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Executive summary

Bioprinting

- There are a number of parameters that should be considered before undertaking a bioprinting project: cell viability, resolution, print speed, and 3D range.
- Bioprinting is advantageous to scaffold-based tissue engineering due to precise placement of cells of multiple lineages.
- Bioprinting presents a highly-reproducible method to create complex tissue-engineered constructs.

Bioprinting approaches

- Four major bioprinting approaches and their applications are discussed:
    - Inkjet bioprinters are fast and inexpensive with high resolution, but are limited to low-viscosity bioinks and low cell-densities due to droplet-formation requirements.
    - Laser-assisted bioprinters have extremely high in-plane resolution, but are limited in the z-dimension. They are nozzle free, and are therefore able to print with high-viscosity fluids.
    - Extrusion-based bioprinters are inexpensive, and are especially suited to printing larger-scale constructs. They are able to print most gels, and are highly adaptable.
    - Stereolithographic bioprinting is not limited by bioink viscosities as there is no ejection, but currently they are only able to print one bioink at a time. Due to the use of light to crosslink, high-resolution prints may be achieved in all dimensions. Care must be taken to avoid cytotoxicity arising from UV-light exposure and photoinitiators.

Bioink design

- Discussion of popular bioinks and their applications:
    - Hydrogel-based bioinks are popular due to their biocompatibility; their porosity allows excellent oxygen and nutrient perfusion, and their morphologies are similar to that of natural extracellular matrices. They are typically easily extrudable and may be crosslinked under mild conditions.

- Natural biopolymers such as collagen and gelatin may be used, but are more immunogenic than hydrogel-based bioinks. However, they present biochemical cues for processes such as adhesion, migration, and proliferation.
- Composite bioinks may be used to combine benefits of different gels:
    - Pluronic F127 composites produce a thermally reversible gelation. This may be used to enhance printing of the hydrogel before being removed after crosslinking of other components. The shear thinning behaviour of Pluronic F127 may also be beneficial.
    - Alginate composites are easily and stably crosslinked under mild conditions with divalent metal ions such as calcium or barium.

Challenges and future directions

- Production of complex, hierarchical tissues with native-like mechanical properties is still problematic, in part due to the difficulty associated with vascularising tissues.
- Adaptations of assays standardised for 2D tissue constructs can lead to spurious results. New techniques such as micro-CT and multiphoton microscopy must be adopted by the bioprinting community to produce comparable results.
- The catalogue of cell-compatible bioinks needs to be expanded to address properties beyond viability and proliferation such as adhesion and migration.

1. Bioprinting

Bioprinting is emerging as the premier technique for the construction of complex 3D tissue-like architectures. To a large extent, the expeditious progress in bioprinting can be reconciled by the strong coupling of the technique with advances in automated spatial control systems and rapid prototyping. Moreover, the foundation science developed for 2D printing (e.g. inkjet, laser) has provided the inspiration for several analogous approaches in bioprinting. These approaches have relative advantages and disadvantages, which can be parameterised by considering several key factors, including cell viability, resolution, print speed, and 3D range. Accordingly, the new user must carefully consider which technique is most appropriate for their target tissue system. For example, approaches that favour high in-plane resolution are likely to be most appropriate for structures such as skin or membranes. Conversely, high-resolution–slow-printing techniques may favour situations where the cells are extremely precious.

Scaffold-based tissue engineering allows the crude geometry of a tissue construct to be defined and cell–biomaterial interactions to established, but it does not provide a mechanism for the precise placement of cells for the generation of complex hierarchical tissue, nor does it allow for the rapid assembly of macroscopic tissue morphologies. Conversely, bioprinting provides the opportunity for the fine tuning of structure and the addition of multiple cell lineages and other components in pre-determined sites in the same construct without using scaffolds.[1] Perhaps the most exciting potential application of bioprinting is the engineering of tissue and organs. Here, the high throughput, flexible bottom-up approach provides a mechanism to fabricate 3D cell patterns and tissue-like structures with multiple cell types.[2] Moreover, bioprinting provides a possible future solution to the problems associated with creating large vascularised tissues and organs. Previous methods of vascularisation have focussed on a single approach, such as scaffolds, cells, or growth factors, with limited success.[3] Printing directly with cells allows complex vascular structures such as veins to be printed simultaneously with the rest of the surrounding tissue construct.[2]

In bioprinting, living cells, biomaterials and associated biochemical cues are precisely positioned layer-by-layer to fabricate 3D tissue structures. Spatial control of the functional components is of the upmost importance when attempting to recapitulate functional tissues with *in vivo*-like biological function and mechanical properties. Moreover, this automated approach offers a new way of producing highly scalable and reproducible tissue-engineered

constructs, which are likely to pave the way for the development of new organ-on-chip models for big pharma. However, there are still major challenges associated with 3D bioprinting that must be overcome, such as the ability to adapt printing technologies designed to fabricate with plastic and metals for the printing of sensitive cells and biomaterials. To this end, new hydrogel-based bioinks are currently being developed, which support cell growth, and can be extruded to fabricate complex stable 3D structures. Perhaps the greatest challenge in the bioprinting field, and indeed the tissue engineering field, is the reproduction of the sophisticated, hierarchical cell and extracellular matrix (ECM) structures in organs; this has been extensively reviewed by Murphy and Atala,[4] and Kim *et al*.[5] Although this holy grail of regenerative engineering appears to be a long way off, it is likely that bioprinting will feature heavily in the eventual realisation of this ultimate goal.

In this review, we summarise the current state-of-the-art in bioprinting through a description of the various approaches to bioprinting, both conceptual and technical. In doing so, we highlight advantages and disadvantages of the various techniques, which should inform the reader who is new to the field. This is followed by a brief discussion centred predominantly on composite hydrogel-based bioinks, looking at both naturally derived and synthetic gelling agents. We conclude with a look at the major challenges still ahead for the broader biofabrication community.

2. Bioprinting approaches

2.1 Conceptual differences

There are several different conceptual bioprinting approaches that fall broadly into three categories: biomimicry, directed-assembly and tissue building block construction (Fig. 1) In biomimicry, the bioprinting process aims to reproduce the cellular and ECM components of tissue. In practice, this is achieved by reproducing specific 3D structures and cellular components that simulate the target tissue.[6] For example, a number of approaches seek to mimic the branching networks of vasculature, or by recreating growth factor gradients to generate the cartilage to bone transition.[7] Here, to accurately mimic the tissue, the microenvironment, including placement of cells, mechanical forces, gradients of biomolecules and ECM composition needs to be carefully considered, so that the tissue can be replicated on the microscale.[4]

Directed-assembly approaches use embryonic development as a guide, wherein self-assembling cellular spheroids that undergo cellular organisation are fabricated.[8] These cell

aggregates already display appropriate cell signals and biomolecule secretions such that the cells organise and fuse, mimicking developing tissues.[9] This approach relies on the inbuilt programming of the cells to drive the self-organisation of the tissue, meaning that an in-depth knowledge of developmental mechanisms of embryonic tissue is required.[10]

Finally, tissue building block construction involves assembly of the smallest structural functional components of tissues, for examples the nephron in the kidney.[11] These building blocks can then be assembled into larger structures by placement, fusion or both.[12] One approach is the self-assembly of cell spheroids, which are printed next to each other and fused to create a macro-tissue.[13] These have been used to create vasculature by fusing spheroids in branched vascular networks, and to create organ-on-a-chip models.[14]

2.2 Bioprinting Techniques

The four predominant techniques used for bioprinting are inkjet, extrusion, stereolithography, and laser-assisted printing (Fig. 2). As discussed above, each has advantages and disadvantages with respect to resolution, speed, cell viability and print height.

2.2.1 Inkjet Bioprinting

Inkjet-based bioprinting was developed in the early 2000s and was one of the first approaches used for biofabrication.[15] The first inkjet bioprinters were modified desktop 2D ink-based printers, where the ink cartridge was simply replaced with a cartridge containing cells and biomaterials (bioink).[16] Here, the bioink was expelled from a noncontact nozzle, using thermal pressure or piezoelectric pulses to create a droplet (Fig. 3). In general, inkjet-based bioprinters have a high resolution, low cost and high speed. A variety of droplet sizes have been printed, from less than 1 pL to more than 200 pL in volume,[17, 18] with rates of 1 – 10,000 drops per second.[19] Moreover, single droplet printing has produced bioink line widths of less than 50 μm.[20]

Thermal inkjet bioprinters use heat to generate a pressure pulse that forces droplets from the nozzle, where the localised heating does not significantly affect viability, and the printers have a high print speed, low cost and are extremely versatile.[21] However, there are a number of disadvantages, including non-uniform droplet size, fouling of the nozzle and incomplete cell encapsulation. Piezoelectric inkjet printers create acoustic waves inside the nozzle to eject droplets, where the parameters such as pulse, amplitude and wave duration can be controlled to change the size and rate of droplet ejection.[22] Shear stress can be reduced by using an open-pool system without a nozzle, reducing the loss of viability and preventing nozzle

clogging.[19] However, the frequencies used by piezoelectric inkjet printers have the potential to damage cells and the printers have a limited number of materials that can be used as a bioink, due to the requirement for low viscosity fluids.[23]

Examples of inkjet bioprinting include *in situ* approaches where fibroblasts and chondrocytes were printed directly into skin or cartilage wounds and the bioink was subsequently crosslinked *via* chemical or photo-initiated crosslinking.[24, 25] Inkjet bioprinting has been used to print bone constructs, which were grown *in vitro* before being implanted into mice. The bone constructs matured *in vivo* and produced tissue that was highly mineralised with similar density to natural bone tissue.[26] Inkjet printing has also been combined with electrospinning to make layered cartilage constructs that support cell function and have composite mechanical and structural properties.[7]

In general, the bioinks for inkjet bioprinting need to be low viscosity liquids to allow microdroplet formation, which can be subsequently crosslinked or solidified to form 3D structures.[23] This somewhat limits the selection of bioinks that can be used. Moreover, chemical or light-induced crosslinking can reduce the overall print velocity and can be cytotoxic.[27] Another limitation of inkjet bioprinting is the low cell density (less than 10 million cells mL^{-1}), as increasing cell density to limits approaching biologically relevant cell densities inhibits droplet nucleation.[21]

2.2.2 Laser-assisted Bioprinting

Laser-assisted bioprinting (LAB) is modelled from laser-induced forward transfer, which was initially developed to print metals.[28] This is a less common form of bioprinting as the high cost of these systems is prohibitive for basic tissue engineering research.[4] LAB uses a pulsed laser beam which is focused on a donor support, usually made from glass, covered with an energy absorbing layer and a biological material layer. The laser pulses on the absorbing layer (gold or titanium) and a high-pressure bubble is nucleated that propels the biomaterial and cell layer towards the collector substrate. The process can deposit cell densities of around 10^8 cells mL^{-1}.[29] High resolutions approaching single cells per droplet can be achieved with high deposition speeds of 1,600 mm per second.[30] This method is also nozzle-free, which enables the usage of high-viscosity bioinks.

LAB has been used to print skin constructs, depositing clinically relevant cell densities in layers, however it remains to be seen whether the system can be scaled to larger tissue sizes.[31] LAB has also been used *in situ* to print nanoparticles of hydroxyapatite in a 3 mm

wide, 600 μm deep, mouse calvaria defect model.[32] Mammalian cells have been printed as 50–100 μm stacks or as individual cells on a hydrogel substrate[33] and laser-based bioprinting has been used to make a cardiac patch of polyester urethane urea seeded with MSCs and human umbilical vein endothelial cells.[34]

Although LAB has an extremely high in-plane resolution, there are a number of disadvantages. For example, the heat from the laser energy may damage cells and affect their functionality if printing parameters are sub-optimal.[35] Accordingly, the cell viability in LAB is generally lower when compared to inkjet or extrusion-based bioprinting.[36] Moreover, LAB is severely limited in the third dimension, which defines the height of the bioprinted construct.[37] The preparation of the donor substrate, which often needs to be changed for each cell or hydrogel type, is time consuming.[4] It can also be difficult to accurately position cells on the collector substrate. Finally, there are often metallic residues present in the printed construct arising from vaporisation of the metallic absorption layer.[38]

2.2.3 Extrusion Bioprinting

Current bioprinting methods such as ink jet and laser-induced forward transfer (LIFT) often damage the cells[39] and have not yet been used to successfully produce large 3D structures.[40] Extrusion-based bioprinting is ideally suited to the fabrication of large tissue constructs. For example, in 2013 Campos *et al.* printed into a high-density hydrophobic fluid, which allowed an almost unlimited number of printed layers, resulting in reproducible and high fidelity structures.[40] Extrusion systems are also the most cost effective bioprinters, and accordingly, academic institutions are increasingly using extrusion bioprinting technology in tissue engineering research.[41] Extrusion printers use pneumatic or mechanical pressure to deposit continuous filaments of materials rather than individual droplets, building up 3D structures layer-by-layer.[4] Mechanically-aided extrusion using a piston or screw-drive system generally provides high levels of control of the bioink flow through the nozzle, which is essential for bioinks with high viscosities.[42] However, in certain cases, mechanical systems can generate large backpressures along the nozzle, which can be harmful to cells.[43] Pneumatic systems use air pressure to dispense the bioink, which can be adjusted to suit a range of viscosities. Structural integrity of extruded bioink structures are generally better than the other techniques, due to deposition of continuous filaments, and physiological cell densities have been used to bioprint tissue-like structures.[35] It is therefore considered the most convenient, inexpensive and facile technique for bioprinting 3D cell-laden constructs.

Extrusion bioprinting has been used to deposit hepatocytes and adipose-derived stromal cells in gelatin/chitosan hydrogels to engineer liver tissue constructs.[44] Here, channels were created throughout the constructs to allow the delivery of nutrients and removal of waste from the cells. These bioprinted structures retained structural integrity for over 2 months in culture, with a cell survival rate of over 95%. Sun and colleagues printed rat heart endothelial cells in an alginate hydrogel that were able to proliferate six fold to infill the hydrogel scaffold.[45] Perhaps the most exciting application of extrusion bioprinting is the fabrication of branching vascular networks. Forgacs and colleagues achieved this by extruding multicellular spheroids, layer-by-layer, with supporting agarose rods.[46] The spheroids fused post printing into double-layered vasculature tubes of 0.9 to 2.5 mm in diameter (Fig. 5).

Vasculature bioprinting has also been achieved using fugitive inks, which can be removed by reverse temperature-dependent crosslinking, leaving behind a channel that can be seeded with endothelial cells.[47] Alternatively, coaxial needles can be used to print vasculature by printing alginate-based, endothelial cell-seeded bioink through the outer nozzle, and a calcium-containing solution or fugitive ink through the inner nozzle.[48] Lewis and co-workers developed a fugitive Pluronic F127 (F127) ink that was used to print vasculature networks that were embedded in a gelatin methacrylate (GelMA) bulk matrix containing human neonatal dermal fibroblasts (HNDFs). The F127 was then removed by cooling the constructs below 4°C and the open channels were seeded with human umbilical vein endothelial cells (HUVECs). In 2016, the Lewis group took this one step further by bioprinting renal proximal tubules on chips.[49] These open lumens were seeded with proximal tubule epithelial cells (PTECs) and after being perfused with medium, formed a tissue-like polarized epithelium. Other groups have demonstrated angiogenesis within fibrin networks by sprouting endothelial cells.[50, 51] These techniques should enable media exchange systems to be developed and allow thicker tissues to be fabricated in the future. Another considerable advantage to extrusion-based bioprinting is the range of compatible materials and viscosities. However, the identification of appropriate materials and bioinks then becomes a major challenge, which is discussed below.

2.2.4 Stereolithographic bioprinting

Stereolithographic (SLA) and projection stereolithographic (PSL) bioprinting use photocurable bioinks to form constructs *via* exposure to either lasers or projections (PSL). These systems can print structures with 50 μm resolution,[52] and produce constructs with >90% cell viability.[53] PSL technology allows a complete layer to be printed at once, and so print times depend only on layer thickness.[52]

Bioink formulations for SLA bioprinting require a photoinitiator to produce free radicals for polymerisation, and suitable monomers. Many photoinitiators are cytotoxic, particularly due to the production of reactive oxygen species. Although 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959) has been shown to cause minimal cell death across several cell types,[54] the ultraviolet (UV) light irradiation required for crosslinking can have adverse effects on viability and proliferation, and can cause DNA damage.[55] Accordingly, Wang *et al.* developed a visible-light sensitive photocurable bioink containing 2′,4′,5′,7′-tetrabromofluorescein disodium salt (eosin y) as a photoinitiator (Abs_{max} @ 517 nm) to mitigate this risk.[52]

Methacrylated polymers frequently used to generate crosslinkable hydrogels include alginate methacrylate,[56] GelMA,[57] and hyaluronic acid methacrylate (HA–MA).[58] Here, the degree of methacrylation can be tuned to alter the mechanical properties of the crosslinked hydrogel.[56, 57]

A major advantage of SLA is that the print quality is to a large extent unaffected by the rheological properties of the bioink as there is no extrusion and the printed structures are supported by the surrounding bioink reservoir. However, this makes multi-material SLA printing almost impossible within a single process, limiting the ability to print complex tissues. It is possible to perform sequential prints with different bioinks, as demonstrated by Zhu *et al.* who produced prevascularised tissue contracts.[59]

3. Bioink design

The development of suitable bioinks remains one of the most challenging aspects of the bioprinting field. Here, cells must be suspended within a fluid to form a bioink, which needs to fulfil multiple requirements. The bioink must supply sufficient biological cues and mechanical support for the cells, but must also meet the physical and mechanical demands to complete the bioprinting process, known as ‘printability’.[35] Often these requirements are in conflict, as a material that can be extruded from a nozzle and undergo a transition to form a solid 3D structure, may not be cytocompatible. Moreover, different cell types may be more or less sensitive to constituents in the bioink formulation, which can create problems when more than one cell type is being used in the same construct.

3.1 Hydrogel-based bioinks

Hydrogels are excellent candidates for 3D cell culture and bioink design. They exhibit similar morphologies to some ECMs (soft to medium tissue) and have tuneable mechanical properties.

Perhaps most importantly, hydrogels are stabilised by networks of water-swollen polymers that can be crosslinked by either covalent or non-covalent intra- and intermolecular attractions.[60] The ability to absorb large quantities of water (99% w/w), coupled with their porous network structure, make hydrogels an extremely attractive class of materials for regenerative engineering.[61, 62] Moreover, hydrogels are largely biocompatible, with high oxygen and nutrient permeability making them ideal for cell encapsulation. Hydrogel materials are also injectable/extrudable, and many of the crosslinking methodologies are mild enough to be carried out in the presence of living cells.[63] Hydrogels can be biodegraded in a controlled manner, and properties such as swelling and crosslinking can be influenced by their environment, for example through changes in temperature, ionic strength, pH, and shear.

Generally, a hydrogel bioink will have a temporal crosslinking mechanism such that a self-supporting structure can be built up layer-by-layer. Moreover, it must be biocompatible, not elicit an immune response, have short and long term stability, and have the ability to be remodelled by cellular processes. The bioink should also support cell function, and in some cases, it is beneficial to have material constituents that support cellular attachment and proliferation.[4] Many hydrogel bioinks are shear-thinning, allowing them to be extruded through a nozzle, yet hold their shape in a 3D structure.

3.2 Natural biopolymer components for hydrogel bioinks

Biogenic polymers have long been used to synthesise hydrogels for tissue engineering, due to their inherent biocompatibility and functionality. Hydrogels have been constructed from proteins, polysaccharides and DNA, however, their widespread adoption is somewhat restricted due to immunogenicity and poor mechanical properties. Some of the most commonly used proteins in hydrogels for tissue engineering are collagen and gelatin. Collagen is biodegradable and abundant in mammalian tissue, meaning it can be sourced readily.[64] Gelatin is a denatured form of collagen, is less immunogenic, and promotes cellular adhesion, migration and proliferation.[57] Another commonly used hydrogel for tissue engineering is Matrigel, a gelatinous protein hydrogel secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells.[65] Matrigel is composed of laminin and collagen type IV, as well as many other proteins and growth factors, and it is widely used as a 3D scaffolding material due to its wide spectrum cytocompatability.[66] However, the complex and somewhat poorly defined composition of Matrigel can introduce variability in experimental results.[67]

Polysaccharides such as hyaluronic acid (HA), heparin, chitosan, and alginate have been used to produce hydrogels for tissue engineering.[68, 69] HA is an ECM component made from glycosaminoglycans (GAGs), which is a major constituent of connective tissue, and it plays a vital role in processes such as nutrient diffusion, hydration and cell differentiation. However, HA structures often have to be modified, either through coating with cell-adhesive biomolecules or by treating with UV light, to increase cell attachment due to the smooth surface and anionic charge.[70] In general, polysaccharides are attractive due to their chemical addressability, where functional groups such as acrylate and amines can be easily and efficiently introduced, which can drive hydrogel formation and provide sites for bioconjugate chemistry.[71] In addition, polysaccharide hydrogels can be combined with proteins such as collagen[72] and fibrin[73] to form composite hydrogels, with composite mechanical properties.

A variety of different hydrogels based on constituents derived from eukaryotes are used in extrusion bioprinting, including Matrigel, collagen I,[1] and synthesised gels such as GelMA.[47] By utilising these naturally occurring gelation agents, it is possible to generate microenvironments displaying many copies of ECM specific sequences. For example, collagen forms a hydrogel at physiological temperatures and pH by triple helix formation, and has RGD sequences that stimulate cell adhesion.[74] Conversely, gelatin has a partially unfolded triple helix, which imparts thermo-responsive properties, forming a gel at around 30°C.[75] Hyaluronic acid is a glycosaminoglycan found in the ECM, which can be modified to introduce crosslinkable moieties.[76] Generally, bioinks constituted from natural polymers are preferred, due to their inherent biocompatibility, however synthetic polymers such as poly(ethylene) glycol (PEG) are also used in bioprinting, and can be modified with binding sequences, such as RGD, to increase cellular adhesion.[77]

3.3 Composite, hybrid bioinks

This composite or hybrid approach to gel formation represents a promising new way to improve the printability and persistence of bioinks without sacrificing biocompatibility.[78] In general, high-viscosity gels provide the printed construct with structural support and low-viscosity gels provide a better environment for cell viability and function.[4] Accordingly, a variety of composite bioinks have been reported, including collagen/fibrin gels and alginate/gelatin gels.[79] Composite gels can either be deposited on top of one another, often to induce crosslinking, or mixed together before printing. Li *et al.* developed a two-nozzle inkjet-based bioprinter that combined two DNA-based hydrogels.[80] One hydrogel was a polypeptide-

DNA conjugate and the second was double stranded DNA with sticky ends, the sticky ends being complementary to the DNA polypeptide sequences of the first bioink. This meant when the two bioinks were printed on top of one another the solution crosslinked, cells were encapsulated and were viable after 48 hours. Rutz *et al.* used a PEGX crosslinker with gelatin, fibrinogen, GelMA, PEG amine and other gels, to create a range of bioink formulations.[81] Gels that were strongly crosslinked were unable to be extruded, but softer gels could be built up into hydrogel constructs. PEGX crosslinking allowed properties of the bioink to be controlled, such as viscosity and biodegradability.

The combination of hydrogels to create a composite bioink can confer useful properties from both hydrogels. Markstedt *et al.* combined shear-thinning properties of nanofibrillated cellulose with fast crosslinking of alginate and the resultant hydrogel printed constructs were stable over 7 days.[82] A composite alginate, gelatin and hydroxyapatite hydrogel was shown to have both instantaneous and long-term structural stability, conferred by a two-step printing mechanism combining the thermosensitive response of gelatin with the alginate chemical crosslinking.[83] The addition of gelatin increased the viscosity of the bioink and improved print fidelity. HA–MA and thermosensitive polymer poly(N-isopropylacrylamide) (pNIPAAM) have been combined to increase mechanical integrity.[58] HA–MA has a low viscosity and cannot be printed by itself, therefore pNIPAAM was conjugated to HA–MA to make HA–pNIPAAM, which acted as a thermoresponsive structural support, while the HA–MA could be crosslinked, after which the HA–pNIPAAM was washed away.

3.3.1 F127 composite bioink systems

Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO; Pluronic®) copolymers form temperature responsive materials that have been widely studied for biomedical applications.[84] Pluronics or poloxamers are water soluble, biocompatible and certain molecular weights are FDA approved.[85] F127 is one such poloxamer, which when dissolved in water at high concentrations (≥16 wt%), exhibits a temperature dependent sol–gel transition at physiological temperatures.[86] The sol–gel transition results from the formation of nanoscale micelles *via* hydrophobic interactions, which are closely packed when the temperature rises above the low critical solution temperature (LCST), resulting in a change of rheological properties. At low temperatures the block copolymers exist as dissolved monomers in solution, but self-assemble into micelles under conditions defined by the critical micelle temperature (CMT) at constant concentration and critical micelle concentration (CMC) at

constant temperature. This reversible sol–gel transition, along with the micelles ability to solubilise hydrophobic solutes, has generated interest in using F127 for injectable carriers for drug delivery.[87]

Although F127 is biocompatible and non-cytotoxic, it is known to be poorly adhesive to cells, and has to be modified with anionic polymers such as poly(acrylic acid) (PAA) to improve cellular adhesion.[84] Pluronic hydrogels also show low mechanical strength, rapid degradation and fast drug release *in vivo,* due to dilution of the hydrogel and therefore lowering of the concentration to below the CMC.[86] Therefore, chemical modifications or combination with other polymers and hydrogels is needed to enhance stability and adhesion properties.

F127 has received recent renewed attention as a bioink constituent due to its predictable temperature-dependent sol–gel transition. As discussed above, Lewis and colleagues used it as a fugitive ink to templates vessel structures, before washing away.[47] Armstrong *et al.* used F127 as a fugitive component in their bioink to produce microporous structures capable of producing bone and cartilage tissues.[88] Kang *et al.* used a multi-head bioprinter to fabricate an interwoven scaffold of polycaprolactone (PCL) and F127, where the F127 acted as a sacrificial material.[89] Here, the bioink was also printed at the same time, comprising gelatin, fibrinogen, HA and glycerol. Gelatin was included to improve the viscosity of the bioink, whereas fibrinogen afforded good cell adhesion. The fibrinogen was crosslinked using thrombin before the F127, gelatin, HA and glycerol were removed by washing. F127 has also been used in bioprinting for cardiovascular applications, mixed with collagen I to enable a double gelling system.[43]

F127 exhibits good shear thinning behaviour, which is advantageous for extrusion bioprinting, due to shear-induced back pressures. Pluronic hydrogels have also been shown to produce nanostructures when combined with other hydrogels, such as PEG-Fibrinogen, which increases the storage modulus of the hydrogels.[90, 91] Muller *et al.* used this property to combine diacrylated Pluronic F127 (PF127-DA) with F127 at a high concentration to print with chondrocytes.[92] After bioprinting and photoinitiated crosslinking of the PF127-DA, the F127 component was washed away leaving behind a nanoporous hydrogel. Interestingly, they found that the F127 sterically hindered photoinduced crosslinking of other hydrogel combinations, such as HA–MA. Cells were viable for 14 days in the bioink, however the hydrogel was still relatively weak, with a compressive modulus of just 1.5 kPa.

3.3.2 Alginate as a bioink constituent

Alginate is a naturally occurring polysaccharide normally obtained from brown algae which has been used extensively in many industries, such as food, textiles and pharmaceuticals, because of its biocompatibility, low cost and mild gelation.[93] Alginate consists of linear block copolymers of (1,4)-linked β-D-mannuronate (M) and α-L-guluronate (G) residues, which participate in crosslinking with divalent cations, such as Ca^{2+}, and form hydrogels.[94] This gentle ionic crosslinking makes alginate hydrogels cytocompatible, and the gelation is reversible, meaning cell recovery is possible. A range of divalent cations can be used to crosslink alginate (calcium, magnesium, barium) and although it can be covalently crosslinked, most approaches use ionic crosslinking.[93] The most popular method of crosslinking involves the addition of using calcium chloride ($CaCl_2$), which can give rise to limited long-term stability in aqueous media. Alternatively, immersion-bioprinting of alginate into a $CaCl_2$ bath causes the alginate to crosslink immediately upon extrusion, allowing the printing of overhanging structures. Hinton *et al.* printed into a bath of $CaCl_2$-doped gelatin slurry to crosslink the alginate bioink whilst providing support to give excellent structural fidelity (Fig. 6).[95] Commercial alginate-based hydrogel kits are available, such as the NovaMatrix-3D cell culture system, which has air-dried alginate foams contained in well plates which can be mixed with culture media and cells to create hydrogels.[96]

Cells normally have a rounded morphology in alginate hydrogels, and unlike collagen or fibrin, alginate hydrogels are normally modified with adhesive biomolecules, such as RGD, to facilitate cell attachment and spreading.[97] Though in some cases, such as cartilage tissue engineering, the rounded morphology can be an advantage.[98] Alginate is used extensively in cell and tissue culture due to its lack of receptors and low protein adsorption, meaning the hydrogels serve as a "blank slate".[93, 99] Highly specific amounts of biomolecules can then be incorporated to study the effect of particular cellular adhesion receptors or growth factors. For example, MSC differentiation was shown to be controlled by RGD–alginate gels' elastic moduli, where more rigid gels promoted osteogenesis, and less rigid gels promoted adipogenesis.[97] Tumour microenvironments have also been studies using alginate gels, RGD–alginate gels altered cancer cell signals in recruiting blood vessels, which could pave the way to developing new anti-angiogenic cancer treatments.[100]

Sodium alginate has been used as a component bioink in many different applications.[82, 101, 102] Jia *et al.* bioprinted adipose-derived stem cells lattice structures using oxidised alginate hydrogels to control the degradation rate of the bioink. In general, however, the printability, cell viability and mechanical properties of alginate bioinks are often not stable unless cross-

linked using $CaCl_2$.[103] Shu and colleagues used a three stage crosslinking process to inkjet-print U87-MG human brain tumour cells for cancer disease models.[104] Firstly, the gel was crosslinked with calcium ions before printing to generate the flow properties for the bioprinting, secondly the alginate was crosslinked immediately after printing for to increase stiffness, and finally barium ions were used to crosslink for long term stability. This gave way to tubular structures with a minimum diameter of 7.7 mm with cell high viability over 11 days of culture.

By combining alginate with other gels, such as gelatin, the properties of alginate can be enhanced and well-defined structures can be printed.[7, 101] Often printed cells cannot degrade surrounding alginate matrices, causing them to proliferate poorly and remain dedifferentiated.[105] To counter this problem alginate has been combined with collagen and gelatin to print human corneal epithelial cells (HCECs) in macroporous structures.[106] Cell viability was over 90% and degradation of the constructs could be controlled using concentrations of sodium citrate.

4. Challenges and future directions

Bioprinting represents a facile and reproducible path for the rapid construction of tissue-like constructs and is quickly emerging as the must-have tool for the tissue engineer. That being said, many of the challenges associated with scaffold-based tissue engineering still remain. For example, there are few if no examples of bioprinted 3D tissue constructs exhibiting the complex hierarchical structures or mechanical properties of native ECMs, even for avascular tissue such as cartilage. Although examples of printed constructs containing perfusable vasculature are starting to emerge, these are still rather primitive when compared with the genuine article.

The development of standardised assays for the phenotypic interrogation of bioprinted constructs is also a major challenge. Essentially, all the in vitro-based assays have been developed for 2D culture and adapting these to 3D culture models can be challenging. For example, the presence of the bioink may give rise to spurious results when using fluorescence-based approaches. Moreover, the majority of microscopes available to the user have limited working distances. Accordingly, the field must work hard to address this issue by embracing new experimental methodologies, both instrumental (e.g., two-photon, micro- and nano-CT) and chemical (e.g., new probes and antibodies) if it is to build a body of literature with comparable data.

The range of cell-compatible bioinks is now starting to expand to a level that meets the needs of most cell types, and contains constituents that are derived from eukaryotes, other biota or purely synthetic origins. However, the biocompatibility of bioinks are predominantly benchmarked by cell viability and proliferation, with little emphasis placed on cell adhesion and motility. Accordingly, if more complex tissue architectures displaying ECM structures are to be realised, these other factors that affect cell fate must be built in to the design philosophy.

The large number of challenges that need to be overcome may make the bioprinting of organs seem to be a far-off goal. However, the technology is still extremely new and the rate of development over the last five years has been staggering. It is an extremely exciting field to work in, and bioprinting is perhaps the most inherently interdisciplinary research area. With that in mind, it is essential that a convergent approach be used to assemble the best research team, where chemists, engineers, biologists and medical practitioners are all focussed on achieving the global objective of functional synthetic tissue.

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Keywords:

Bioprinting, biofabrication, bioinks, tissue engineering, hydrogels, scaffolds, regenerative medicine

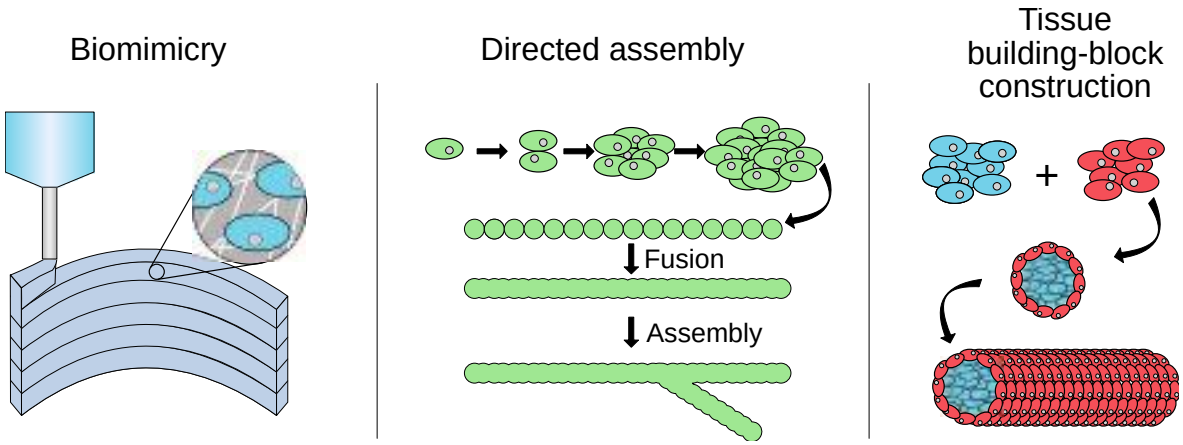

Figure 1. Three current bioprinting approaches: biomimicry aims to recapitulate tissue macro- and micro-structures with cells and ECM components in the bioink; directed assembly uses cell aggregates to form structures before assembly structures; tissue building-block construction involves co-culturing cell types to form micro-tissues that can then be assembled.

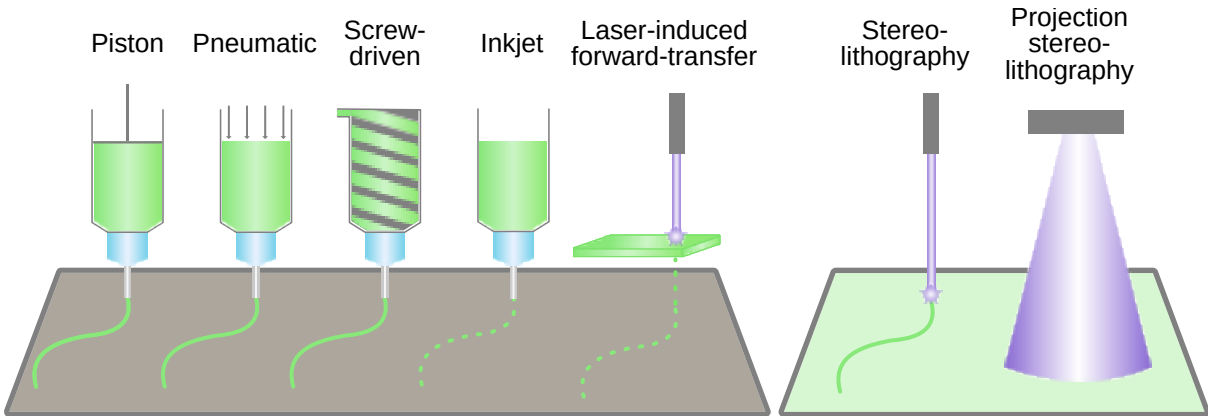

Figure 2. The main bioprinting technologies: microextrusion bioprinters use a piston, air pressure, or screw drives to extrude material; inkjet bioprinters use thermal or piezoelectric actuators to emit droplets, or Rayleigh instability of a continuous flow through a small orifice; laser-assisted bioprinters use a laser focused on an energy absorbing substrate to propel biomaterials and cells onto a collecting plate; photocuring bioprinters use light to polymerise the surface of bioink reservoirs.

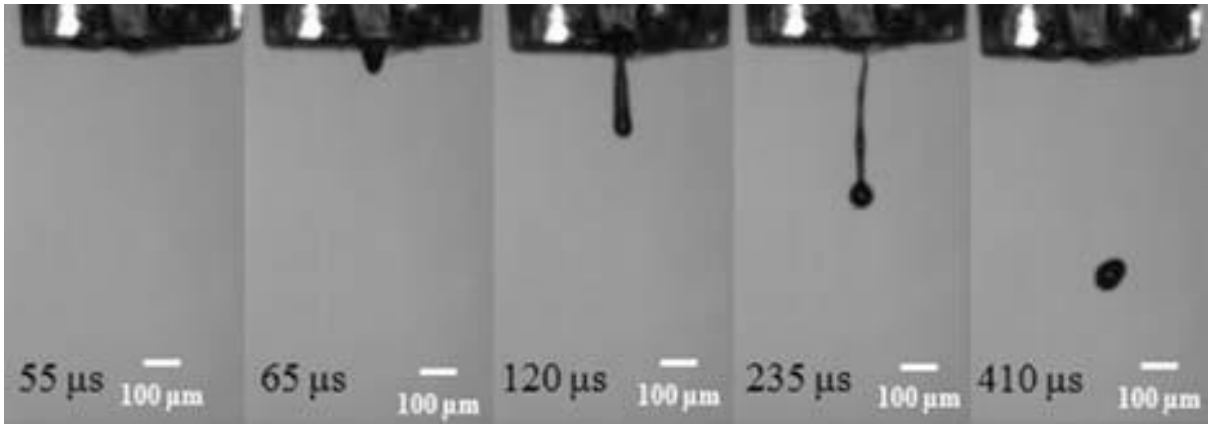

Figure 3. Cell-laden bioink being ejected from an inkjet nozzle. Reprinted with permission from Wiley Periodicals, Inc.[107]

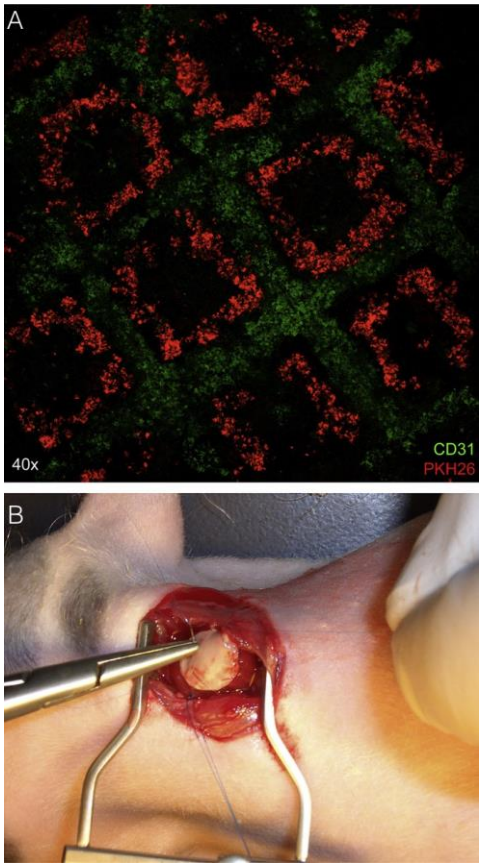

Figure 4. Cardiac patch produced via laser-assisted bioprinting. (A) Micropatterned hMSCs (red) and hUVECs (green). (B) Cardiac patch implanted into rat myocardium. Reprinted with permission from Elsevier Ltd.[34]

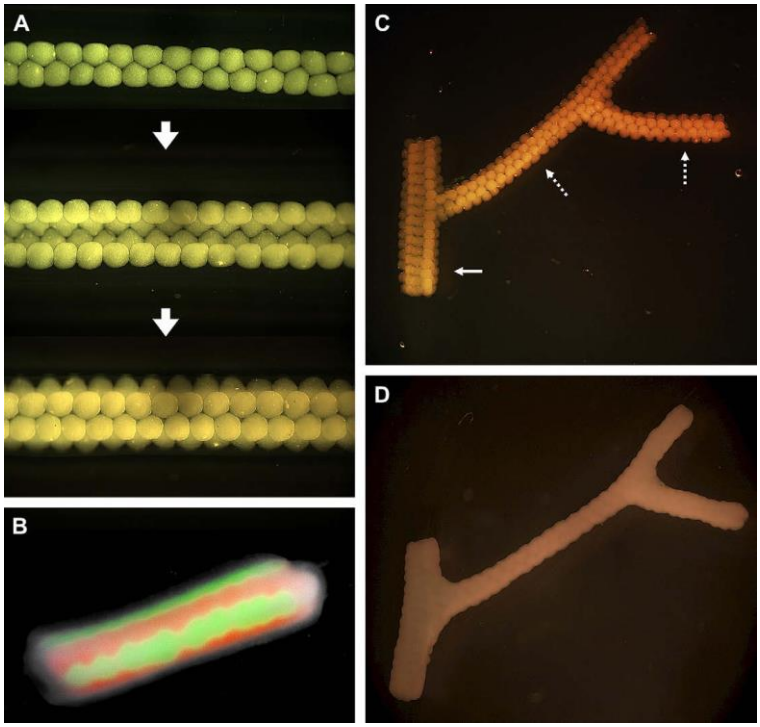

Figure 5. Extrusion-based bioprinting of multicellular spheroids to produce vascular networks. (A) Spheroids deposited in hollow cylindrical pattern on day 0 and (B) day 7. (C) Printed branched vascular network with 1.2 mm diameter (solid arrow) and 0.9 mm (dashed arrow) branches on day 0 and (D) day 6. Reprinted with permission from Elsevier Ltd.[46]

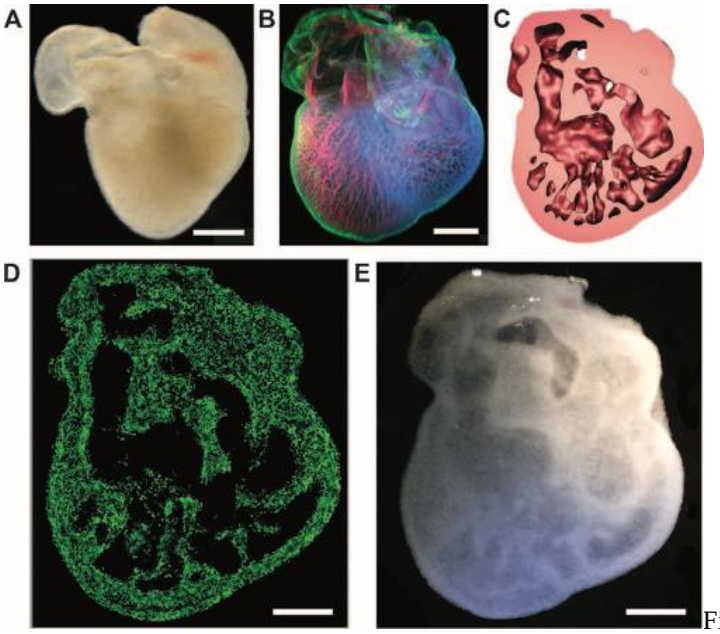

Figure 6. Embryonic chick heart printed in alginate. (A) Dark field image embryonic chick heart. (B) The heart stained for fibronectin (green), nuclei (blue) and f-actin (red), and imaged using confocal microscopy. (C) Cross section of the CAD model based on the confocal data. (D) Cross section of the printed heart in fluorescent alginate. (E) Dark field image of the printed heart. Scale bars: 1mm (A,B) and 1 cm (D,E). This work is licensed under the Creative Commons Attribution 4.0 International License. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/ or send a letter to Creative Commons, PO Box 1866, Mountain View, CA 94042, USA.[95]