live/dead staining of HepG2 cells cultured in gels with different stiffness (soft, medium, stiff) for 1 (left) or 7 days (right). B) Fluorescence (top, cell trackers: red, green and yellow for soft, medium and stiff gel, respectively) and brightfield (bottom) images of the printed liver analog. C) Percent area of cell outgrowth originating from three different regions (soft, medium, stiff) over time (Scale bars: 500 μ m). Adapted with permission from ref 423. Copyright 2018 Elsevier Ltd.

Some studies have taken advantage of the high resolution of projection VP printing to mimic microarchitecture and mechanical heterogeneity of tissue. Ma et al. biofabricated a liver disease model where regionally varying mechanical properties and biomimetic hexagonal microarchitectures are found.⁴²³ Liver dECM and Gel-MA-based bioresins were used to bioprint hydrogels with three different stiffnesses (0.5, 5, 15 kPa corresponding to softer than normal tissue, healthy tissue, and cirrhotic tissue, respectively) by varying irradiation time. They confirmed that HepG2 cells in the stiff gel showed reduced *in vitro* growth over 7 days (Figure 42A), as well as higher expression of an apoptosis marker gene (CASP8) and lower expression of hepatic function marker genes (ALB, AFP) compared to the soft or medium gel. In addition, expression of marker genes related to metastasis or ECM degradation (MMP2, MMP9, TWIST1) was upregulated in the stiff gel compared to the other two gels, which resembles characteristics of cells in cirrhotic liver tissue. Using a DLP bioprinter and three bioresins, they printed lobule-like structures with varying local stiffness, where higher outgrowth of cells was observed in the stiff region compared to the other two regions (Figure 42B,C). The printed liver model showed good promise as a biomimetic platform to study the invasion of hepatic cancer cells from cirrhotic tissue into adjacent healthy tissue. In the follow-up study, they printed heart analogs using a bioresin based on heart dECM and Gel-MA.⁴¹⁵ The bioresin containing hiPSC-derived cardiomyocytes was printed to form parallel lines with 60 μ m of width and spacing. Compared to the 2D control, bioprinted cells showed a significantly increased expression of myosin regulatory light chain 2 ventricular isoform (MLC-2v), a marker of force generation caused by the movement of actin filaments, which was attributed to biophysical cues provided by printed lines. In addition, significant upregulation of troponin T, a mature cardiac marker regulating contraction of the heart muscle, was observed for the bioprinted tissue compared to the 2D control and the bioprinted tissue without dECM but with collagen I.

Figure 43. Biofabrication of vascularized tissue based on projection VP. A) Left: Bioprinted vascular structures with varying vessel diameters ranged from 50 to 250 μm (Scale bar: 250 μm). Right: Three fluorescent images of cells (HUVECs and supportive MSCs stained as green and purple, respectively) aligned within the channels with different diameters (Scale bars: 100 μm). B) I) Schematic and II) fluorescence staining image of bioprinted hepatic aggregates on pre-vascularized networks (Scale bar: 1 mm). III) A macrograph and IV) H&E staining image of bioprinted constructs infiltrated with host blood after 14 days in mice (Scale bar: 40 μm). A: Adapted with permission from ref 416. Copyright 2017 Elsevier Ltd. B: Adapted with permission from ref 401. Copyright 2019 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science.

Biofabrication of vascularized constructs has also been of interest in the field of projection VP. Zhu et al. biofabricated vascularized tissue constructs using Gel-MA and glycidyl methacrylate-HA (GM-HA).⁴¹⁶ A sheet-like first layer was printed using a resin composed of Gel-MA (5 w/v%), on top of which a cell-laden vascular pattern (Figure 43A, left) was bioprinted with a bioresin comprised of Gel-MA (2.5 w/v%), GM-HA (1 w/v%) and cells (HUVECs and 10T1/2 cells at the ratio of 50:1). The vascular pattern was then enclosed by printing a third layer of Gel-MA (5 w/v%). Considering the much lower elastic modulus of the crosslinked bioresin (Gel-MA + GM-HA)(ca.3 kPa at 0.1 Hz) than that (ca. 5 kPa at 0.1 Hz) of the crosslinked resin (Gel-MA) and presence of hyaluronidase in the *in vivo* environment, the authors hypothesized that the printed cells would preferably line along the edge of the vascular pattern, inducing the formation of channel-like structures. Indeed, the vascular pattern was eventually endothelialized by the printed cells, leading to the formation of lumen-like structures with a range of diameters (50-250 μm) after one week of *in vitro* culture (Figure 43A). They also confirmed endothelial network formation of the bioprinted construct after two weeks of subcutaneous implantation. More recently, generation of a complex multivascular network within a monolithic hydrogel with high resolution (voxel resolution of 50 μm) was demonstrated using food dyes as photoabsorbers.⁴⁰¹ Hepatic aggregates encapsulated in a fibrin gel with 3D printed hydrogel anchors were combined with a 3D printed vascular construct (Figure 43B). In a rodent chronic liver injury model, the hepatocytes in the implanted vascularized hydrogel expressed albumin, indicating the presence of functional hepatocytes. Furthermore, the microchannels in the hydrogel were perfused with host blood, which was confirmed by H&E staining (Figure 43B) and immunostaining of Ter-119, which detects red blood cells and those of erythroid lineage.

4.1.3 Limitations and Outlook

Projection VP provides high resolution as well as a high degree of freedom of printable structures, which enables the biofabrication of tissue analogs recapitulating microarchitecture, multicellularity and mechanical heterogeneity of native tissue. Still, several issues should be addressed before the method can be broadly applied to bioprint functional cell-laden constructs.

So far, most of the bioprinted cell-laden structures are limited to thin constructs composed of a few layers, which is partly due to the slow layer-by-layer multistep printing procedure which causes possible cell death and/or sedimentation when printing a tall structure. Layer-less volumetric printing shows great promise by bioprinting centimeter-scale structures within a minute. However, for all types of VP printing methods, bioprinting tissues with high cell density, such as epithelial tissue, is challenging due to cell sedimentation, possible light scattering by cells, and the very high number of cells required to fill the vat,

not all of which are used in the product. More importantly, for bioprinting, a vat should be filled with a bioresin, and uncrosslinked bioresin volume is discarded. Considering the cost and effort of securing clinical grade cells, this aspect could greatly impede the practical use of the technique in the clinic. Furthermore, the process for printing multimaterials using projection VP is extremely slow due to the necessity of repeated washing steps after printing each layer. Therefore, strategies for printing multimaterials with high speed should be developed, to achieve the biofabrication of biomimetic tissue analogs within a reasonable timeframe.

Another challenge is to bioprint a tissue analog with mechanical properties matched to native tissue. The bioresins developed for projection VP are mostly based on Gel-MA and/or PEG-DA, which limits the range of attainable mechanical properties. Therefore, diversification of resin materials and crosslinking chemistry will widen possible applications of projection VP. As mentioned in Section 4.1.1, a silk-based DLP bioresin was successfully printed into various structures with high mechanical strength.⁴¹⁰ Recently, a nanocellulose-reinforced PEG-based resin was reported by Li et al.⁴²⁴ To increase the dispersibility of nanocellulose in PEG, 1,3-diglycerolate DA (diGlyDA)-modified PEG (PEG-diGlyDA) was mixed with PEG-DA and used as a polymeric matrix. The nanocomposite resin composed of nanocellulose PEG-DA/PEG-diGlyDA exhibited high printing fidelity as well as enhanced mechanical strength, which was attributed to the controlled swelling and mechanical reinforcement given by nanocellulose. Incorporation of nanoparticles can provide not only mechanical enhancement but also anisotropic architecture in a hydrogel. In the study by Yang et al., an electrically assisted DLP printing method was developed to print 3D nanocomposite constructs retaining anisotropic fiber alignment.⁴²⁵ They used a resin composed of surface-modified multiwalled carbon nanotubes (S-MWCNTs) embedded in a commercially available polymer resin (Polymer resin A, 3D Systems). Alignment of the S-MWCNTs was induced by an electric field generated by electrodes installed in the printing vat. At the same time, the direction of alignment was precisely controlled by rotating the printing platform. As a result, they printed a meniscus with biomimetic radial and circumferential alignment of S-MWCNTs, which retained tensile and compressive moduli comparable to a native meniscus.

A construct with large x-y dimension can easily be printed with projection VP methods. However, in terms of resolution, for a given DMD size, a larger x-y dimension will lead to decreased x-y resolution due to the principle of light projection by a mirror (i.e., pixel size). Therefore, printing, for example, an HT array of microtissue of several tens of μm would be challenging given that the largest available DMD size is 2560*1600p.⁴²⁶

Still, projection VP bioprinting has recently shown remarkable progress and achieved technical innovations toward rapid, layer-less biofabrication of cell-laden constructs. The layer-less printing also provides high structural integrity without delamination between layers. In addition, microfluidics-assisted approaches showed great promise for printing multimaterials with projection VP. Together with recent advances in biocompatible photochemistry and photo-activated materials, the generation of biomimetic organ or tissue structures with desired resolution within a reasonable timeframe is within reach.

4.2 Two-Photon SL

During the last 15 years, another light-mediated 3D fabrication technique named two-photon stereolithography (2P-SL) (also named two-photon lithography or direct laser writing DLW) has seen impressive growth in interest from the research community. Among the current additive manufacturing technologies, 2P-SL is the only one able to offer 3D control over complex structures with sub-micrometer precision. These unique features are based on a non-linear photophysical phenomenon known as two-photon absorption (2PA). In the following section, the principles of 2PA and its exploitation for biofabrication are presented, with special emphasis on relevant concepts for biological applications.

4.2.1 Principles of 2P-SL

2P Absorption

2PA process was first predicted by Maria Göppert-Mayer in her doctoral dissertation in 1931.⁴²⁷ It was, however, only experimentally proven 30 years later, once the laser had been invented. It was Werner Kaiser who finally demonstrated 2P-excited fluorescence using an europium-doped crystal.⁴²⁸ 2PA is a third-order non-linear process whose probability “p” scales quadratically with the light intensity “I” ($p \propto I^2$), and therefore a high intensity light source such as a laser is needed to trigger it. In the linear one-photon absorption process, a molecule can be excited from the ground state (S_0) to an excited electronic state (S_n) if the photon energy matches the energy gap between the two states. However, this molecular

electronic transition can also occur when two photons having half the energy (twice the wavelength) of the corresponding one-photon transition are simultaneously absorbed⁴²⁷⁻⁴²⁹ (Figure 44). It follows that 2PA takes place with red-shifted wavelengths, more commonly in the near-infrared (NIR) and infrared (IR) region.

Figure 44. One-photon and two-photon excitation. On the left side, one-photon absorption and subsequent fluorescence emission take place in the whole UV-Vis excitation light cone that encounters the sample. However, in two-photon infrared (IR) excitation (right side), the non-linear nature of the absorption process defines a very confined excitation and subsequent fluorescence limited by the focal volume of the laser beam. One-photon vs two-photon fluorescence images adapted with permission by Steven Ruzin and Holly Aaron, UC Berkeley.

In short, when a first photon interacts with the molecule, an intermediate and extremely short-lived virtual state ($\sim 10^{-15}$ - 10^{-16} s) is formed above the ground state. The electronic transition to the real excited state takes place only if a second photon interacts with the molecule within this short time frame (Figure 44). Exploiting 2PA allows the use of NIR light sources instead of UV-Vis ones, which brings two relevant advantages to biofabrication in the presence of cells, namely less cytotoxicity due to photodamage and higher penetration depth into the sample thanks to lower absorption and scattering. Biological tissues have indeed a low absorption window around 800 to 950 nm with water starting to act as a light filter above it.⁴ This optical “transparency window” strongly reduces the risk related to photodamage and therefore represents an intrinsic biocompatible feature of 2P-SL when compared to one-photon absorption-driven biofabrication approaches (e.g., SLA and DLP).⁴³⁰ Moreover, the biocompatibility of non-invasive *in vivo* 2P imaging^{4, 431-434} indicates that *in vivo* remote physicochemical control of photo-activated material might also represent a future direction of 2P-SL.

2P microscopy setups generally make use of tightly focused NIR femtosecond (fs, 10^{-15} s) pulsed lasers. The alternation of high peak intensity pulses with duration of ~ 100 fs with breaks (zero laser intensity) in the nanoseconds range (MHz frequency) gives mild average power, thus reducing the possible damage of sensitive biological specimens. Its non-linear behavior makes 2PA several orders of magnitude weaker (less probable) than linear one-photon absorption. Therefore, the probability of 2PA rapidly decreases away from the intensity maximum and the excitation remains localized within the 2P focal volume generated by a tightly focused laser beam (Figure 44). This intrinsic confocality basically eliminates out-of-focus photodamage to cells and undesired photochemical reactions. Upon 2PA, the excited PIs can decay through an ISC transition to a long-lived triplet state (T_1), from which active species (ions, radicals) are generated. Therefore, photochemical reactions such as radical chain growth polymerization, step-growth polymerization, or photo-cleavage can be triggered in the confined ellipsoidal focal volume (Figure 47). Thus, 3D structures/patterns can be obtained within the photo-activated material by precisely scanning the focal spot in x, y and z. It follows that 2P-SL is not limited by the layer-by-layer constraints typical of most of the 3D fabrication techniques, but in principle it enables a purely 3D freeform processing. A system suitable for 2P-microfabrication typically includes photosensitive monomers/polymers (e.g., acrylate, epoxy or vinyl derivatives) transparent to the excitation wavelength and a molecule, a 2PI, which is efficiently excited by 2PA and produces active

species. In other strategies, such as 2P-patterning, which will be discussed later, a photocage that can be cleaved from the substrate upon 2PA is present.

Instrument Setup and Fabrication Parameters

The instrument setup for 2P-SL generally comprises a laser source, beam control elements, focusing optics, sample motion systems, and control software. Other optional components such as spatial light modulators (SLMs)⁴³⁵ or microlens arrays⁴³⁶ can also be implemented. All together, these elements contribute to define key processing parameters.

Figure 45. 2P-SL setup. A) Common setup of 2P-SL apparatus showing the beam path from the fs-pulsed laser source to the sample. B) Detail of the sample and fabrication process in which a solid spiral structure (blue) is formed in a drop of liquid photoresin (light blue).

Figure 45A describes the main components and parameters of 2P-SL, further explained in the following section. It does not claim to be an exhaustive explanation of the technical and physical concepts underlying 2P-SL, but rather a short overview of the main concepts to give the non-expert readers a general background and a state-of-the-art perspective of this technique prior to discussion of its biofabrication-related applications. More comprehensive and detailed technical dissertations can be found elsewhere.^{131, 437-439}

Laser source: As previously discussed, high-intensity light sources such as fs-pulsed lasers are necessary to drive the 2P excitation. In this context, Ti:sapphire fs-pulsed lasers represent the standard for most of the instrument setups. Other light sources such as second harmonic fiber lasers and amplified ultrafast yttrium-aluminium-garnet (YAG) lasers have been used but represent a substantial minority for the topic covered in this review. Titanium sapphire doped lasing medium (Ti_2O_3 into a melt of Al_2O_3) is used to construct highly tunable lasers that can operate in a wavelength range that spans from about 660 to 1100 nm.⁴⁴⁰ Therefore, this solid-state laser emerged as an ideal light source for biomedically-oriented applications, for its ability to conveniently operate around the biological “transparency window” (~800 nm), where it exhibits its maximum gain and efficiency (Figure 46B). The generation of ultrashort fs pulses (10^{-15} s) relies on the so-called mode-locking.⁴⁴¹⁻⁴⁴² In short, when a fixed-phase relationship between the stationary waves (modes) that oscillate in the laser optical cavity is established, constructive interferences can occur periodically, giving rise to laser pulses. In this context, the laser is defined as phase-, or mode-locked. The mode-locking can be either passive, where non-linear phenomena are introduced in the cavity with elements such as natural or artificial saturable absorbers, or active, when externally driven modulators are used.

Laser parameters: The pulsed laser is mainly defined by means of repetition rate, pulse width, wavelength, and laser power (peak P and average P_{av}), which are all crucial parameters for an optimal 2P-SL platform.

Figure 46. Laser parameters and spectra. A) Characteristics of the laser pulse train and respective equations. The time scale is not realistic but meant to illustrate the main concepts of temporal focusing of peak intensity. B) Absorption and emission spectra of Ti:sapphire lasing medium. Ti:sapphire is normally pumped with argon or frequency-doubled Nd:YVO4 lasers which emit in its absorption range. Then, the emission spectrum shows a peak at around 800 nm, where the laser has its maximum output intensity.

The repetition rate (frequency f_{rep}) of the train of laser pulses depends on the cavity round trip time (τ) of the laser (time taken for the light to make a round trip of the optical cavity), which is determined by resonator size (L) and light speed (c) (Figure 46A). The most common repetition rate of the lasers employed for 2P-SL is ~ 80 MHz, which corresponds to 12.5 ns separation ($\tau = 1/f_{rep}$) between consecutive pulses. The laser pulse duration or pulse width (t_p) is instead determined by the number of phase-locked modes and, for Ti:sapphire lasers operating in a mode-locking regime, it normally spans from 20 to 150 fs. The power output of the peaks (P) increases with the cavity length (L) since more and closer oscillating modes are generated. However, since pulse separation depends on the resonator size (L), an increase of the latter also determines an increase of the repetition rate (f_{rep}).

The pulsed nature of the laser enables a concentration of the excitation in the time domain, with the energy per voxel (E) easily calculable as product of power (P) and pulse duration (t_p) (Figure 46A), while tightly focusing high numerical aperture (NA) objectives concentrate the excitation in space. Additionally, the concentration in time of high-intensity peaks (I_{peak}) determines an overall lower average intensity resulting in lower risks of thermal- and photo-damage. For fabrication or patterning applications, one pulse per pixel is necessary to trigger a photochemistry reaction. Therefore, scanning speed, dwell time (time that the laser spends on every pixel), f_{rep} , and t_p should always be chosen so that at least one peak per pixel is assured. With writing speeds varying from a few $\mu\text{m/s}$ to 1 m/s, common values ranges for Ti:sapphire laser-driven 2P-SL are 0.5-200 mW (P_{av}), 50-150 fs (t_p) and ~ 80 -100 MHz (f_{rep}).

Regarding the wavelength, the broad tuning range of Ti:sapphire lasers generally enables the user to choose the 2PI maximum absorption. However, the user should be aware that this modulation affects the laser beam output power. The maximum power output is indeed different at the center (~ 800 nm) or at the edges of the tuning curve (Figure 46B). Since this value can strongly influence the whole fabrication process, an optimal 2P-SL setup requires the implementation of a power meter in the beam path to control the power fluctuations and thus improve the process reproducibility and comparability with the work of other groups.

Laser beam path:

Shutter system. The rapid blanking of the beam during the scanning process is guaranteed by optical modulators. Since the maximum frequency modulation of optical choppers (rotating mechanical shutters) is limited to the kHz range, fast acousto-optic modulators (AOM) or electro-optic modulators (EOM) with responses in the MHz range are commonly preferred. When the modulators are switched on (acoustic wave generation for AOM and electric field for EOM), the first-order diffracted laser pulses selectively pass through the shutter aperture and continue toward the beam path, while the undiffracted ones (zero-order) are dissipated onto the shutter (Figure 45A). When the modulators are switched off, the laser is not diffracted (blanked) and therefore is not directed toward the sample.

Laser power. A combination of half-waveplate ($\lambda/2$ waveplate) and a polarizing beam splitter is typically included in the beam path to control the laser power. Neutral-density filters or variable attenuators have also been used to reduce the light intensity with no wavelength selectivity. The average energy that is directed to the focal spot and encounters the photosensitive material can be easily estimated (Figure 46A). With the most common 2P-SL setups (80 MHz, 1-200 mW), energy from about 10 to 200 pJ can

be applied at the focal volume. Other factors, such as energy loss along the beam path and the addition of other optical components such as SLM affect the final output power and need to be evaluated with the implementation of laser power meters.

Focusing and scanning. The laser beam is manipulated in shape, size and direction through a system of lenses and mirrors. It commonly includes a beam expander system, scanning mirrors, and objective lens. In order to minimize power loss and distortions, NIR-corrected and optimized optics need to be used. Beam expander lenses are used to adjust the laser beam size to fill the back aperture of the objective. This enables the user to maximize the power transmission and reduce the focal volume.⁴³⁴ Several types of objective have been used, from 10x water immersion to 100x oil immersion with numerical apertures (NA) ranging from 0.3 to 1.4. The focus spot generated from these objectives is moved within the sample to create 3D structures. As explained in more detail in the next paragraph, higher NA leads to a smaller focus spot and therefore better resolution. Conversely, higher magnification power leads to a reduction of the objective field of view (FOV). Galvanometer mirrors are commonly implemented to increase the laser scanning speed within the objective FOV. However, to fabricate structures with dimensions that exceed the FOV a motion system is necessary.

Stage/Objective movement. Commonly, the objective (or the stage) moves in the z direction thanks to piezoelectric actuators, while the movement in the x,y plane of the sample stage is produced either by piezoelectric actuators or linear motors. Depending on the requirements, linear motors that guarantee high speed and long travel range but low precision, or piezoelectric actuators that reach nanometer precision but are limited to short travel range (hundreds of μm) and low speed (hundreds of μm per second) are chosen. To fully profit from 2P-SL resolution, piezoelectric-controlled movements are necessary. This aspect also hinders potential *in vivo* applications. With an objective moving in the three dimensions by piezoelectric actuators, even small movements/vibrations of the patients will result in a substantial error in the biofabrication or patterning process. In *in vitro* systems, however, coordinated combinations of the aforementioned technologies allow, thanks also to an efficient photocurable system, writing speed up to 1 m/s to be achieved.^{140, 179, 443}

Resolution. Characteristics of the laser, optics, photoresin, PI and motion system together determine the minimum feature size, the overall dimension of the biofabricated structure, and the processing writing speed. To describe the accuracy of the biofabrication process, attention should be paid to the terms used to describe the resolution⁴⁴⁴:

- *Linewidth resolution:* defines the minimum size feature that can be generated.
- *Writing resolution:* defines the minimum distance at which two adjacent features can be biofabricated without overlap.

Both types of resolution can be specified in the *axial* (z) and *lateral* (x-y) directions.

Figure 47. Relationship between laser intensity, polymerization voxel dimension and linewidth. Top left) Detail of the focal volume in which 2PA takes place and inner polymerization voxel where the polymerization threshold is exceeded and the photochemical reaction occurs. Bottom left) Narrowing dose-response profile for the 2PA process compared to one-photon absorption (light grey). The arrows and the three black curves indicate the increase in polymerization voxel dimension (and therefore also linewidth) with an increase of laser intensity. Thus, the minimum intensity that exceeds the threshold leads to the minimum linewidth resolution. Bottom-right) Jablonski diagram showing the inter-system-crossing transition to the triplet state T_1 and subsequent radical generation that takes place in the 2PA volume and triggers the polymerization in the inner polymerization voxel where the radical concentration exceeds the threshold.

The linewidth mainly depends on light diffraction, thus on objective NA and laser wavelength. In the x-y plane the diffraction limit, d , (distance at which two distinct elements can be resolved) is generally defined by the Abbe's equation:

$$d = 0.61 \frac{\lambda}{NA} \text{ (Rayleigh criterion coefficient)} \quad (4)$$

Therefore, higher NA objectives decrease the feature size and are preferred. Considering a NA value of 1.4 and a λ of 800 nm, the value of d_{xy} would be approximately 350 nm. In the z axis the d_z value is roughly 3 times bigger, giving an ellipsoidal excitation volume (voxel). However, the dependence of 2PA on the square of light intensity determines a narrower dose response profile by a factor of $\sqrt{2}$ (Figure 47).⁴⁴⁴ For Gaussian like pulses, the voxel dimension intended as full width at half maximum (FWHM) can be calculated as follow⁴³¹:

$$FWHM_{x,y} = \frac{32\lambda}{\sqrt{2NA}} 2\sqrt{\ln 2} \quad (5)$$

$$FWHM_z = \frac{0.532\lambda}{\sqrt{2}} \left[\frac{1}{n - \sqrt{n^2 - NA^2}} \right] 2\sqrt{\ln 2} \quad (6)$$

For example, as shown by Dobos et al. at 720 nm, a 10x /0.3NA objective results in a $31.1 \mu\text{m}^3$ voxel while a 2.5x/0.085NA objective results in a 2.67 mm^3 voxel.⁴⁴³ Also, an increase in laser intensity (Figure 47) and exposure time results in a larger voxel size.⁴⁴⁵

The sub-diffraction limit linewidth is also a result of a threshold response of the photo-activated material. Taking the radical polymerization process as an example, its threshold is overcome only when the active species (radicals) generated by PI excitation are enough to drive the polymerization of the liquid resin.

The radicals can be quenched by oxygen or other additional quenchers.⁴⁴⁶ Therefore, the actual volume where the threshold is exceeded (polymerization voxel) can be made smaller or larger by varying the inhibiting species type and concentration. It follows that a common rule to minimize the feature size is to set the laser intensity slightly above the threshold (Figure 47). To date, it has been possible to obtain feature sizes down to few tens of nanometers.⁴⁴⁷ Beside “conventional” 2P-SL setups, the implementation of a doughnut-shaped inhibition beam inspired by stimulated emission depletion (STED)⁴⁴⁸ has already shown the possibility of going down to 9 nm linewidth resolution, thus achieving a scale that was previously only obtained with electron beam lithography.⁴⁴⁹

The writing resolution in the axial or lateral direction is instead generally much larger than the linewidth (Figure 47). The active species generated in the peripheral volume in which the intensity is below the threshold do not contribute to the fabrication process. However, these peripheral active species can accumulate with the ones generated during the polymerization of an adjacent feature and thus, by activating the chemical reaction in undesired regions, lower the resolution. It follows that active species half-life, diffusivity and writing speed influence the system resolution.⁴⁵⁰

It is noteworthy that, for a number of years, several companies have provided commercially available, desktop-sized and user-friendly 2P-SL systems: Photonic Professional GT2 (Nanoscribe), μ FAB-3D (Microlight3D), NanoOne (UpNano) and Holograph X (Cellink). The possibility of having access to ready-to-use instruments without the need for customized microscopes led to a notable increase in publications, in particular on microfabrication with commercially available resins.⁴⁵¹⁻⁴⁵⁶

Figure 48. 2P-microfabrication and 2P-patterning. General illustration of the fabrication and patterning approaches that have been exploited and are covered in this review. 2P-microfabrication can be either obtained by bond formation through radical mediated polymerization/crosslinking (top-left) or by bond degradation when a photocleavable linker is used (top-right). 2P-patterning can be either obtained by direct immobilization of a bioactive signal through radical mediated conjugation (bottom-left) or indirect immobilization following the removal of a photocage from a functional group that can then bind the bioactive signal (bottom-right).

2P-SL can be used by means of bond formation and bond cleavage. For clarity, the use of these two strategies for both 2P-microfabrication and 2P-patterning is presented in separate respective sections.

From this point forward, the term 2P-microfabrication will be used to refer both to polymerization/crosslinking of liquid resins (negative photoresists) and to degradation of solid structure (positive photoresists), which gives spatiotemporal control over hydrogel stiffness and degradation kinetics¹⁴². Both these approaches of spatiotemporal control of phase transition can indeed be used to microfabricate 3D structures. On the other hand, for 2P-patterning we refer to the spatially and temporally controlled immobilization of bioactive signals within hydrogels. This can take place in different ways, generalizable into direct photocrosslinking (bond formation) or indirect uncaging mediated (bond cleavage) selective immobilization (Figure 48).

4.2.2 2P-Microfabrication Applications

The main achievements of 2P biofabrication by means of bond formation and bond cleavage are herein presented with particular interest paid to biocompatible and cell-laden photosensitive systems.

Bond Formation in Microfabrication

Figure 49. 2P-microfabrication of micro/nano scale features. A) First example of 3D 2P-microfabrication with urethane acrylate photoresin SCR500. B) Sub-micrometer printing of a bull model (Scale bar: 2 μm) with urethane acrylate photoresin SCR500. C) Castle model fabricated on the tip of a pencil. A: Adapted with permission from ref 457. Copyright 1997 The Optical Society B: Adapted with permission from ref 458. Copyright 2001 Springer Nature C: Adapted with permission from ref 131. Copyright 2016 John Wiley and Sons

The first example of 3D 2P-microfabrication dates back to 1997, when Maruo et al. used a commercially available acrylate-based resin (SCR500) to polymerize a spiral structure with micrometer size linewidth (Figure 49A).⁴⁵⁷ A sub-diffraction linewidth was achieved just a few years later with the manufacturing of a ~10 μm bull model showing outstandingly small, detailed features down to 120 nm⁴⁵⁸ (Figure 49B), which inspired the advent of a new and exciting research field based on this technique. The fabrication of cellular/sub-cellular-sized structures represents an ability that is still unmatched by any other biofabrication technique and whose potential has probably not yet been fully exploited.

Acrylate based materials:

Synthetic polymers. Acrylic (e.g., SR9008, SR368, IP-L 780) and hybrid organic-inorganic resins (e.g., ORMOCER®) have been extensively used in 2P-microfabrication.⁴⁴⁶ The high reactivity of acrylate groups and the use of highly efficient PIs enable fast fabrication of 3D CAD models with impressive accuracy⁴⁵⁹⁻⁴⁶⁰ (Figure 49C), extending linewidth down to 65 nm.⁴⁴⁷ In most of these cases, the reaction is performed in organic solvents and extensive washing steps to eliminate un-crosslinked resin are necessary. Clearly, these harsh conditions hinder the possibility of a direct encapsulation of cells and limit the use of these systems to a post-fabrication cell seeding approach. However, the faithful replication of almost any CAD shape and the high printing reproducibility obtainable with synthetic resins

are a significant advantage for systematic *in vitro* cell studies.⁴⁶¹⁻⁴⁶² Moreover, the thermal, chemical and mechanical stability of the final structures make synthetic resins suitable for various biomedical applications.⁴⁶³

Figure 50. 2P-microfabrication of biomimetic structures with synthetic polymers. A) Bone trabeculae fabricated with organic-inorganic hybrid photoresin OrmoComp® (Scale bar: 50 µm). B) Cardiomyocyte alignment on ORMOCER® fabricated fibers with different diameters and spacing (Scale bar: 50 µm and 100 µm). C) SU-8 biomimetic blood-brain barrier with dimension comparable to those that can be found *in vivo* and 1 µm pores to mimic mass transport (Scale bar: 20 and 2 µm). A: Adapted with permission from ref 451. Copyright 2014 Elsevier Ltd. B: Adapted with permission from ref 465. Copyright 2013 Elsevier Ltd. C: Adapted with permission from ref 466. Copyright 2017 John Wiley and Sons.

Ovsianikov et al. first introduced the use of 2P-microfabrication for 3D tissue scaffolds.⁴⁶⁴ Since then, several materials, architectures, and cell types have been exploited for the generation of 3D scaffolds with negligible cytotoxicity.^{451, 465-467} Nevertheless, the absent or reduced cytotoxicity was achieved by extensive washing of generally toxic un-reacted monomers/oligomers and PIs. Since these washing steps are undertaken with non-cytocompatible solvents such as ethanol, isopropanol, acetone, or even more nonpolar mixtures, one then needs to be sure that all the toxic components are washed away before seeding cells. Bio-mimetic structures such as bone trabeculae⁴⁵¹ (Figure 50A), cardiac tissue model⁴⁶⁵ (Figure 50B) and retina model⁴⁶⁷ have been manufactured by means of 2P-microfabrication with organic-inorganic hybrid resins (ORMOCER®s). The precise reproduction of trabeculae-like structures from human bone μ -CT scans with a cell adhesive ORMOCER® resin named OrmoComp® seemed to positively affect the osteogenic differentiation of SaOS-2 bone-like cells compared to flat control surfaces. This biomimicry at the nanoscale has also been recently exploited by Marino et al. to reproduce an *in vitro* real-scale human model of the blood-brain barrier with the epoxy-based photoresin SU-8 (Figure 50C).⁴⁶⁶ Post-fabrication co-culture of endothelial-like and glioblastoma cells rendered this system suitable for biological studies or HT drug screening on disease models.

During the last 10 years, many acrylate derivatives of synthetic polymers such as biodegradable methacrylated polylactide (PLA),⁴⁶⁸⁻⁴⁶⁹ acrylamide,^{122, 470} organometallic polymers⁴⁷¹, poly(2-ethyl-2-oxazoline) diacrylates⁴⁷² and stimulus-responsive poly(ionic liquid)s⁴⁷³ have been investigated as potential materials for tissue-engineered scaffolds made by 2P-SL. To get specific features that resemble natural systems, one could also rely on the preparation of nano-composites. Carbon nanotubes,²⁸⁵ zinc oxide nanowires⁴⁷⁴ and nanoparticles^{122, 470, 475-477} are just a few examples of composite materials that have been used for 2P-microfabrication.

Nevertheless, acrylate-based and hybrid resins have also been widely exploited to generate scaffolds for single-cell force measurements and cell migration studies. With the spread of user-friendly 2P-SL systems and photopolymerizable resins, this field has become increasingly important. Excellent reviews on this topic have recently been published.⁴⁷⁸⁻⁴⁷⁹ Furthermore, it is noteworthy that 2P-microfabrication with synthetic acrylates and hybrid resins has been used to develop a new generation of biomedical devices such as stents⁴⁵⁴, needles⁴⁸⁰⁻⁴⁸¹ and valves⁴⁸² with outstanding precision. To give an example, recently Lölsberg et al. developed a microfluidic 2P vertical flow lithography to fabricate free-standing thin (<1 µm) microtubes with tunable diameter (1-400 µm), wall porosity (pore size 1-20 µm), and length (hundreds of micrometers) with potential uses including stents or nerve guides.⁴⁵⁴ Another relevant potential biomedical application of 2P-SL that is not discussed here is the manufacturing of nano/micro-

robots. In recent years, micromotors,⁴⁸³ mobile magnetic^{452, 476, 484-486} and biodegradable microrobotic swimmers⁴⁸⁷⁻⁴⁸⁹ have been fabricated, opening up new perspectives for diagnostics and targeted drug and cell delivery.

Figure 51. 2P-microfabrication of 3D scaffolds with PEG-DA. A) 2P-microfabricated PEG-DA scaffold seeded by means of LIFT with two cell types: ovine vascular smooth muscle (vSMCs, green) and endothelial cells (EC, red) (Scale bar: 1 mm). B) *C. elegans* trapped in 2P-microfabricated woodpile hydrogel structures made of PEG-DA (Scale bar: 20 and 10 μm). A: Adapted with permission from ref 492. Copyright 2010 IOP Publishing Ltd B: Adapted with permission from ref 139. Copyright 2012 SPIE. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC) <http://creativecommons.org/licenses/by-nc/4.0/>.

Among the various synthetic polymers used for 2P-microfabrication of cell scaffolds, PEG-DA certainly has been one of the most promising and most investigated.^{204-205, 490-491} Ovsianikov and colleagues combined the accuracy of 2P-microfabrication of PEG-DA with cell seeding by laser-induced forward transfer (LIFT) in what is, to our knowledge, the first hybrid biofabrication technique that includes 2P-SL.⁴⁹² A highly porous periodic scaffold was obtained by 2P-SL, and thanks to precise LIFT cell deposition, a multicellular construct was obtained (Figure 51A). Precise deposition of vascular smooth muscle-like cells (vSMCs) in the outer part of the PEG-DA scaffold and ECs in the inner part resembled the arrangement of natural blood vessels. LIFT was used to achieve higher cell density and a better homogenous distribution over the whole structure height (300 μm). However, this concept is limited to thin and highly porous constructs and therefore cannot replace biofabrication in the presence of cells, which has the advantage of reaching high density and homogenous cell distribution within the hydrogel.

As the focus of this review is on biocompatible systems for cell-laden approaches, the use of this technique to fabricate acellular tissue scaffolds,^{453, 468-469, 490, 493-498} cell and drug delivery carriers^{476, 499-500} and other 2P-microfabrication applications are not addressed in depth. Therefore, the interested reader is invited to read the previously-cited reviews and papers.

Successful cell encapsulation within a photo-activated material (bioresin) depends on many factors. Undoubtedly, the first requirement is to be able to perform the biofabrication process in an aqueous environment. The first example of 2P-microfabrication in an aqueous medium in which a 2P specific PI was used comes from Jhaveri et al.⁵⁰¹ In this case, the hydrophobic PI AF240, which has a large 2P cross section ($\sigma^{2\text{PA}} \sim 100 \text{ GM}$ at 800 nm), was dissolved in aqueous media by using the non-ionic surfactant Pluronic F127 (PF127). 2P-SL was then performed in a water-soluble monomer solution composed of 2-hydroxyethyl methacrylate (HEMA) and PEG-DA. A few years later, Torgersen et al. successfully performed 2P-microfabrication in the presence of a whole living organism (*C. elegans*) (Figure 51B).¹³⁹ The PEG-DA solution in combination with the highly efficient water-soluble 2PI, WSPI ($\sigma^{2\text{PA}} = 120 \text{ GM}$ at 780 nm) enabled fast processing speed (10 mm/s), comparable to organic-based systems. This study again showed the importance of high water content systems to reach good biocompatibility. The above-mentioned work, together with the 2P-microfabrication of waveguides for selective single yeast cells' immobilization and illumination⁵⁰² in PEG-DA hydrogel, is, to our knowledge, the only study in which cell-laden synthetic polymers have been used. Importantly, the writing process exhibited high cytotoxicity that can be attributed to the Irgacure 819 which, being an inefficient 2PI, requires higher laser doses. It is therefore clear that efficient water-soluble 2PIs play a crucial role for biocompatibility. In the past decade, different strategies for their development have been employed, ranging from host-guest interactions^{134, 491} or tailoring hydrophobic cores with hydrophilic moieties^{22, 133}

to the use of semiconductor-metal hybrid nanorods.⁴²⁶ For a detailed discussion of PIs, the reader is referred to Section 2.2: Water-Soluble Photoinitiators (PIs).

The substantial absence of 2P-driven biofabrication with synthetic polymers can be attributed to the fact that a majority of synthetic photoresins are better or only soluble in organic solvents. Even if they can be easily used for acellular scaffold manufacturing because of their conveniently tunable mechanical and chemical properties, they do not accurately resemble the composition in native ECM, and they generally need to be loaded or conjugated with bioactive molecules. Given the above, most of the 2P biofabrication studies employed natural polymers, such as polysaccharides or peptides that have been made photoactive by functionalization with various vinyl moieties (e.g., acrylates, methacrylates, vinyl esters) as discussed in the next paragraph.

Naturally derived polymers. To date, as also seen for other photo-mediated biofabrication techniques, Gel-MA is the predominantly used photo-active naturally derived polymer. It was first used in the presence of I2959 and at an excitation wavelength of 515 nm to fabricate a woodpile microstructure-mimicking cellular microenvironment.⁵⁰³ These constructs successfully supported adhesion and proliferation of porcine bone marrow-derived MSCs seeded on top of them. This study, like all others employing post-fabrication cell seeding, avoids the potential toxicity of radical mediated crosslinking that was shown in a following study.⁵⁰⁴

Figure 52. 2P-microfabrication in the presence of cells with Gel-MA bioresins. A) Live/dead staining. Low viability (red-stained cells) in the region exposed to the laser in Gel-MA matrices (Scale bar: 500 μ m). B) Viable (green-stained) MC3T3 cells after 2P-microfabrication of yin-yang CAD model with Gel-MA bioresin (Scale bar: 50 μ m). C) Live/dead staining. 2P-microfabrication of a CAD model (left) done with 2PI DAS shows higher cell viability after 1 and 5 days (left top and bottom) when compared to 2P-microfabrication done with P2CK (right top and bottom) in Gel-MA bioresin. Viable cells in green and dead cells in red. (TU Wien logo dimension: 500 x 500 x 125 μ m). A: Adapted with permission from ref 504. Copyright 2013 American Chemical Society B: Adapted with permission from ref 141. Copyright 2017 Royal Society of Chemistry C: Adapted with permission from ref 140. Copyright 2018 Royal Society of Chemistry

The first cell-laden hydrogel microfabrication revealed significant cytotoxicity on human osteosarcoma cells (MG63) resulting from reactive oxygen species (ROS)-induced cell damage (Figure 52A). Notably, IR laser radiation (800 nm) alone showed no impact on cell viability. The use of highly efficient water soluble 2PIs (G2CK, P2CK) enabled a writing speed of up to 7 mm/s to be reached with a formulation

consisting of 80% cell culture medium content. Later, the source of the cell damage was partially reduced by suppressing the diffusion of PI into cell cytoplasm with a hyaluronan-PI (HAPI) conjugate.¹⁴¹ Limiting ROS generation within the cells led to a considerable improvement in cytocompatibility. Successful direct encapsulation of mouse calvaria-derived preosteoblasts cells (MC3T3) into Gel-MA 3D microstructures was obtained at a printing speed of 100 mm/s (Figure 52B). Another approach used to limit the main source of cytotoxicity, 2PI-generated radicals, relied on the development of a cleavable diazosulfonate (DAS) initiator.¹⁴⁰ By using the latter in combination with Gel-MA bioresin, ADSCs maintained normal metabolic activity and good viability within a microstructure biofabricated at the record speed of 1 m/s (Figure 52C).¹⁴⁰ This demonstrates the promising potential of this biofabrication technique as a powerful tool in tissue engineering, namely to construct stem cell/organoids niches with sub-cellular features or tissue models for drug discovery.

To date, 2P biofabrication based on photoactivated natural polymers has mainly been done with Gel-MA formulations and simple constructs. However, other methacrylated polysaccharides, such as dextran,⁵⁰⁵ HA,⁴⁹³ chitosan,⁵⁰⁶⁻⁵⁰⁷ cellulose⁵⁰⁸ and pullulan,⁵⁰⁹ or those with other types of functionalization, like tyramine conjugation,¹⁰⁹ have been successfully used for 2P-SL. The choice of natural polymers over synthetic ones is driven by their biocompatibility, biodegradability, potential bioactivity and in general by their ability to better resemble the native ECM. However, the modification of such polymers does not come without costs. The functionalization with photosensitive moieties inevitably increases their toxicity¹⁹² and changes the properties of these materials to a certain degree. As an example, a high degree of methacrylation of primary amines (97%) and carboxylic acids (56%) of gelatin results in a better processability, but also in the loss of thermal gelation.¹⁷⁷ In general, increasing the DF can help to get a faster processing speed and indeed most of the papers cited presented a DF > 60% and up to 95%.¹⁴⁰ Another approach, again on the material side, to increase printing speed and tunability of mechanical properties without a dramatic loss of native polymer properties relies on the addition of a crosslinker. Photosensitive crosslinkers such as PEG-DA^{493, 510} have been widely used to tune the crosslinking of naturally derived polymers. Different stiffnesses can be obtained by varying their content in the initial formulation of naturally derived polymers and result in a tunable ability of cells to invade the hydrogel and migrate into it.⁵¹⁰

To date, the formation of cell scaffolds with synthetic and naturally derived acrylated/methacrylated polymers have represented, in many cases, an acceptable tradeoff between processability and biocompatibility. However, although these polymers still represent the standard for light-induced microfabrication, their use for biological applications is limited because of their irritancy and cytotoxicity, in particular for highly reactive acrylates.⁵¹¹ Attention should be also paid to the fact that a certain degree of unreacted potentially toxic monomers/oligomers will be generally present in the biofabricated structures in the absence of long and extensive washing steps and can therefore hamper cell viability. Alternative approaches based on click chemistry or on native unmodified polymers have been investigated and will be discussed in the following sections.

Step-growth thiol-ene chemistry: Photoinitiated thiol-ene click chemistry for hydrogel formation was first introduced by Anseth group.^{84, 512} It was shown that the fast kinetics and the mild reaction conditions, together with the orthogonality toward other reactive groups, make them excellent candidates for cell-laden hydrogels. In addition to the possibility of being performed in an aqueous environment without being inhibited by oxygen, another clear advantage for cell-laden systems made by the step-growth mechanisms is the low amount of generated radicals, which leads to higher cell viability.^{204, 513} The advantages of thiol-ene chemistry over the commonly adopted radical-chain polymerization of acrylates and methacrylates have become increasingly evident.^{142, 514} However, despite the fact that thiol-ene reaction for light-driven additive manufacturing and 2P-patterning (see Section 4.2.4) is increasingly explored, its use in 2P-microfabrication is so far limited to the work of Liska and Ovsianikov.^{179, 192, 515}

Figure 53. Microfabrication of 3D structures by photoclick thiol-ene chemistry. A) Construct made of Gel-VE/BSA (Scale bar: 500 µm). B) Octopus structure made of HA-VE/DTT (Scale bar: 100 µm). C) Reproduction of CAD mouse model (left) with PVA-ene/PVA-thiol resin visualized with light microscopy (middle) and SEM (right) (Scale bar: 25 µm). D) Z-stack of Gel-NB grids biofabricated in the presence of mCherry labelled L929 fibroblasts after 1,3 and 5 weeks showing cell spreading (week 3) and starting of hydrogel degradation and cell migration (week 5). A: Adapted with permission from ref 515. Copyright 2013 John Wiley and Sons. B: Adapted with permission from ref 192. Copyright 2014 Royal Society of Chemistry. C: Adapted with permission from ref 516. Copyright 2016 John Wiley and Sons D: Adapted with permission from ref 179. Copyright 2019 John Wiley and Sons

Gel-VE in the presence of reduced bovine serum albumin (BSA) acting as a macrothiol donor was firstly used and showed notable processability.⁵¹⁵ Highly efficient water-soluble 2PI (WSPI) at 0.5 wt% enabled writing speeds as high as 50 mm/s at relatively low laser power (20 mW) to be obtained. As expected, a direct correlation between hydrogel stiffness and higher thiol to alkene ratio was observed, as well as low cytotoxicity and good retention of gelatin cell adhesion properties. Another naturally derived photoclickable polymer, HA vinyl ester (HA-VE), was obtained by lipase-catalysed transesterification.¹⁹² A comparison with HA acrylate (HA-AC) and HA-MA in the presence of fibroblasts revealed significantly lower cytotoxicity of the HA-VE system and a comparable or higher crosslinking efficiency. The use of another water-soluble efficient 2PI (P2CK) in the presence of dithiothreitol (DTT) as thiol donor enabled fast 2P-microfabrication with a large processing window (20-100 mW average laser power, 1-56 mm/s writing speed) and relatively small feature size (4 µm). In both cases, the fabrication of complex 3D microstructures was demonstrated, but only in the absence of cells (Figure 53A, B). Combination of PVA-ene and PVA-thiol also enabled 2P-microfabrication (Figure 53C) at relatively low laser power and high writing speed (1-100 mW, 1-178 mm/s) in the presence of water soluble 2PI E2CK-THEMA.⁵¹⁶

However, to date, Gel-NB appears as the most promising material for photo click-based 2P-microfabrication. First, Van Hoorick et al. showed the advantages of using highly reactive norbornene functionalities. Highly substituted Gel-NB (DF up to 90%) showed better processing capabilities when compared to commonly used Gel-MA.⁵¹⁴ In particular, this led not only to faster processing and wider processable concentration range, but also to lower laser power needed to exceed the fabrication threshold. Only recently, Dobos et al. exploited the high reactivity of such systems to perform the first photoclick-based 2P-microfabrication in the presence of cells.¹⁷⁹ By using high DF (53%) Gel-NB, low molecular weight dithiol donor DTT, and thanks to the efficient water-soluble 2PI DAS, a record speed of 1 m/s was reached. Cell-embedded (L929 mouse fibroblasts) hydrogel constructs showed high cell viability when laser power below 100 mW was used. However, it is important to mention that in the regions exposed to the laser (from 50 mW to 90 mW), a strong decrease in proliferating cells was observed.¹⁷⁹ Despite this, after 3 to 5 weeks, fibroblasts partially degraded the gelatin structure and migrated into it (Figure 53D). Clearly, as this technique is paving its way to biofabrication, there is an urgent need to better understand at a molecular level what determines such cell response to the biofabrication process.

In general, the better processability of thiol-ene-based systems and the more homogeneous network formation compared to chain-growth photochemical processes (see Section 2.1) represent key advantages for 2P-microfabrication of cell-laden hydrogels. Even though this photo click-chemistry based approach has not been widely adopted so far for 2P-microfabrication, there is an expectation that the pioneering work in this direction will guide future efforts on the use of thiol-ene bioresins in 2P-SL as a high-resolution biofabrication technique.

Native Proteins: A small number of studies have demonstrated that 2P-induced biofabrication is also possible with native (unmodified) proteins, in the presence of photosensitizers such as Rose Bengal⁵¹⁷⁻⁵²⁰ or flavin adenine dinucleotide.⁵²¹⁻⁵²³ Following the excitation of the PI to the T1 state, two photo-oxidation mechanisms are believed to be responsible for the crosslinking process: generation of singlet molecular oxygen and hydrogen abstraction from amino acid residues. In the first case, crosslinking takes place when the singlet oxygen oxidizes a photo-oxidizable amino acid residue (e.g., histidine, cysteine, tryptophan, and tyrosine) that then forms a covalent bond with a second electron-deficient amino acid.⁵²⁴ In the absence of quenchers, since the cell-containing aqueous environment is not oxygen-free, the singlet oxygen formation is the most plausible mechanism.

Figure 54. 2P-microfabrication with unmodified proteins. A) Dynamic trapping, incubation and release (arrow) of *E. coli* in crosslinked BSA (Scale bar: 5 μ m) B) *In situ* encapsulation of *S. aureus* (blue) and *P. aeruginosa* (green) by microfabrication with gelatin/BSA (Scale bar: 10 μ m (left), 20 μ m (right)). C) Post-fabrication seeded HUVECs in 3D microvasculature channels generated in collagen hydrogel (Scale bar: 100 μ m (top,middle), 10 μ m (bottom)). A: Adapted with permission from ref 522. Copyright 2008 National Academy of Sciences, U.S.A. B: Adapted with permission from ref 520. C: Adapted with permission from ref 15. Copyright 2016 John Wiley and Sons

2P-SL with collagen type I, II and IV^{518, 524} was performed in the presence of Rose Bengal diisopropyl amine or benzophenone dimers (BPDs). Unlike xanthene dyes, BPD can be used in acidic conditions, which facilitates the solubilization of collagen. Simple 1.5 μ m collagen lines were biofabricated under physiological conditions and showed bioactivity retention (cell adhesion) and enzymatic degradation. Other native proteins such as BSA,^{517-518, 521-523, 525} fibrinogen,^{517, 525} avidin,⁵²¹⁻⁵²² and laminin⁵²¹⁻⁵²² have been used for 2P-microfabrication in biologically compatible aqueous environments and have shown good bioactivity retention. Notably, functional protein microstructures were biofabricated in the presence of cells by Kaehr et al. to guide neurite outgrowth of rat brain cortical neurons.⁵²¹ The guidance structures were obtained without cytotoxic effects on the neurons. However, the potential harmful effect of FAD photoexcitation was not fully explored, since no direct irradiation on the cells was required. Later, Kaehr et al. exploited 2P-mediated BSA crosslinking to realize cell chamber⁵²³ and dynamic hydrogel microactuators,⁵²² proving effective *E. coli* trapping, incubation and release (Figure 54A). Similarly, gelatin and BSA were used in the presence of Rose Bengal to arrange polymicrobial communities within entrapping containers (Figure 54B).⁵²⁰

As a type II PI, Rose Bengal needs a co-initiator and TEA is commonly used. However, it was demonstrated that the proteins themselves can act as co-initiators.^{507, 517} Following a microinjection of Rose Bengal only, Basu et al. were able to fabricate simple structures within the cytoplasm of live starfish

oocytes, demonstrating potential compartmentalization of cellular processes.⁵¹⁹ PI-free 2P structuring of un-modified proteins (collagen and fibrinogen) and liquids (fetal calf serum and human blood) was also investigated and grids with micrometer width lines were obtained. However, the underlying structuring mechanism remains unclear.⁵²⁶

To date, the 3D structural complexity and accuracy generally obtained with unmodified proteins does not yet achieve the standard reached with acrylate polymers and other modified materials, mainly because of the lower density of reactive groups (e.g., oxidizable amino acids such as tyrosine) and lower reactivity. In addition, the crosslinking of oxidizable residues in an aqueous environment with water-soluble dyes and amine co-initiators is heavily limited by the need for high laser intensities and low writing speeds, generally $\sim 5 \mu\text{m/s}$. To the best of our knowledge, the only exception is represented by the fast (400 mm/s) 3D microfabrication of biomimetic collagen microvasculature structures produced by fluorescein photobleaching by Skylar-Scott et al.¹⁵ High laser power (up to 2.5 nJ/pulse) was adopted to trigger the crosslinking process upon fluorescein photobleaching in a bulk collagen gel and form the channels wall. Upon post-fabrication seeding of HUVECs via hydrostatic pressure-driven flow, the microvasculature network was readily covered by a confluent monolayer of VE cadherin positive cells (Figure 54C). This demonstrated the retention of cell adhesion properties of collagen after crosslinking. This simple setup, consisting of off-the-shelf polymers and fluorescein, holds great potential for 2P-microfabrication, since it is readily accessible and does not involve complex synthesis or functionalization steps. However, it must be emphasized that biofabricated collagen structures were in this case limited to a cell-seeding approach, because they need to be post-processed in acetic acid or Tris buffer.

Commercially available hydrophilic dyes (e.g., Eosin Y, Rose Bengal, fluorescein) are mainly used as UV-visible fluorophores and therefore show low σ^{2PA} and high fluorescent quantum yields. The use of efficient 2PIs also represents in this case the need to avoid compensating these sub-optimal properties with high laser power, low writing speed or high number of scans. Given the above, it is expected that the recent synthesis of highly efficient water-soluble 2PIs (e.g., WSPI, P2CK, E2CK, DAS) will renew interest in unmodified protein 2P-microfabrication.

Bond Cleavage in Microfabrication

The cell's extracellular environment is extremely complex and dynamic, undergoing continual remodeling. It is well established that mechanical signals of the extracellular milieu are as crucial in determining cell fate⁵²⁷ as GFs and other signaling molecules. Therefore, 3D microfabrication often does not sufficiently mimic the diverse and mutable properties of the native ECM. Real-time manipulation of light-induced degradation has proven to be a useful tool for addressing this limitation.⁵²⁸ Important advances in the design of dynamic hydrogels have been made in the past decade thanks to 2P-SL. Using photolabile crosslinkers, a 3D spatiotemporal control over hydrogel mechanical properties can be achieved. Pioneering work on locally induced changes in the network stiffness has been made by DeForest and Anseth.^{28, 149, 529} A major part of the studies on multi-photon-assisted photoscission rely on oNB derivatives as photolabile moieties. However, photocleavage strategies employing more efficient photolabile moieties such as coumarins¹⁶⁶ and ruthenium complexes⁵³⁰ have also been investigated.

oNB photodegradation: An oNB-based 2P-degradable hydrogel was first reported in the pioneering work by Kloxin et al.¹⁴⁹ Site-specific degradation of a PEG hydrogel in the presence of cells allowed remote control over properties such as stiffness, water content and diffusivity. The erosion of interconnected 3D channels was used to study cell migration of encapsulated fibrosarcoma cells. The possibility of studying cell migration and connectivity was further demonstrated in the presence of MSCs.²³ The precise control of cell adhesion sites by photolysis showed the utility of this material for 3D dynamic cell studies.

Figure 55. *ortho*-nitrobenzyl (oNB) mediated degradation. A) 3T3 fibroblasts (green) confined outgrowth within oNB mediated photodegraded channels patterned with RGD adhesion peptide (left branch) via thiol-ene photoclick coupling. Rounded shaped human mesenchymal stem cells in blue in the outer region. Click-based PEG hydrogel in red (Scale bar: 100 μ m). B) Motor axon extension (green) in oNB mediated eroded channels in PEG hydrogel (red) (Scale bar: 100 μ m, 10 μ m). C) HUVECs endothelialized 60x60 μ m and 45x45 μ m channels generated by oNB photodegradation in MMP-sensitive PEG-based hydrogel (Scale bar: 50 μ m). A: Adapted with permission from ref 28. Copyright 2011 Springer Nature B: Adapted with permission from ref 16. Copyright American Chemical Society C: Adapted with permission from ref 14. Copyright 2017 John Wiley and Sons

A similar system based on oNB-containing crosslinker was made more biocompatible by replacing acrylate-based crosslinking with cytocompatible SPAAC click-chemistry.²⁸ SPAAC reactions proceed rapidly, without a catalyst, in physiological conditions. Together with being remarkably bio-orthogonal and non-toxic, click-based hydrogel formation represents an advantageous approach for biological applications. The irreversible 2P cleavage of oNB (740 nm) was used to direct *in situ* growth of fibroblasts (3T3) contained within a fibrin clot. The authors reported a strong preference of 3D-directed cell outgrowth when channel erosion and subsequent functionalization with fibronectin adhesion peptides (RGDs) were used in combination (Figure 55A). In contrast, human MSCs encapsulated in the hydrogel without erosion and RGD maintained a round morphology. Notably, the patterning of the short peptides was achieved with photoclick thiol-ene chemistry in the presence of Eosin Y showing wavelength orthogonality with oNB cleavage. Therefore, remote post-gelation control of both chemical and physical properties was achieved with orthogonal light-based chemistry.

The possibility of forming channels within a 3D hydrogel and the observation that cell growth can be directed toward the eroded regions inspired the use of this approach for the biofabrication of perfusable vasculature networks¹⁴ and neural networks¹⁶ in cell-laden hydrogels. Embryonic stem cell-derived motor neurons axonal extensions were directed upon 740 nm fs-pulsed laser-induced hydrogel degradation. The system was exploited to investigate the dependence of neuron migration on channel dimension, erosion degree and angle (Figure 55B). Besides the ability to study the influence of biophysical cues with 3D control, this biocompatible strategy was employed to direct the formation of neuromuscular junction in the presence of two cell types. C2C12 myotubes were co-encapsulated with ESMN embryoid bodies. Thanks to the photo-cleavage of oNB-based linkers, channels connecting the two cell types were generated, allowing the neuron extension toward the muscle cells to then show evidence of neuromuscular junction formation. Another application of such systems to produce microchannels was presented by Arakawa et al.¹⁴ Endothelialized vasculature networks with different channel diameters ranging from hundreds of micrometers down to 10 microns were generated. Successful attachment of HUVECs on the channel wall was then obtained by post-fabrication seeding (Figure 55C). To date, possibly in combination with other techniques such as one-photon SL to improve the scalability and reduce the processing time, the generation of multiscale vascular networks appears to be one of the most appealing applications of 2P-SL for tissue engineering purposes.

Recently, the DeForest group combined the established oNB photocleavage strategy in copper-free SPAAC crosslinked PEG hydrogels with other stimuli-degradable moieties to generate a complex "boolean logic"-responsive biomaterial.⁵²⁹ Di-azide crosslinkers were engineered to respond to redox, enzyme, and light stimuli by the inclusion of disulfide bonds, MMPs-sensitive sequences, and oNB respectively. The linker architecture was designed to achieve material degradation only in response to

a specific combination of the environmental stimuli (reducing agent, proteolytic activity, and light exposure). The system showed a high degradation specificity, which has been tested by means of doxorubicin release in a cancer *in vitro* model and release of live cells. This platform offers unique opportunities for targeted drug delivery, tissue engineering and *in vitro* spatiotemporal control over stem cells' or organoids' differentiation and development. The potential for the cells to modify the surrounding environment with the production of certain stimuli is a crucial aspect for an advanced 3D cell culture system. In addition, the inclusion of a photolabile group allows the operator to maintain the ability to remotely control the engineered matrix, moving her/his role on the cell fate from "passive" to "active".

Even if largely employed for photodegradable systems, oNB linkers exhibit low σ^{2PA} , which significantly reduces their efficiency in 2P degradation. Recently, Lunzer et al. proposed a strategy to enhance the two-photon triggered cleavage of oNB.¹⁵³ The addition of a 2P active cyclic benzylidene ketone-based (P2CK) photosensitizer significantly improved the oNB ester cleavage upon NIR (800 nm) laser exposure and enabled cell-laden hydrogel degradation under moderate laser power. Micro-loop channels were photoeroded from a thiol-ene click-based HA hydrogel at a notable writing speed (200 mm/s) in the presence of hTERT-immortalized ADSCs. The system showed high cell viability after one and two weeks and therefore represents a good solution for oNB-based photocleavable hydrogels in which fast processing is needed. Another approach to improving the efficiency of two-photon degradation relies on the use of photolabile molecules with higher σ^{2PA} such as ortho-nitrobiphenyl⁵³¹ or coumarin derivatives.

Coumarin photoscission: Coumarin-based photodegradation has two main advantages compared to the oNB-based systems. First, as previously mentioned, coumarin's optical properties are more suitable for 2P lithography and generally represent a better choice for cytocompatible platforms (red-shifted absorption). Secondly, coumarin byproducts are less reactive than the aldehyde or ketones generated by nitrobenzyl systems.¹⁴⁸ However, unlike oNB, only a few examples of photolabile coumarin linkers for 2P hydrogel degradation can be found.¹⁶⁶ Coumarin was exploited by Zhu et al. as a hydrophobic photolabile block for constructing a linear triblock copolymer in combination with PEG hydrophilic blocks.⁵³² 2P-induced uncaging of the coumarin changes the properties of the block copolymer, leading to disintegration of the physical crosslinking. A similar approach was used by Yoshii et al., who presented the first two-photon responsive supramolecular hydrogel.²⁴ By conjugating a coumarin derivative to a phenylalanine dipeptide, the authors were able to self-assemble a fibrous gel and subsequently induce a transition to solution through site-specific 2P uncaging. Notably, the entangled fiber network rapidly disassembled upon 2P-uncaging, leaving a highly fluid void space. In fact, the small molecule (dipeptide) composition of the fibers leads to a drastic change in viscosity upon light-mediated degradation. The self-assembly of supramolecular fiber hydrogels and their fast, complete, and precise degradation differ from most of the studies in this field, which make commonly use of crosslinked polymer networks, and therefore represents another interesting approach for spatiotemporal-controlled tissue engineering.

Coumarin uncaging was also employed to control the hydrogel crosslinking. The protection of a reactive site with a 2P sensitive photocage is a widely used approach for 2P-patterning (see Section 4.2.4). However, it can also be used to trigger fast and cytocompatible gelation through thiol-ene Michael addition reaction.⁵³³ This approach enabled the build up of cell-laden hydrogels with negligible cytotoxicity. It combines the 3D control given by 2P-microfabrication at 800 nm with rapid thiol-ene click-chemistry in a physiological environment (PBS, pH 7.4) and overcomes the radical-induced damage typically observed in photoinitiator-based microfabrication. Thiol-ene microfabrication obtained upon thiol photo-uncaging can be applied to almost any hydrogel system. Thiol functionalization of the polymer chain and use of maleimide, or even better norbornene-terminated linkers (or viceversa), render this approach a powerful tool for 2P-microfabrication in the presence of cells.

In addition to oNB and coumarin derivatives, photolysis by 2PA was also shown in ruthenium complex containing polyurea gel.⁵³⁰ Other mechanisms such as photoablation, upconverting nanoparticle (UCNP)-assisted photochemistry or one-photon processes¹⁵⁵ have also been investigated. However, these approaches lie outside the scope of this review. Another strategy for achieving photodegradation is via radical addition-fragmentation chain transfer (AFCT) as proposed by Brown et al.⁵³⁴ In this study, radical initiated thiol-ene exchange reaction was employed to tune the mechanical properties of a SPAAC based PEG hydrogel in the presence of hMSCs. Even though this approach leads to faster bulk

hydrogel degradation compared to oNB or coumarin-containing hydrogels, the presence of a soluble photoinitiator and the generation of potentially harmful radicals remain major concerns.

Spatiotemporal control over local network crosslinking by 2P-SL holds great promises in dynamic 3D cell culture systems, single cell studies, drug or cell delivery and tissue engineering. Nevertheless, as comprehensively discussed by Brown and Anseth¹⁴², the design of a photodegradable material should take into consideration different aspects such the chemistry of chain-growth or step-growth networks, σ^{2PA} of the photolabile moiety and possible toxic or reactive byproducts that together define the material processability and biocompatibility.

4.2.3 2P-Microfabrication Limitations and Outlook

Beside the recent notable results achieved with 2P-microfabrication, several limitations still need to be overcome. The most pressing of these is the low scalability of the technique when relevant size constructs are considered. Even at the max speed reported to date (1 m/s)¹⁴⁰, the production of a 1 cm³ (100% infill) structure with 1 μ m linewidth would require almost 12 days. On the material side, the writing speed depends on the PI efficiency in generating radicals and the density and reactivity of photocrosslinkable cues. Therefore, the synthesis of novel, water-soluble and highly efficient 2PI would further improve the 2P-microfabrication processability. On the other hand, since the use of highly substituted polymers generally presents a relevant source of cytotoxicity (see Section 2.5), increasing the DF may not be a viable route for cell-laden constructs dependent on the biological properties of the polymer. Among the various chemical strategies, step-growth thiol-ene click-chemistry appears in general to be a good tradeoff between reactivity and biocompatibility. However, since thiols or disulfides are also normally present in proteins, such systems are not fully bioorthogonal.

On the instrumentation side, the maximum processing speed is obtainable with non-stop single-line, single-pass laser scanning.⁴⁹⁴ To do so, the laser intensity needs to be high enough to trigger the photochemical reaction during each single pixel dwell time.

Figure 56. 2P-microfabrication of cm size structures. A) PEG-DA tube structures fabricated by wider objective working range 2P-microfabrication show the possibility to go beyond the limited objective working distance (Scale bar: 1 mm). B) Terra Cotta Warrior CAD model (left) and SU-8 2P-microfabricated cm tall structure (right). The laser scan along the contour 3D profile was performed at 400 μ m/s and took 15h. A: Adapted with permission from ref 535 Copyright 2013 Springer Nature B: Adapted with permission from ref 536 Copyright 2018 John Wiley and Sons

Technical solutions have also been investigated to enable the manufacturing of tall (mm to cm) 3D structures, which is normally limited by the use of short working distance for high NA objectives. Obata et al. proposed a setup in which the objective and the coverglass can be moved together in the liquid photoresin, therefore resulting in a wider objective working range.⁵³⁵ Structures with height (7 mm) beyond the objective working distance have been fabricated with micrometer resolution (Figure 56A). More recently, Chu et al. generated a centimeter-height 3D structure with 10-40 μm resolution (Figure 56B)⁵³⁶ in 15 h utilizing simultaneous spatiotemporal focusing of the fs-pulsed laser.⁵³⁷ The resolution and the writing speed (400 $\mu\text{m/s}$) were limited by the low laser power (5 mW) and repetition rate (1 kHz) employed, but overall were enough to demonstrate that relatively tall constructs can also be generated by 2P-SL. The intrinsic drawback of being a serial (voxel-by-voxel) technique can be in part overcome by the use of a microlens array. The array of small lenses converts the collimated beam into multi-spots enabling parallel processing.⁴⁵⁰ Splitting the laser beam reduces the maximum energy in the excitation voxel, and therefore also in this case, highly efficient 2PIs are desired to lower the fabrication threshold. While this technique represents a powerful tool for speeding up the microfabrication of microscopic devices (e.g., MEMS) and periodic structures, to the best of our knowledge it has not been applied for the development of cell-containing hydrogel constructs. Recently, Saha et al. introduced a promising projection-based parallelization approach to speed up the printing process and overcome the point-by-point scanning.⁵³⁸ Spatial and temporal focusing of ultrashort laser pulses was used to generate femtosecond light sheets at the desired focal plane. With the use of DMD, 2D arbitrary patterns can be projected in a layer-by-layer fashion, enabling 3D printing process similar to projection VP while maintaining 2P derived sub-micrometer resolution. Another innovative strategy to speed up the printing process, which is also now available on the market (Holograph X by Cellink), is instead based on the generation of holograms by using SLMs. The (bio)fabrication time of this holographic 2P-SL is proportionally reduced with the number of generated focal points.⁵³⁹⁻⁵⁴¹

Another aspect that might limit the achievement of high resolution and structure fidelity, in particular with relatively soft materials such as hydrogels, are the time-dependent swelling and shrinkage that take place upon crosslinking or degradation. As these geometrical changes are often not predictable, an optimized CAD design that takes this aspect into consideration remains an open challenge.¹⁷⁷

Moreover, 2P-SL is normally limited to the use of a single material throughout the whole manufacturing process. Multimaterial constructs can be made by post-printing single or multiple functionalization⁵⁴² or, as recently shown by Mayer et al., with microfluidic based systems.⁵⁴³ After a first microfabrication step with a resin, instead of manually repeating washing, application of a second resin and re-alignment steps, microfluidic based multimaterial printing can be used to automatize this cycle.

Although *in vivo* 2P-microfabrication has not yet been demonstrated, a few papers already showed good *in vivo* biocompatibility of 2P-microfabricated scaffolds upon implantation for cartilage⁵⁴⁴ and myocardial⁵⁴⁵ tissue engineering applications and as cochlear drug release implants⁵⁴⁶. If it were possible to obtain relevant size constructs (cm^3) in a reasonable amount of time, this technique would clearly emerge as an attractive approach for patient-specific scaffold manufacturing. Even if clinical use of 2P-SL may not be ready in the near future, we envision that the unrivaled ability to mimic native ECM in sub-micrometer-resolved grafts will lead to significant improvements in this field.

In conclusion, 2P-driven bond formation and bond degradation are powerful tools for the design of micrometer/sub-micrometer-scale bioengineered matrices. However, due to the above-mentioned limitations, the advantages of this manufacturing technique in tissue engineering have not yet been fully explored. We envision that the exponential increase of research seen in recent years on this topic will hopefully bring forward novel efficient, water-soluble and wavelength-orthogonal PIs and photocages together with technical solutions to address the current limitations. We are also confident that this will shift the attention from the material and processing side to the biological side and contribute to advances in real-world biomedical applications.

4.2.4 2P-Patterning Applications

Cell behavior in 3D culture systems does not only depend on matrix composition, architecture and mechanical properties. The ECM is a highly mutable biochemical environment. During tissue development or regeneration, or in disease, the pattern of bioactive signals changes dramatically. *In vitro*, a fine control in time and space of bioactive cues can be obtained by 2P-patterning. In the last two

decades, several photochemical approaches have been developed to immobilize biologically relevant molecules, ranging from small molecules and short peptides to whole enzymes, in a broad range of hydrogel substrates. Tailoring biochemical properties at the microscale has opened up unique opportunities for developing complex dynamic cell niches and studying fundamental cell behaviors such as migration, differentiation and cell-matrix interactions. The 2P-patterning approaches are divided on the basis of their use of photocages or not. As seen for microfabrication, the excitation of a PI can lead to radical generation and therefore trigger radical mediated bond formation. In the first section (Bond Formation in Patterning) the “direct patterning” approach by radical mediated immobilization is discussed. In the second section (Bond Cleavage in Patterning), we guide the reader toward the recent achievements of the indirect strategies that include uncaging steps. 2P labile molecules are exploited to cage (render inactive) functional reactive sites that are, upon light-mediated uncaging, used to immobilize a bioactive cue.

Bond Formation in Patterning

Figure 57. 2P-patterning by bond formation in PEG-DA hydrogels. A) Human dermal fibroblasts (red) migration within acrylate-RGD patterned region (green) in collagenase-sensitive PEG-DA hydrogel (Scale bar: 100 μ m). B) Patterning of acrylate-RGD peptide (c) in regions of interest (ROI) reconstructed (b) from different tissue vasculature projections (a). Patterning fidelity was assessed by merging (a) and (c), with the yellow colour indicating good overlapping (d) (Scale bar: 5 μ m). A: Adapted with permission from ref 548. Copyright 2008 Elsevier Ltd B: Adapted with permission from ref 549. Copyright 2012 John Wiley and Sons

3D immobilization of biomolecules was first reported by Hahn et al.⁵⁴⁷ In a series of studies, the authors demonstrated the ability to recapitulate complex gradients of adhesion peptides (e.g RGD, CS-1 and IKVAV) within PEG hydrogels. In the presence of 2,2-dimethoxy-2-phenylacetophenone acting as PI, acrylated peptide derivatives were conjugated to PEG-DA gels by means of 2P (720 nm) radical-mediated conjugation. Using PEG-DA as a “blank slate”, the peptide adhesion properties were isolated and studied with different cell types. Notably, pre-encapsulated cells followed the guiding RGD pattern, while they failed to migrate toward the non-adhesive PEG-DA (Figure 57A).⁵⁴⁸ The same principle was adopted to control the formation of microvasculature network architectures derived from native tissues such as the retina, cerebral cortex and subependymal neural stem cell (NSC) niche. 3D images of endogenous vasculature beds were converted to regions of interest (ROI) to define the laser scanning path and intensity (Figure 57B). Excellent peptide patterning fidelity and successfully guided formation of tubule-like network within specified “biomimetic” areas were obtained.⁵⁴⁹

Figure 58. Influence of laser processing parameters on 2P-patterning. A) Higher linewidth resolution of fluorescent RGD patterning with higher magnification objective, from left to right: 10x (NA = 0.45) (Scale bar: 100 μm) and 20x (NA = 0.75) (Scale bar: 25 μm) resulted in a resolution in the order of 5-10 μm while 63x (NA = 1.4) showed 1-2 μm patterned features (Scale bar: 5 μm). B) Controlled concentration of immobilized fluorescent cue (green) by varying scanning speed and laser power. Increasing the scanning speed leads to a diminished light dose per pixel and therefore to a decrease in patterning efficiency. On the other hand, increasing the laser power (P) is associated with an increased immobilization of the fluorescent moiety (Scale bar: 500 μm). C) Importance of step size for the formation of smooth gradients. Diminishing the scanning intervals ensures the formation of continuous gradients in the z axis. A: Adapted with permission from ref 550. Copyright 2010 Royal Society of Chemistry B: Adapted with permission from ref 551. Copyright 2012 John Wiley and Sons C: Adapted with permission from ref 20. Copyright 2018 Elsevier Ltd

In addition, it was shown that pattern resolution range can be tuned, utilizing objectives with different magnification and numerical aperture (Figure 58A).⁵⁵⁰ Furthermore, the concentration of the immobilized molecule depends on the energy applied and therefore can be controlled by varying laser intensity, scanning speed (Figure 58B) and number of scans.⁵⁵⁰⁻⁵⁵² By controlling the light dose and the step size, smooth gradients can be obtained in the three dimensions (Figure 58C).

The Anseth group has explored another “direct patterning” approach based on radical mediated thiol-ene addition. A copper-free click chemistry reaction was adopted to form PEG hydrogels in the presence of cells under physiological conditions and combined with the orthogonal thiol-ene photocoupling click chemistry. Enzymatically degradable crosslinkers with pendant vinyl functionalities enabled photoimmobilization of cysteine-containing RGD peptides upon light irradiation with high cell viability.⁵⁵³ The system was further optimized with the addition of oNB photodegradable linkers, therefore providing the possibility of controlling the arrangement of biochemical (patterning) and physical (photodegradation) signals with “optical orthogonality”.²⁸ 2P-patterning of fluorescent cysteine-containing RGD peptides was achieved with sub-cellular resolution, which represents a relevant aspect for precise tuning of individual cells microenvironment.

Figure 59. 2P-patterning by thiol-ene conjugation A) 3D orthogonal thiol-ene 2P-patterning and subsequent oNB based removal of pre-patterned cues in PEG based hydrogel (Scale bars: 200 μ m). B) Schematic of 2P-patterning guided cell invasion showing thiol-ene mediated RGD immobilization (Left). Confined human fibroblasts invasion within cysteine-containing RGD patterned Y shape in PVA hydrogel (Right, Scale bars: 100 μ m). C) Sequential allyl sulfide based patterning and release (Scale bars: 100 μ m). A: Adapted with permission from ref 554. Copyright 2011 John Wiley and Sons B: Adapted with permission from ref 29. Copyright 2018 John Wiley and Sons C: Adapted with permission from ref 555 Copyright 2018 American Chemical Society and from ref 556. Copyright 2014 John Wiley and Sons

A similar principle was used to independently control the immobilization of a bioactive motif and its release. By synthesizing the bioactive cue with both a thiol (for thiol-ene reaction) and oNB moiety (for photorelease) the authors achieved a “reversible patterning” controllable with λ -orthogonal photochemistries: 860 nm (patterning) and 740 nm (release) (Figure 59A).⁵⁵⁴ Recently, Qin et al. further demonstrated the potential of thiol-ene photoclick chemistry with fast photopatterning of adhesive peptides in preformed PVA hydrogels by using the efficient 2PI P2CK.²⁹ The resulting exquisite control of multicellular invasion from spheroids of human dermal fibroblasts (HDF) (Figure 59B) suggests that this technology has sufficiently improved its speed, biocompatibility and versatility to become a standard tool for biologists in the near future. Lately, the concept of “reversible patterning” was better captured by the elegant introduction of the addition fragmentation chain transfer agent (AFCT) allyl sulfide.⁵⁵⁵⁻⁵⁵⁶ Allyl sulfide crosslinkers enable reversible addition and release of thiol-containing biological epitopes (Figure 59C). Dynamic changes of cell biochemical surroundings, meaning, i.e., exposure to bioactive signals such as growth factors, were proved to be possible with whole signaling proteins and a high degree of control. Unlike commonly used short adhesion peptides such as RGD, IKVAV and PHSRN, the activity and effect of transient presentation of thiolated TGF- β 1 was assessed.⁵⁵⁵ Certainly, this first study on fully reversible whole protein patterning points toward new applications such as complex *in vitro* dynamic disease models.

Another direct 2P grafting strategy relies on photolysis of aromatic azides with high multi-photon absorption cross section.⁵⁵¹⁻⁵⁵² In this case, a single-molecule insertion mechanism is obtained through the decomposition of azide groups to reactive nitrene species upon two or three-photon irradiation. A second azide group or a terminal alkyne was then exploited as CuAAC sites to immobilize a desired clickable molecule in a localized fashion. With the use of organic solvents to form and pattern the PEG-DA gels, a remarkable writing speed of $\sim$ 550 mm/s was reported. Similar approaches, based on the photografting of a universal reaction substrate followed by immobilization of bioactive cues, were presented by Seidlits et al.⁵⁵⁷ and Skylar-Scott et al.¹⁵ Following the photopatterning of a biotinylated molecule (BSA or fluorescein), NeutrAvidin or Streptavidin was diffused into the gel to then enable the physical binding of a second biotinylated active motif. Notably, Skylar-Scott successfully combined 2P-patterning and 2P-microfabrication within the same system. Demonstration of P-selectin-mediated HL-60 human leukemia cells homing and capturing in 50 μ m collagen microchannels was reported.

In general, the use of potentially toxic acrylate moieties and PIs, long incubation times, and radical generation give rise to concerns about deleterious effects of this patterning strategy on encapsulated cells. In this context, the use of photocages is a “milder” alternative to obtain localized patterns of

bioactive epitopes. Nevertheless, as with any other strategy, advantages and disadvantages exist that will be discussed in more detail in the following section.

Bond Cleavage (Uncaging) in Patterning

Bond degradation upon light exposure offers remote and bioorthogonal control over photosensitive systems. This type of photochemistry has been widely explored for controlled release of neurotransmitters and signaling molecules and, as discussed in Section 4.2.2, to control the stiffness of matrices for 3D cell culture. Photolabile molecules have also been used to protect functional reactive groups. Upon light exposure, the photocage is removed and functional groups such as thiols or amines are rendered available for site-specific reactions. It follows that photouncaging can be exploited for 2P-patterning. In this review, the literature regarding this application is divided into three categories called *covalent*, *physical binding*, and *pre-loaded latent patterning*. While patterning with covalent bonds used direct immobilization through covalent bond formation between the bioactive molecule and the uncaged reactive site, patterning with physical binding uses an indirect approach based on affinity binding pairs. With pre-loaded latent patterning, on the other hand, photocaged bioactive molecules are conjugated to the polymer backbone and activated *in situ* without further reactions.

Covalent patterning: Light-mediated localized uncaging in a 3D hydrogel system was first introduced by Luo et al.⁵⁵⁸ Upon UV laser exposure, an oNB photocage was cleaved, leaving free thiol groups to react with maleimido-modified adhesion peptides. Thiol deprotection and subsequent Michael-addition coupling were extensively adopted and optimized by the Shoichet group in agarose^{145, 559-562} and HA^{20, 563-564} based matrices. To achieve a 2P-sensitive system, coumarin- and dibenzofuran-based protecting groups were used instead of oNB because of their higher efficiency for 2P cleavage. Agarose was covalently modified with 6-bromo-7-hydroxycoumarin (Bhc) sulfide derivatives and these were used to immobilize maleimide-activated vascular endothelial growth factor (VEGF) in concentration gradients and to study their effect on cell activity.^{560, 562, 565} These works demonstrated the usefulness of chemically defined 3D culture systems in investigating the role of GFs in processes such as migration and development. Immobilized VEGF-A was found to be more efficient than soluble VEGF-A in the induction of embryonic stem cells toward blood progenitor cells.⁵⁶⁰ Moreover, it was found that ECs follow the GF gradients forming tubular-like structures.⁵⁶⁵ Later, the co-culture of ECs, retinal stem and progenitor cells (RSPCs) on VEGF-A gradients revealed an unexpected EC inhibitory role on RSPC fate.⁵⁶² 2P-patterning upon thiol deprotection was also studied with furan-modified HA.⁵⁶³

As previously explained in Section 2.3.2, it is important to mention that even if Bhc has been the one of the most frequently adopted photocages for thiol groups, it is not the most efficient. In recent years, it has been shown that Bhc can undergo photoisomerization to a dead-end product (4-methylcoumarin-3-yl thioether)¹⁶² that limits its cleavage yield. 6-bromo-7-hydroxy-3-methyl-coumarin (mBhc)⁵⁶⁴ and NDBF^{20, 162} were then found to lead to a more efficient thiol photodeprotection.^{20, 162}

Figure 60. Strategies for 2P-patterning based on photocleavage followed by covalent bond formation. A) Schematic of 2P-patterning based on photomediated oxime-ligation and subsequent release based on oNB photolabile linker. B) Cycles of 3D patterning of singly functionalized proteins and release based on oxime ligation and oNB cleavage. A: Adapted with permission from ref 163. Copyright 2015 Springer Nature B: Adapted with permission from ref 21. Copyright 2019 Springer Nature

The same principle presented above for thiol caging can be translated to other functional groups, such as amines,⁵⁶⁶ and conjugation chemistries. As an example, DeForest and Tirrell exploited an oxime-ligation chemistry to immobilize full-length proteins (BSA, collagenase, mouse anti-6xHis monoclonal antibody and GF Delta) in a synthetic PEG matrix.¹⁶³ Upon cleavage of low 2P efficient NPPOC photocage, alkoxyamines were rendered available for oxime-ligation with aromatic aldehyde-modified

proteins. Interestingly, the aldehyde was introduced at the terminus of a photocleavable linker (oNB), thus making it possible to release the patterned protein upon a second light exposure (Figure 60A). Mimicking the dynamic nature of native matrices is crucial to capturing the *in vivo* complexity and studying biological phenomena *in vitro*. Notably, the authors used the mentioned strategy to obtain a spatially defined reversible differentiation of human MSCs by the photoreversible immobilization of vitronectin. However, it is important to highlight that the native proteins need to be modified with an aldehyde linker in the latter example,¹⁶³ or with a maleimide-terminated linker for the Michael-type addition patterning described previously.^{560, 562, 565} Therefore, attention should be paid to the bioactivity retention of the derived protein. To this end, Shadish et al. recently used a chemoenzymatic sortase-mediated transpeptidation strategy to install single functional handles on fluorescent proteins, enzymes and GFs.²¹ With this elegant one-step single functionalization and purification strategy named sortase-tag enhanced protein ligation (STEPL), proteins showed a near-native activity. Moreover, by exploiting the NPPOC photodeprotection-oxime-ligation and oNB-based photorelease strategy presented above, the authors achieved 2P-induced reversible patterning of entire functional proteins (Figure 60B). Mild and specific enzyme crosslinking can also be used to directly immobilize the desired molecule if the latter is previously tagged with the appropriate substrate.^{22, 55} By photo-liberating a first substrate immobilized into an MMP-sensitive PEG matrix, Mosiewicz and colleagues were able to pattern recombinant proteins through photo-uncaging and subsequent enzymatic tethering of proteins by FXIIIa.⁵⁵

Another interesting strategy for obtaining a bioorthogonal chemical patterning was developed by McNitt et al.⁹⁶ Cyclopropanone-masked dibenzocyclooxetynes were found to be decarbonylated, and therefore available to a mild and specific click-chemistry conjugation with azide-terminated species, upon two- and three-photon absorption. With a maximum efficiency at 690 nm, 3D photoclick patterning was reported into PAAm hydrogels.⁹⁶

Physical binding patterning: Physical patterning is referred to here as the use of high affinity binding pairs for spatio-temporally defined immobilization of bioactive signals. A first covalent immobilization is still needed to pattern the first member of the binding pair. Then the introduction of the bioactive signal linked to the second binding partner enables the achievement of a site-specific patterning. Since the immobilization of these binding pairs is based on physical interactions, many chemical side-reactions can be avoided. Moreover, the fact that it is performed in purely physiological conditions with no need of catalysts, pH changes or potentially cytotoxic chemicals makes this step fully cytocompatible. In addition, the orthogonality of different binding partners guarantees higher specificity than most of the organic chemistry approaches and can contribute to a final lower background due to unwanted side-reactions. The application of this concept to 2P-patterning was introduced for the first time by Wosnick et al.⁵⁵⁸ Upon localized thiol deprotection, the authors immobilized maleimide-derivatized biotin and then added a fluorescent streptavidin derivative that selectively bound the immobilized biotin.

Figure 61. 2P-patterning based on physical binding. A) Schematic of nitrodibenzofuran (NDBF) uncaging of thiol reactive groups and subsequent streptavidin-biotin based patterning of EGF. The ability to generate EGF gradients in a 3D matrix was exploited to study cancer cell invasion and cetuximab (EGF receptor inhibitor) response. B) Patterning of multiple bioactive signals: CNTF (red) and SHH (green) (Scale bar: 400 μ m). C) Schematic of coumarin uncaging of thiol reactive groups and subsequent albumin-albumin binding domain-based 3D patterning. D) Schematic of Sortase A mediated avidin-biotin patterning strategy (top) and fluorescence imaging showing axon (red) guidance into patterned (blue) regions (middle). The two Sortase A substrates threonine donor peptide (SAT) and glycine donor peptide (SAG) can be ligated upon 2P uncaging of the latter. Biotinylated nerve growth factor (NGF) is immobilized through the binding with avidin bearing SAT. Bottom row shows delayed patterning, with neurons (red) following a first pattern (white dashed line) introduced after 1 day of culture and then a second one (blue dashed line) introduced after 4 days (Scale bars: 50 μ m). A: Adapted with permission from ref 20. Copyright 2018 Elsevier Ltd B: Adapted with permission from ref 561. Copyright 2011 Springer Nature C: Adapted with permission from ref 145. Copyright 2011 American Chemical Society D: Adapted with permission from ref 22. Copyright 2020 John Wiley and Sons

Recently, Fisher et al. took advantage of the highly efficient thiol cage NDBF and immobilized maleimide-streptavidin in a concentration gradient fashion within a HA hydrogel (Figure 61A).²⁰ Adding biotinylated epithelial growth factor (EGF) resulted in a bioactive GF pattern that was used to elucidate its influence on breast cancer cell invasion. Notably, the authors showed that EGF gradients can differentially influence the cellular response to a drug, therefore highlighting the need for and usefulness of such complex drug screening platforms. By the same principle, Wylie et al. developed a system based on barnase-barstar and streptavidin-biotin, two orthogonal physical binding pairs.⁵⁶¹ The maleimide modified binding peptides (barnase and streptavidin) were first patterned sequentially in different regions of an agarose hydrogel. Afterward, fusion proteins barstar-SHH (sonic hedgehog) and biotin-CNTF (ciliary neurotrophic factor) were added together and left to interact with their respective partners. The orthogonality of the binding partners enabled a “one-step” final process which leads to a precise “multi-color” patterning (Figure 61B). Similarly, fibroblast growth factor (FGF2) expressed as an albumin binding domain fusion protein was physically immobilized into an agarose hydrogel previously patterned with human serum albumin (HSA) (Figure 61C).¹⁴⁵

Avidin-biotin affinity pair was also recently exploited to direct the neurite outgrowth of encapsulated dorsal root ganglia (DRG) from chicken embryo in transglutaminase crosslinked hyaluronan matrix²² (Figure 61D). 2P-uncaging of a Sortase A substrate was followed by enzyme-mediated patterning of avidin bearing the second substrate. This, also with biotinylated-NGF binding, were performed as a one-step procedure, therefore circumventing the multiple incubation and washing steps needed in traditional 2P-patterning methods. The use of bacterial transpeptidase Sortase A gives full orthogonality to mammalian matrices and enabled free-shape 3D patterning of NGF in the presence of cells with no

cytotoxicity and negligible background. Broguiere et al. also demonstrated a time-controlled patterning, potentially applicable to any amine-containing matrices, with multiple rounds of growth factor immobilization at different time points following a pre-culture time in non-functionalized matrix.²² As it is probably the best-known example of physical high affinity binding in biological systems, DNA hybridization represents another elegant tool for 2P-patterning. To date, there are only a few examples of work in this direction.⁵⁶⁷⁻⁵⁷⁰ Notably, Becker et al. achieved 3D patterning upon photorelease of NDBF protected oligonucleotides.⁵⁷⁰ Uncaging of immobilized DNA strands allowed the hybridization with a second, complementary fluorescent DNA strand. However, this study did not explore the potential biological implications, such as the possibility to load the second DNA strand with bioactive signals. In addition to this, it is envisioned that the high compatibility and flexibility of DNA/RNA in terms of synthesis, modification, multiple dynamic interactions (e.g., toehold displacement) and capabilities (e.g., DNazymes, Ribozymes, aptamers, miRNA, siRNA) may bring new unexplored solutions into this field.

Pre-loaded latent patterning: Although many different materials have been developed to obtain spatiotemporal control over the presentation of bioactive ligands, none of them was tested *in vivo*. In addition to the complexity and generally high cost synthesis of small amounts of these materials, the major limitation lies in the unavoidably lengthy (30 minutes to days) incubation and washing steps. Notably, to overcome these limitations, Lee et al. “pre-loaded” caged-RGD adhesion peptides in PEG hydrogels with protease-degradable crosslinks.¹⁴⁴ Transdermal injection of this material allowed researchers to temporally and spatially control the presentation of the bioactive signals *in vivo* upon light exposure. Thus, in combination with the controlled release of incorporated VEGF, transdermal upregulation of adhesion, inflammation, and vascularization was shown. Using 3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl ester (DMNPB) as a photolabile cage limited the study to the use of UV light (351 nm) which, as expected, showed a strong attenuation due to tissue absorption and scattering and may raise concerns about photodamage.

Figure 62. Pre-loaded latent 2P-patterning. 2P uncaging of cyclic RGD adhesion peptide in MMP degradable PEG hydrogel (left). Light-guided cell migration from fibroblasts-laden fibrin clot (red) confined in the laser exposed region (yellow dashed box) after 3 days of culture (right) (Scale bar: 50 μ m). Adapted with permission from ref 571. Copyright 2018 John Wiley and Sons

Recently, Farrukh et al. optimized the system by incorporating the efficient 2P cleavable group p-methoxynitrobiphenyl (PMNB).⁵⁷¹ Hence, the release of the photocage with NIR light (740 nm) having high penetration depth and low damage potential was performed in the presence of cells (HUVECs and fibroblasts aggregate) and showed again the potential to direct adhesion, invasion (Figure 62) and angiogenic morphogenesis with spatiotemporal control. To date, the incorporation of caged bioactive ligands represents the closest strategy to *in vivo* 2P-patterning applications. However, it is worth mentioning that this approach appears to be limited to small molecules, since immobilization and photocaging of whole proteins and enzymes without impairing their structure and activity represents a difficult task.

4.2.5 2P-Patterning Limitations and Outlook

The precise positioning of bioactive cues by 2P-patterning offers a unique opportunity for *in vitro* micrometer control of the cells' extracellular environment. Technical developments and increasingly advanced chemical solutions are pushing this approach toward novel applications, both *in vitro* and *in vivo*. However, limitations remain, leaving room for improvement. The main issues of this technique are related to the long incubation and washing steps that normally require hours or days. Hydrogel dimension and porosity, diffusing signal dimension and static or shaking conditions contribute to define the length of these steps. Therefore, the use of relatively large hydrogels and high molecular weight bioactive signals such as proteins leads to an exponential increase in the incubation time. To give an example, for a reaction-diffusion model of BSA into a 1 mm PEG hydrogel, 6.5 h are needed to ensure diffusion into the top 80% of the gel.¹⁶³ Recently, the mixing of patternable signals directly with cells and gel precursor showed that a one-step patterning approach without incubation steps is also possible.²²

In general, being a diffusion-limited process restricts the possibility of patterning multiple cues with multiple cages at orthogonal excitation wavelengths. In this case, by caging different reactive sites with selective cages of different wavelength, one could immobilize multiple bioactive signals without the need for lengthy sequential incubation steps. We expect that the development of such a "multi-color" patterning strategy will strongly enhance the potential use of this technique.

Another aspect that needs to be considered is that immobilizing bioactive signals in a cell-laden matrix necessarily means that an excess of the soluble signal needs to be added in the first place. As a consequence, during this incubation step the encapsulated cells are exposed to an uncontrolled burst of the bioactive signal. Only after several washing steps is the pattern revealed, and the cells can sense its different distribution in their surroundings. In this respect, the "pre-loaded" approach appears to be a valuable solution.

Another problem that may arise from the diffusion of the signal in the matrix is the undesired background as a result of off-target non-specific binding to the matrix which might interfere with the patterned guidance cues. The use of anti-fouling inert polymers such as PEG and alginate helps, as well as the choice of more hydrophilic cages.²² However, choosing the chemical strategy that better fits with these requirements does not necessarily lead to the desired processability. Therefore, it is important to first test and determine the processing window. For each system, the material response is different and needs to be evaluated in terms of scanning speed, laser power, and number of scans (Figure 58). In addition, identifying the processing window is necessary in understanding how to tune the parameters to generate chemical gradients.

In general, the ability to incorporate multiple biochemical signals in relative bio-inert hydrogels will enable elucidation of many crucial biological processes such as chemotaxis, vasculogenesis, and neurite outgrowth in a biomimetic 3D environment. We envision that *in vitro* spatiotemporal manipulation of the cell/organoid environment will contribute to answering fundamental biological questions. The versatility of 2P-patterning also relies on the possibility of forming gradients of bioactive motifs at a scale (micrometers) that potentially enables single cell manipulation. Mimicking the true complexity of healthy and diseased tissues also holds great promise for several other applications, including drug discovery and regenerative medicine. Moreover, while *in vivo* applications remain unexplored, the state-of-the-art material/chemistry/instrument palette makes it a fascinating path to follow in the near future.

5. OUTLOOK

In the previous sections, we have shared our honest perspectives on the strengths and limitations of photochemistries, bioinks/bioresins, and their use in photo-activated biofabrication. The progress in this field has been extensive, and for the first time in science, it is possible to print models of complex and challenging cellular interactions such as those found in the air-blood barrier,⁵⁷² blood-brain barrier,⁴⁶⁶ epithelial-mesenchymal transition,⁵⁷³ models of thrombosis,⁵⁷⁴ and cancer metastasis,⁵⁷⁵ and to achieve continuous mechanical and signaling gradients.³²⁴ Photo-activated materials have played an important role in this progress, and the benefits (and disadvantages) of this approach are summarized in Table 5.

Table 5. Advantages and disadvantages of photo-activated materials in bioprinting.

Advantages	Disadvantages
<ul style="list-style-type: none"> • Spatiotemporal control • Applicable to wide ranges of materials • Mild crosslinking conditions 	<ul style="list-style-type: none"> • Potential cytotoxicity of PIs, radicals and photo-reactive groups

<ul style="list-style-type: none"> • Applicable to all bioprinting techniques • Commercial availability of photo-activated printing materials • Enables physical/chemical gradients by tuning light intensity 	<ul style="list-style-type: none"> • Need for modification of polymers with photo-reactive moieties • Limited penetration depth • Dependency on optical transparency of materials
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Although light-induced approaches have led to substantial advances in the biofabrication of tissue and ECM mimics, key challenges remain. As an example, radical chain-growth photocrosslinking has dominated the field of light-responsive bioprinting, even though the toxicity of radicals, ROS, and PIs might be an issue in biological applications. Nevertheless, novel photoclick chemistry has emerged as a promising, more cell-friendly alternative. Despite its attractive features, the use of photoclick chemistry for bioprinting has been limited to date. Perhaps the most demanding challenge in the development of non-radical based photocrosslinking in bioprinting is the need to expand useful ligation reactions further into red-shifted regions. Visible and NIR light with upconverting nanoparticles will be a useful alternative to chromophore-based red-shifting. Besides using biologically benign light, longer wavelengths allow for greater penetration depths for novel volumetric printing. Moreover, wavelength selective λ -orthogonal photo-activation systems will allow for immobilization of multiple spatiotemporally controlled signals and for investigation of combinatorial effects, which may occur in diverse ECM. In addition, photoswitchable reactions have been widely explored in the last decade and could be used to add an additional level of spatiotemporal control for photo-activated materials. Beside the systems based on small molecules, such as azobenzene and ruthenium complexes, the exploitation of optogenetics proteins such as LOV2-J α (light-oxygen-voltage-sensing domain 2) and PhoCI showed promising results.⁵⁷⁶⁻⁵⁷⁷ An excellent review on this topic, with a focus on visible and NIR light activatable systems, has recently been published by Rapp and DeForest.⁵⁷⁸

Another important consensus from the current field is that highly substituted and highly concentrated photoresins are required for optimal processing parameters (faster processing speed, need of lower energy light source), yet the resulting hydrogels are highly crosslinked and dense with suboptimal biological properties. It is now well accepted that cells need relatively soft and porous permissive matrices to spread better and invade the material and to facilitate diffusion of nutrients and catabolic waste,⁵⁷⁹⁻⁵⁸⁰ as also shown by highly porous granular bioinks^{298-301, 303-304}. Functionalization can negatively influence the bioactivity of a polymer⁵⁸¹ or even determine the emergence of cytotoxicity/irritancy.⁵⁸² The particular photoresins used in the publications of the review have been optimized for fabrication, but not as *ad hoc* cell matrices. An important future direction of the field is to generate bioprinting materials which preserve more of the key biological properties of the unmodified polymers. It is interesting to note that the most significant results with deposition bioprinting have been achieved with non-modified biological polymers (collagen, fibrin, PRP, matrigel).

The major advantages and disadvantages of each of the biofabrication techniques described in this review are listed in Table 6. An important potential downside of orifice-dependent deposition bioprinting is the possible effects of biofabrication-associated stress on phenotypic expression of the cells, with broad changes in gene expression reported.^{330, 332, 382, 386} More *in vivo* evidence is required to judge the impact of processing and chemical modifications of cells on their immunogenicity. Still, deposition printing is technically more compatible with printing multimaterials compared to VP printing, which is critical for fabrication of native tissue analogs with multimaterials and multiple cell types. Despite the difficulty in printing multimaterials at reasonable speed, the VP techniques allow biofabrication of more complex structures at higher resolution compared to deposition techniques. And thus, the VP techniques have high potential for mimicking the highly resolved nature of the ECM.

Table 6. Summary of advantages and limitations of each bioprinting technique

Advantages		Limitations
Deposition	Extrusion	<ul style="list-style-type: none"> • Printing anisotropy • Excellent multimaterial printing • Enable printing clinical-sized grafts
		<ul style="list-style-type: none"> • Shear stress to cells causes potential phenotypic changes • Low resolution • Clogging of nozzles • Layer-layer and line-line defects

	Droplet	<ul style="list-style-type: none"> • Facile generation of HT arrays • Excellent multimaterial printing • Control over the deposition pattern (voxelated printing) • Low cost of printers 	<ul style="list-style-type: none"> • Restricted to low viscosity inks • Clogging of nozzles • Mechanical stress to cells • Not suitable for printing tall structures • Stress to cells causes potential phenotypic changes
VP	Projection VP	<ul style="list-style-type: none"> • Fast printing speed in x-y plane • High resolution • High degree of freedom of printable structures (no need for supports) 	<ul style="list-style-type: none"> • Waste of cells and resins • Slow speed for multimaterial printing • Need to remove unreacted materials
	2P-SL	<ul style="list-style-type: none"> • Sub-cellular resolution • Use of cell-friendly IR light • High resolution patterning of biomolecules 	<ul style="list-style-type: none"> • High instrument cost • Small size of printable objects • Need to remove unreacted materials

Hybrid Bioprinting/Convergence of Biofabrication Techniques

One noticeable trend in the field is the emergence of contactless bioprinting modalities (i.e., acoustic⁵⁸³, ultrasound⁵⁸⁴, magnetic levitation,⁵⁸⁵ and convergence of existing modalities to span length scales and achieve more accurate tissue models. For example, Liu et al. demonstrated that extrusion printing combined with droplet-based printing could provide multiscale control of cells in a gel, allowing for generation of *in vitro* tumor models with controlled relative positions of peripheral cells to cancer cell spheroids within a gel.⁵⁸⁶ A bioink containing the spheroids was firstly printed by an extrusion printer, followed by deposition of single cells next to the spheroids with a resolution of 100 μm . Such constructs could not have been achieved either by an extrusion printer or a droplet-based printer, considering the limitations of each technique (Table 6). In addition, DLP,⁵⁸⁷ extrusion⁵⁸⁸ or inkjet⁵⁸⁹ bioprinting combined with FDM printing have shown good promise in fabrication of mechanically reinforced tissue constructs.

Another hybrid approach that has emerged in recent years is the combination of different laser printing technologies,⁵⁹⁰ such as 2P-SL together with LIFT⁴⁹² or multiphoton ablation.⁴¹⁹ Laser ablation makes use of high laser power (10-100 times the P_{av} commonly used in 2P-SL) to induce optical breakdown in the material which results in permanent structural changes such as the formation of void spaces (subtractive method). Both single-photon ablation⁵⁹¹ and 2P-ablation⁵⁹² have been used to generate vasculature networks in biological matrices. However, 2P-ablation⁵⁹³ appears as a more powerful tool because of the intrinsic 3D confinement given by the non-linear 2PA phenomenon. Recently, Kunwar et al. interestingly reported the use of UV-Vis CLIP in combination with multiphoton ablation and 2P-microfabrication to achieve multiscale and multimaterial structures with construct size ranging from mm to cm and resolution from $\sim 25 \mu\text{m}$ (CLIP) down to $\sim 3 \mu\text{m}$ (multiphoton ablation and 2P-microfabrication).⁴¹⁹ Nevertheless, it is noteworthy to mention that the 2P-SL key limitation of slow point-by-point laser scanning was not overcome by this work, since the 2P processing of a $600 \mu\text{m}^3$ cube in a $\sim 72 \text{ mm}^3$ pyramid construct made by CLIP required 62 min out of 66 min of the total fabrication.

The expanding palette of photo-activated materials shared between various biofabrication techniques represents a relevant strength of this field, because it gives multiple possibilities for hybrid strategies that, to date, have only barely been explored. We envisage that well-designed hybrid bioprinting would allow generation of tissue/organ analogs with highly biomimetic multiscale architecture.

Notes

The authors declare no competing financial interest.

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KEYWORDS

Bioprinting, Biofabrication, Photo-activated materials, Bioinks, Bioresins, Extrusion, Inkjet, Stereolithography, DLP, Two-photon stereolithography, Gel-MA, Hydrogels, Rheology, Photochemistry, Photoinitiators

ABBREVIATIONS

[Ru(II)(bpy) ₃] ²⁺	Tris(2,2'-bipyridyl)-ruthenium(II) chloride hexahydrate
1PI	Single-photon initiator
2PA	Two-photon absorption
2PI	Two-photon initiator
2P-SL	Two-photon SL
3D	Three-dimensional
ADH	Adipic acid dihydrazide
ADSC	Adipose-derived mesenchymal stem cell
AFCT	Addition fragmentation chain transfer
AGE	Allyl glycidyl ether
AHPG	Acrylated hyperbranched polyglycerol
Alg-MA	Alginate-methacrylate
Alg-NB	Alginate-norbornene
Alg-Ph	Phenyl-alginate
AEMA	Amino ethoxy methacrylate
AOM	Acousto-optic modulator
APi-180	2-hydroxy-1-[3-(hydroxymethyl)phenyl]-2-methyl-1-propanone
BAPO	Bisacylphosphine oxides
Bhc	6-bromo-7-hydroxycoumarin
BMP-2	Bone morphogenetic protein 2
BPD	Benzophenone dimer
BSA	Bovine serum albumin
CAD	Computer-aided design
CJP	Continuous jetting printing
CLIP	Continuous liquid interface printing
CMC	Carboxymethylcellulose

Col-MA	Collagen-methacrylate
DAS	Diazosulfonate initiator
dECM	Decellularized ECM
DF	Degree of functionalization
dHTz	Dihydropyridazine
DIW	Direct ink writing
DLP	Digital light processing
DMD	Digital micromirror device
DMNPB	3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl ester
DOD	Drop-on-demand
DRG	Dorsal root ganglia
DTT	Dithiothreitol
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epithelial growth factor
EHD	Electrohydrodynamic
EOM	Electro-optic modulator
FDA	Food and drug administration
FDM	Fused deposition modelling
FGF2	Fibroblast growth factor 2
FOV	Field of view
FXIIIa	Transglutaminase factor XIIIa
G	α -L-guluronic acid
GAGs	Glycosaminoglycans
GC	Glycol chitosan
Gel-AGE	Gelatin-allyl glycidyl ether
Gel-MA	Gelatin-methacryloyl
Gel-NB	Gel-norbornene
Gel-VE	Gel-vinyl ester
GF	Growth factor
GH-VE	Gelatin hydrolysate vinyl ester
GM	Göppert-Mayer
GRAS	General recognized as safe
H&E	Hematoxylin and eosin

HA	Hyaluronic acid
HA-MA	HA-methacrylate
HA-NB	HA-norbornene
HAPI	Hyaluronan-photoinitiator
HA-VE	HA-vinyl ester
HEMA	2-hydroxyethyl methacrylate
HMVEC	Human dermal microvascular endothelial cell
HRP	Horseradish peroxidase
HSA	Human serum albumin
HT	High-throughput
HUVEC	Human umbilical vascular endothelial cells
I2959	α -hydroxyketone 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone
IC ₅₀	Half maximal inhibitory concentration
iPSC	Induced pluripotent stem cell
IR	Infrared
ISC	Intersystem crossing
LAP	Phenyl(2,4,6-trimethylbenzoyl)phosphinate salts
LC ₅₀	Median lethal dose
LCD	Liquid crystal display
LIFT	Laser-induced forward transfer
LOV2	Light-oxygen-voltage-sensing domain 2
LOV2-J α	Light, oxygen and voltage domain-J alpha-helix
M	β -D mannuronic acid
MAA	Methacrylic anhydride
maleimide-VEGF	Maleimide activated vascular endothelial growth factor
MAP	Microporous annealed particle
MAPO	Monoacrylphosphine oxides
mBhc	6-bromo-7-hydroxy-3-methyl-coumarin
MC3T3	Mouse calvaria-derived preosteoblasts cells
MEW	Melt electro-writing
MG63	Human osteosarcoma cells
MLC-2v	Myosin regulatory light 2
MMP	Matrix metalloproteinase

mPDLLA	Methacrylated poly-D,L-lactic acid
MSC	Mesenchymal stem cell
NA	Numerical aperture
NDBF	Nitrodibenzofuran
NIR	Near-infrared
NITEC	Nitrile imine-mediated tetrazole-ene cycloaddition
NLT	Nonlinear transmission
NPPOC	2-(2-nitrophenyl)propyloxycarbonyl
NSC	Neural stem cell
NVP	<i>N</i> -Vinylpyrrolidone
o-Cl-HABI	Bis[2-(ochlorophenyl)-4,5-diphenylimidazole]
OHA	Oxidized hyaluronic acid
oNB	Ortho-nitrobenzyl
PAAm	Polyacrylamide
PCL	Polycaprolactone
PEDOT/PSS	Poly(3,4-ethylenedioxythiophene)/poly(styrenesulfonate)
PEG	Polyethylene glycol
PEG-DA	PEG-diacrylate
PEG-DMA	PEG-dimethacrylate
PEG-MA	PEG-methacrylate
PEO	Poly(ethylene oxide)
PF127	Pluronic F127
Ph	Phenolic hydroxyl
PI	Photoinitiator
PIL	Poly(ionic liquid)
PLA	Poly lactide
PMNB	p-methoxynitrobiphenyl
PPG	Photoremovable protecting group
PVA	Poly(vinyl alcohol)
PVA-A	PVA-acrylate
PVA-MA	PVA-methacrylate
PVA-NB	PVA-norbornene
RGD	Arg-Gly-Asp

ROS	Reactive oxygen species
RSPC	Retinal stem and progenitor cell
SE	Skin equivalent
SL	Stereolithography
SLM	Spatial light modulator
SPAAC	Strain-promoted azide–alkyne cycloaddition
STEPL	Sortase-tag enhanced protein ligation
TEA	Triethanolamine
TGF- β 1	Transforming growth factor beta 1
UCST	Upper critical solution temperature
VA-086	2,2'-Azobis[2-methyl-N-(2-hydroxyethyl)propionamide]
VHM	Variable height micromixer
VP	Vat polymerization
vSMC	Vascular smooth muscle-like cell
WSPI	1,4-Bis(4'-(N,N-bis(6'-(N,N-trimethylammonium)hexyl)amino)styryl)-2,5-dicyanobenzene tetraiodide

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Mihyun Lee studied chemical and biomolecular engineering at the Korea Advanced Institute of Sciences and Technology (KAIST, South Korea). In 2010, she moved to the Department of Chemistry in the same university and started her PhD under the supervision of Prof. Haeshin Lee. During her PhD, she studied mussel-inspired dopamine chemistry in depth and surface functionalization for stem cell engineering and biomedical interfaces. She earned her PhD degree in 2015, which was followed by 8 months of postdoctoral research in natural flavonoids-based drug formulation in the same group. Afterwards, she

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František Surman studied polymer materials science and engineering at the Faculty of Chemistry, the Brno University of Technology in the Czech Republic. During the graduation studies, he has been focused on the development of affinity biosensors based on hydrophilic polymer brushes, surface-initiated radical polymerizations, and surface characterization under the supervision of Dr. Zdenka Sedlakova. He received his Ph.D. in macromolecular chemistry in 2018 at the University of Chemistry and Technology in Prague and Institute of Macromolecular Chemistry of the Czech Academy of Sciences. He is currently a postdoctoral researcher at ETH Zürich in Switzerland in the Tissue Engineering and Biofabrication group led by Prof. Marcy Zenobi-Wong. His research interest involves the development of novel bioinks for two-photon polymerization.

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