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Engineering Bioinks for 3D Bioprinting

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Abstract

In recent years, three-dimensional (3D) bioprinting has attracted wide research interest in biomedical engineering and clinical applications. This technology allows for unparalleled architecture control, adaptability and repeatability that can overcome the limits of conventional biofabrication techniques. Along with the emergence of a variety of 3D Bioprinting methods, bioinks have also come a long way. From their first developments to support bioprinting requirements, they are now engineered to specific injury sites requirements to mimic native tissue characteristics and to support biofunctionality. Current strategies involve the use of bioinks loaded with cells and biomolecules of interest, without altering their functions, to deliver *in situ* the elements required to enhance healing/regeneration. The current research and trends in bioink development for 3D Bioprinting purposes is overviewed herein.

Keywords: Additive manufacturing, 3D Bioprinting, bioinks, biomaterials, hydrogels.

1 Introduction

3D Bioprinting has resulted from the convergence of biofabrication, additive manufacturing, tissue engineering and regenerative medicine. The term 3D Bioprinting regroups different biofabrication techniques that use additive manufacturing technologies in tissue engineering to overcome limitations that conventional 3D scaffolds fabrication methods encounter. Using a computer-aided design software, a living 3D structure is designed then produced by stacking and assembling successive layers of living and/or non-living materials using robotic computer-aided manufacturing processes. This aims to artificially develop equivalents of living tissues and organs for their use in tissue engineering, regenerative medicine, pharmacokinetics and biological research.

3D Bioprinting techniques distinguish themselves from other biofabrication methods thanks to some remarkable advantages they can offer, such as their excellent

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control over the spatial organization of biomaterials and cells, their repeatability and general straightforwardness [1]. There are numerous 3D Bioprinting technologies with their own pros & cons [2]. Nowadays, most 3D Bioprinting techniques are scaffold-based methodologies in which cells are embedded into bioinks which are then printed into the desired shape. These bioinks can be defined as "formulations of cells suitable for processing by an automated biofabrication technology that may also contain biologically active components and biomaterials" [3]. The development of novel bioinks should consider several specificities for optimal performance, which raises critical problems. Bioink development face several challenges including the necessity to create innovative bioinks serving multiple purposes, specifically to better protect the cells during their transport, and securing their adequate differentiation and growth after printing.

The literature comprises numerous reviews on 3D Bioprinting and the bioinks used. Recently, articles have been published on current 3D Bioprinting modalities [4], materials used as bioinks [5–7], chemistry of bioinks [8], and possible reinforcement strategies for bioink development [9]. However, the focus of this review is on the structure property relationships of printable materials and how these influence printing performance. We discuss future materials strategies to improve printing which should ultimately improve patient outcomes. In addition, owing to the fast-paced progress of bioink development and wide range of bioink applications, we assess the trajectory of its evolution.

2 Bioinks

The current 3D Bioprinting technologies, as applied to tissue engineering and regenerative medicine, can be divided in two different approaches: scaffold-free and scaffold-based 3D Bioprinting. Scaffold-free bioprinting can be described as a self-assembly technique where living cell aggregates such as cell sheets, spheroids, or tissue strands, act as prefabricated building blocks directly printed into 3D constructs without the use of matrices. After printing, cell aggregates then mature and fuse

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together through secretion of ECM, similarly to self-assembly phenomena in early morphogenesis [10–13]. In scaffold-based 3D Bioprinting, cells are encapsulated into polymeric hydrogel matrices called bioinks. These bioinks are then printed into the desired shape by one of the techniques presented before. Bioinks can be produced from a large variety of materials to answer specific requirements for each application. Both the requirements and materials are discussed herein.

Bioinks also provide an environment that mimics the ECM to maintain cell viability while inducing the desired cellular response. Bioinks differ from "biomaterial-inks" as these latter comprise (bio-) materials which cannot be formulated with cells: fabricated scaffolds must be printed, then subsequently seeded with cells [3]. Implanted cells will vary from an application to another, as well as tissue properties, architecture, etc. Thus, facing the large array of target sites and possible applications, it is very unlikely – if not impossible – that a single bioink can be versatile enough to satisfy all requirements. In fact, the current literature on bioinks is incredibly diverse as bioinks must be closely tuned to each application for optimal results.

2.1 Bioink Development

The development of a bioink relies on a series of tests to assess its performance before, during and after printing. These tests will differ accordingly to the bioprinting technology and the intended purpose of the fabricated construct. Fig. 1 shows the basic steps involved in the development of a bioink, differentiating the material requirements from biological ones and the chronology of their assessment. The first step in bioink development is to consider the biological environment and requirements of the targeted tissue in terms of mechanical properties and cues to direct cell functionality [14]. This will lead to various possible materials from which the bioink can be formulated. The regulatory approval status of all components used, and their sources, must be approved as early as possible [15]. This will also ensure the cytocompatibility of the materials used. The degradability of these materials and the cytocompatibility of their degradation by-products must also be considered. Then,

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bioink prototypes will be formulated and evaluated on their printability and the properties of the constructs they can yield. These tests can assess the printing quality (resolution, clogging issues, etc.), the structural viability of fabricated constructs, and/or their biological function. Naturally, it is very unlikely that the first formulations display optimal performance, thus numerous prototypes will be formulated in order to achieve structures with the desired properties and characteristics. Subsequent optimisation of the printing parameters and gelation mechanisms is essential to maximise these performances and repeatability [14]. Finally, thorough assessment of the resulting bioinks must be performed to ascertain the mechanical & biological performances of fabricated scaffolds, their resolution, cell viability and functionality post-printing and over time to determine the most suitable bioink formulation.

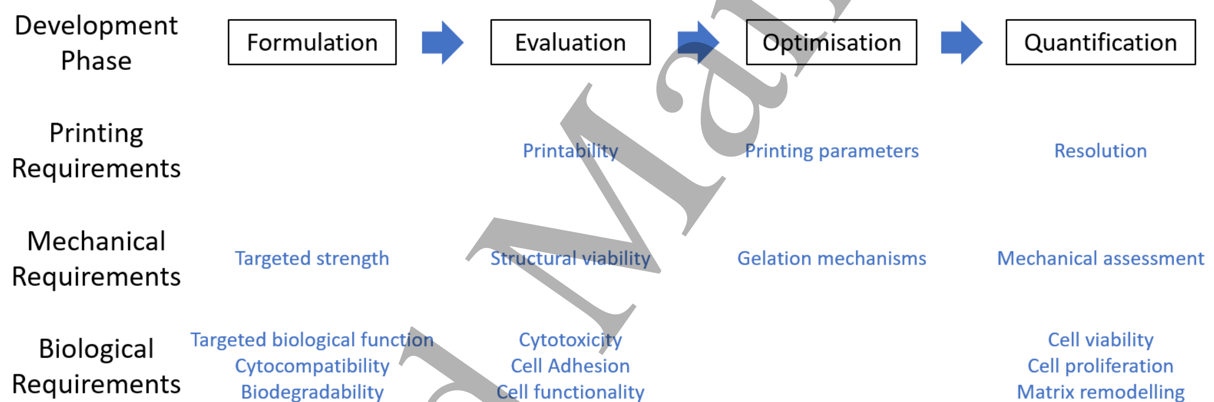

Fig. 1: Overview of the steps involved in the development of a bioink.

2.2 Bioinks Requirements

2.2.1 Primary 3D Bioprinting Requirements

All 3D printed structures must comply to several primary mechanical requirements: firstly, they must be printable and hold their shape after printing. However, they must also combine structural stability with effective biological performance. As a basic principle, successful biological substitutes mimic native tissues. Similarly to any structure used in tissue engineering, regenerative medicine or pharmaceutical research, 3D bioprinted constructs must be biocompatible,

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cytocompatible, and bioactive: bioinks should be non-inflammatory and non-cytotoxic for the host and/or transplanted cells, and they should provide an ECM environment able to maintain a high cell viability while supporting the adhesion, proliferation, and differentiation of living cells similarly to the targeted tissues. They must also allow the production of scaffolds with adequate mechanical and structural integrity for their implantation and finally biodegrade in an appropriate manner. The differentiated characteristic of bioinks as compared to other biomaterials is their capability to be printed, or their printability.

2.2.1.1 Printing strategies

Printability is an important property as it encompasses the ability of bioinks to be deposited in a controlled manner to allow for optimal print fidelity. It is largely influenced by physical properties such as viscosity/stiffness, rheological behaviour, and printing parameters. It is possible to improve the printability of a given bioink by tuning its material properties and printing parameters such as the material concentration, the density of encapsulated cells or the printing temperature. However, materials with optimal printability have very different properties compared to natural ECM, thus they are often subjected to poor cell viability and bioactivity [3]. The biofabrication window paradigm (Fig. 2) illustrates that concept: traditional bioinks have been compromising printability for biocompatibility, whereas the next generations of bioinks should aim to overcome these concessions by offering optimal printability and biocompatibility.

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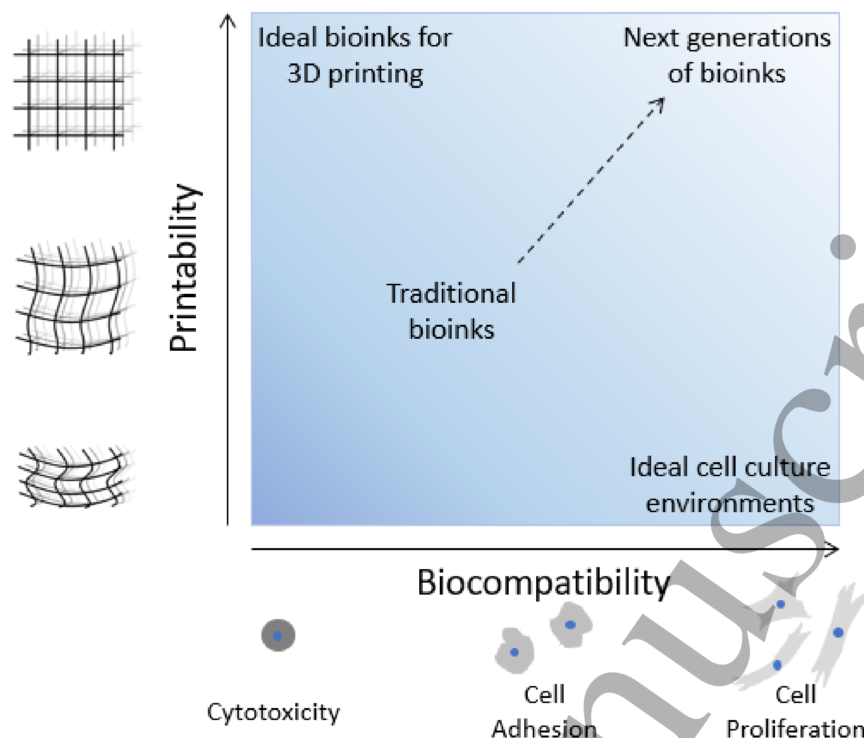

Fig. 2: The biofabrication window.

Viscosity is an essential bioink property which greatly influences printability and biological function. Generally, polymer solutions used in 3D Bioprinting should possess high viscosities and a high thixotropic index in order to retain their shape after processing [16,17]. However, the different 3D Bioprinting modalities lead to different material requirements to achieve optimal performance, and some of these techniques are unsuitable for high viscosities.

In a recent study, Shin *et al.* functionalized hyaluronic acid and gelatin with gallol antioxidant groups to produce a hydrogel with dynamic hydrogen bonds [18]. Gallol moieties are plant-derived antioxidant associated with fruit browning [19]. These bonds induced temporal shear-thinning and self-healing behaviour. Long-term stabilization of the bioinks was then achieved through the auto-oxidation of the gallol moieties over the course of a day, inducing permanent covalent bonds. Depending on the age of the scaffold, gallol functional groups could act both as dynamic and covalent crosslinker. This technique allowed to produced 3D bioprinted constructs by extrusion-

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based technique with an excellent cell viability (≃95%), post-printing proliferation and spreading of cells. On the other hand, droplet-based modalities should use rheopectic bioinks to increase viscosity when stress is applied to trigger droplet formation [20–22]. This property also causes droplets to thicken upon extrusion, which helps maintaining the shape of droplets by reducing spreading, splashing, and deformation. However, a rheopexy too high may prevent droplet formation. Additionally, the low viscosity and non-fibre forming character required for inkjet bioinks limits the usable cell density while doing little to mitigate the shear stress cells experience during printed [22]. Succinctly, not only bioinks properties must be tuned to guarantee an adequate printing fidelity, they must also be tuned accordingly to the selected bioprinting modality.

2.2.1.2 Physical & chemical strategies

Structural integrity and stability are also essential properties to produce scaffolds which can retain their shapes after fabrication. Mechanical properties are inherent from the material composition of bioinks and eventual chemical modifications. Generally, hydrogels produced from naturally derived polymers possess poor mechanical properties compared to their synthetic counterparts. Thus, they are generally chemically modified by increasing their polymer content and/or crosslink density to be strengthened. For example, hyaluronic acid (HA) is a glycosaminoglycan found in the extracellular matrix and numerous biomedical applications. It is a very interesting material because of its immunomodulatory potential [23]. However, pure HA has a poor mechanical properties resulting in a potential lack of structural support [24]. To overcome this limitation, HA can be chemically modified and crosslinked to produce networks of methacrylated hyaluronic acid. In 2005, Burdick *et al.* have produced and reported methacrylated hyaluronic acid networks with compressive modulus ranging from 2 up to over 100 kPa by varying the molecular weight of the initial HA from 50 up to 1,100 kDa, its degree of methacrylation from 6 up to 12% and the macromer concentration from 2 wt.% up to 20 wt.% [25]. More recently, Spearman *et al.* demonstrated that the mechanical and physical properties of methacrylated hyaluronic acid can also depend on the methacrylation technique [26]. They assessed

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the performances of methacrylated hyaluronic acid hydrogels modified by glycidyl methacrylate HA (GMHA) or methacrylic anhydride HA (MAHA). They demonstrated that these two different methacrylation techniques result in varying degrees of methacrylation and moduli, and that mechanical properties plateau with increasing crosslink groups, as shown in Fig. 3ii. Furthermore, this work showed the possibility to adjust the developed GMHA or MAHA to the mechanical properties of several soft tissues (Fig. 3iii). Bioinks formulated from methacrylated hyaluronic acid have been successfully printed into scaffolds for application in soft and hard tissue engineering with adequate mechanical properties, printability and good cell viability [26,27]. Current literature presents an great number of chemically crosslinked natural polymer hydrogels such as collagen, chitosan, gellan gum, etc. and their various applications in tissue engineering, regenerative medicine and pharmaceutical research [28–34]. However, such modifications should be carried to a limited extend: high polymer content and densely crosslinked hydrogels have poor performances in cell culture applications because of their reduced permeability and porosity that prevent cell spreading and migration, and limit nutrient diffusion [16].

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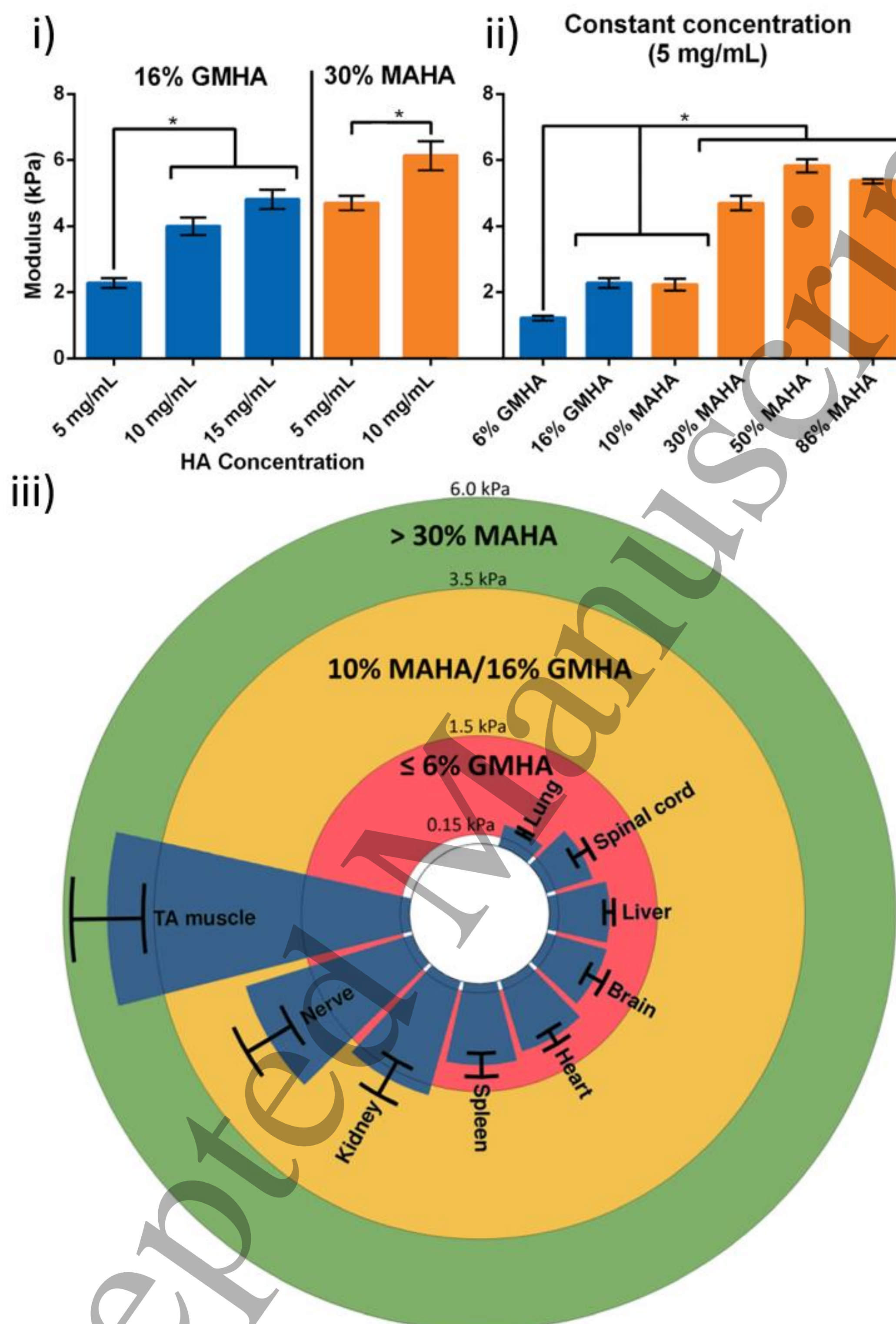

*Fig. 3: Modulus from indentation of various HA-based hydrogels. (i) Varying concentration with constant methacrylation (16% for GMHA or 30% for MAHA). (ii) Constant concentration (5 mg/ml) with changing degrees of methacrylation. The * For Peer Review indicates p < .05. GMHA, glycidyl methacrylate hyaluronic acid; MAHA, methacrylic anhydride hyaluronic acid. (iii) Modulus from indentation on various rat tissues. Coloured regions show the approximate ranges of mechanical properties that are obtainable with the corresponding methacrylated HA. Reprinted with permission from* [26]. *Copyright © 2020 John Wiley & Sons, Inc.*

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Gelation also plays a great part in structural integrity and stability. Gelation refers to the phase change process by which bioinks stiffen to produce stable constructs to avoid structure collapse. The gelation rate greatly affects print fidelity by determining how quickly bioinks can crosslink and reach sufficient stiffness after printing [35]. Ideally, gelation should be rapid for bioinks to retain their shape post-printing and avoid spreading. This process must as well be biocompatible with living cells. The gelation can either be physical (based on reversible interactions such as high molecular chain entanglements, hydrogen bonds, hydrophobic or ionic interactions) or chemical crosslinking (based on formation of covalent chemical bonds) [20,35,36]. There are many currently available gelation processes such as ionic, chemical, physical, thermal, enzymatic or photo-induced that may be used alone or combined [37–40]. For example, physical hydrogels usually possess poor mechanical properties that can be enhanced by introducing chemical functionalities to create covalent crosslinks, thereby resulting in irreversibly crosslinked networks [20,41,42]. Gelation is an essential property of bioinks, especially for energy-based bioprinting techniques such as stereolithography or digital light processing which entirely rely on bioinks ability for photopolymerization. Pure hydrogels are generally unable to crosslink independently. The addition of external initiators or functional groups capable of undergoing free radical polymerization by chemical modification of the molecule allow for independent polymerization of the subsequent polymers [6]. Current cell-loaded bioinks used in these technologies have been developed using cytocompatible photo-initiators and energy doses. Many traditionally used photo-initiators are toxic above a certain threshold and require ultraviolet light for polymerization, which is detrimental to cells at prolonged exposure. These toxic photo-initiators are gradually replaced by their cytocompatible counterparts such as Eosin Y that allow photopolymerization by visible light within small time spans [43–45]. Photo-initiator concentrations, exposure times, and exposure intensities can all be controlled to achieve crosslinking of a photo-reactive material without damaging cells [46]. Gelation processes must be carefully tuned as they greatly influence crosslink densities and subsequent mechanical

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properties. In 2016, Jang and colleagues were able to use sequential thermal gelation and covalent crosslinking induced by the incorporation of vitamin B2 and UVA irradiation, to solidify a decellularized extracellular matrix bioink with a stiffness 33 times superior to that of the control (thermally crosslinked only) [40].

Mechanical properties of fabricated constructs should match those of native tissues to avoid stress concentration once implanted, and because they influence bioactivity properties including cell viability, proliferation & differentiation independently from biochemical cues [47–49]. A major application when controlling the substrate stiffness is to direct the morphology, adhesion, proliferation and differentiation of mesenchymal stem cells into specific phenotypes. It is well known that soft ECMs will direct stem cell differentiation towards chondrogenic lineage, whereas stiffer ECMs promote osteogenic lineage [50–54].

Finding the right balance between the mechanical and the biological properties of a bioink is a critical aspect of its development and subsequent performance. As described, numerous parameters influence the mechanical requirements of bioinks. Succinctly, fabricated constructs must mimic the mechanical properties of the tissues they aim to replace, while displaying appropriate stability to withstand the implantation, natural environment of the tissues, repetitive stress cycles, remodelling processes, etc. However, mechanically strong and stable bioinks exhibit limited biological performance. Finding this balance will depend on the application and on one's perspective when developing a bioink.

2.2.1.3 Biodegradation kinetics

Finally, bioprinted constructs should degrade *in vivo* to be gradually replaced by the new ECM produced by cells [55]. Bioinks are designed only to play a temporary role, such as a sacrificial support structure [14]. For tissue engineering purposes, the degradation rate should ideally match that of new ECM production, and degradation by-products should be non-toxic nor create immunological response. If the scaffolds degrade too quickly, it may result in loss of cell/scaffold function before repair, or even structural failure *in vivo*. On the other hand, cell-proliferation and cell-differentiation

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within the scaffolds may be significantly reduced if the scaffolds degrade too slowly [55]. Biodegradation of the scaffolds can also allow to deliver cells, drugs, and other therapeutics *in vivo*. In such case, the degradation kinetics of the structures should be tuned accordingly to the loaded cargo and intended application. Biodegradation can occur by different processes such as enzymatically, hydrolytically or through ion exchange [16]. It is possible to tune the degradation rate through careful bioink chemistry selection. In 2005, Burdick *et al.* determined that the degradation time could be tuned ranging from 1 up to 38 days by means of modifying the molecular weight, degree of methacrylation and monomer concentration of HA hydrogel networks [25]. More recently, Kim *et al.* developed a methacrylated glycol chitosan (MeGC)/lysozyme hydrogel which enzymatic degradation could be finely tuned in a cell-independent manner [56]. Increasing the concentration of methacrylated lysozyme (MeLyz) allowed to accelerate the degradation rate in a dose dependent manner as seen in Fig. 4i. Their hydrogels displayed adequate performances in terms of cellular behaviour and tissue formation, indicating that it can be used in tissue engineering applications, as seen in Fig. 4ii and 4iii.

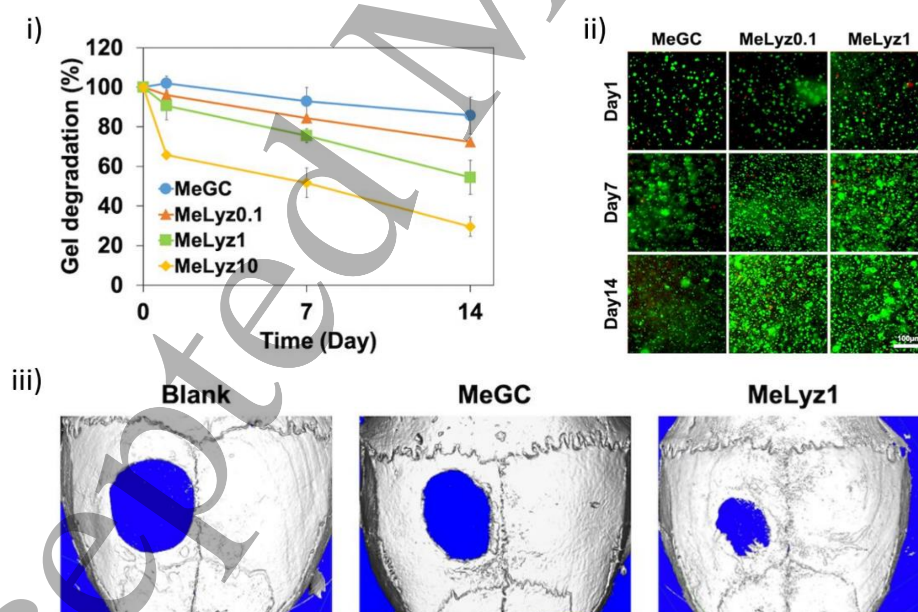

Fig. 4: (i) Degradation kinetics of hydrogels for 14 days by measuring dry weight. MeLyz was incorporated in MeGC with 0, 0.1, 1, and 10 mg/mL concentrations. (ii) Live/Dead staining images of BMSCs encapsulated in MeGC, MeLyz0.1, and MeLyz1 for 14 days and quantified cell viability. (iii) MicroCT images of calvarial defects treated with hydrogels after 6 weeks. Reprinted with permission from [56]. *Copyright © 2018 American Chemical Society.*

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2.2.2 Specific Bioinks Requirements for Additional Purposes

Bioprinting is a promising tool for therapeutic applications. Incorporating bioinks with cells, therapeutic drugs and/or biologics before printing or implantation can allow for their local delivery in a controlled manner [55]. Bioprinted structures loaded with stem cells have also been shown to support positive tissue infiltration, constructive tissue remodelling, and adipose tissue formation without inducing chronic inflammation or cytotoxicity after *in vivo* implantation [57]. Bioinks must have specific physiochemical properties to allow these therapeutic biomolecules to bind to them. Generally, natural polymers used in bioinks are intrinsically bioactive and contain cell attachment sites while synthetic polymers usually lack of these and require chemical modifications for binding [16,53]. However, synthetic polymers allow for controlled degradation processes, an important parameter to create "smart" polymeric hydrogels that can self-regulate the release of bioactive agents in response to specific external stimuli such as pH, temperature, enzymes or biochemical cues, allowing for targeted deliveries [53,58,59]. Embedding nano- and micro-particles into the bioinks allows to also embed drugs & cells while acting as the transducing material [58]. These particles enable to tune the encapsulation efficiency of the drug contained while their density control allows control over the concentration and time course of the target molecule being delivered [53].

An important aspect of implanted bioprinted structures is their ability to be monitored by non-invasive imaging techniques during and after implantation. This allows to ensure their proper retention and/or degradation in the body, to analyse interactions between scaffolds and surrounding tissues, and to trace therapeutics embedded in the scaffolds [60,61]. Clinically applicable imaging techniques such as X-rays, PET or MRI can be used for such applications. Contrary to the other techniques, X-ray imaging is based on phase contrast, which allows it to detect hydrogel structures without requiring contrast agent. However, embedding such particles is an effective method to allow for high-resolution 3D imaging [61]. In addition, some particles can be used as theranostics, meaning that they simultaneously serve therapeutic and diagnostic purposes. Iron oxide nanoparticles for example, can be incorporated into

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hydrogels to act as contrast agents and drugs & cells carriers for delivery [62]. It is known that due to the enhanced permeability and retention effect, nanoparticles accumulate in tumours and inflammation sites. Theranostic iron nanoparticles can then provide a targeted delivery of the loaded drugs and/or cells at tumorous sites, while allowing to monitor them *in vivo*.

Very recently, Lahr and colleagues developed a novel strategy to implant local theranostic scaffolds during osteosarcoma treatments [63]. If clinical evaluations indicate that a patient is developing primary bone cancer, biopsies will be taken from the lesion to grade the cancerous cells and determine the adequate treatment. Patients may then wait up to 6 weeks before the beginning of their treatments [64]. During this “therapeutic gap”, Lahr *et al.* propose to implant a biodegradable, tuneable 3D printed medical-grade polycaprolactone scaffold loaded with Doxorubicin to fill the biopsy voids. This would allow to locally expose tumorous cells to a cytotoxic drug, preventing its diffusion to surrounding tissues, while also being able to evaluate the local tumour behaviour by giving valuable information regarding its grade, invasiveness, dosing requirements and potential resistance to the loaded chemotherapeutic drug. Monitoring the penetration depth of tumours into specifically designed scaffolds for example, is an accurate, repeatable, and easy way to collect essential prognostic information, facilitating decision-making processes to select suitable treatment options. This strategy could be extended to other diseases, facilitating diagnosis and patient-specific treatment development.

2.3 Advanced Bioinks and Formulations

As previously mentioned, bioinks may be produced from a large variety of materials which serve as cell carriers during formulation and processing [65]. Hydrogels are the most viable and commonly used materials for scaffold-based bioinks [3,16]. Hydrogels can be tuned to create suitable bioinks for various applications. Limitations in their properties presented previously have led to the emergence of advanced bioink technologies such as nanocomposites, multi-material hydrogels, interpenetrating

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networks and supramolecular bioinks [7,16]. This new generation of bioinks overcome some of the current boundaries in bioink design to develop biological tissue complexes with improved printability and move further in the biofabrication window. As mentioned, bioink properties and characteristics do not rely on the constituent materials only, but also their concentration within the bioinks, their eventual chemical modifications, their molecular weights, chain architectures etc. Fine optimisation of bioink formulation is generally the last step in its development to reach desired properties. Bioink formulations must consider all parameters that influence performance of bioinks, such as materials characteristics (molecular weights, degrees of crosslinking, viscosities, cell types, densities), and printing parameters (shear rate, temperature, pressure, printer resolutions, etc.). Some of these parameters and the properties they influence have been previously discussed, such as the influence that modifying the molecular weight and degree of methacrylation of a polymer have on its mechanical properties and degradation time. Bioink formulations must also consider parameters that are specific to certain types of bioink technology, such as filler content in nanocomposites, component ratios in interpenetrating polymer networks, and so forth.

2.3.1 Single-Component Hydrogel Bioinks

Hydrogels, as it is well known, are polymeric 3D networks that are insoluble in water and swell to an equilibrium volume while retaining shape [66]. Swelling under biological conditions render them an ideal class of materials for tissue engineering purposes: their high-water content enables them to mimic native tissue ECM, and they resemble natural soft tissue more than any other type of biomaterials [17,67]. In addition, hydrogels display good biocompatibility and high permeability for water-soluble metabolites such as oxygen and nutriments, making them attractive materials for cell encapsulation [6,67–69]. Hydrogels consisting of porous and flexible networks promote cell migration as well as adhesion in 3D environments, allowing for matrix remodelling, which is prerequisite for the normal development of functional tissue

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[6,16,70]. Based on origin, hydrogels can be classified into two groups: naturally or synthetically derived hydrogels. Further classification relies on the crosslinking mechanism: physical crosslinking includes chain entanglement, hydrogen bonding, hydrophobic interaction or crystallite formation that create semi-permanent reversible bonding, whereas chemical crosslinking creates permanent and irreversible junctions formed by covalent bonds [67,71,72].

2.3.1.1 Natural Hydrogels

Also referred to as biopolymers, naturally derived hydrogels are obtained from all types of organisms: animals, plants and microorganisms [71]. They are used for their intrinsic biological properties such as bioactivity, biocompatibility, low toxicity and biodegradation, along with their ease of formulation, ability to self-assemble and low cost. However, they display poor mechanical properties, reproducibility and may induce immunogenic reactions [21,67,71]. The two major types of natural polymers used in hydrogels are: i) proteins (such as silk fibroin, collagen, keratin, elastin, or fibrin) and ii) polysaccharides which can be extracted from both plants (e.g. alginate and agarose) and animals (e.g. hyaluronic acid and chitosan) [71,73]. Proteins used are generally derived from vertebrates and possess inherent signalling molecules to guide desirable cell behaviour, including migration, proliferation, differentiation, and maturation. On the other hand, hydrogels of nonanimal origin are readily available with excellent biocompatibility, low cytotoxicity, are enzymatically biodegradable but lack signalling molecules. They are generally used to avoid sources of viral contamination [6,21,71].

2.3.1.2 Synthetic Hydrogels

Synthetic hydrogels are attractive materials to be used in bioink formulations because they can be tailored to specific applications by varying their molecular weight, chemical structure, and composition [21]. Namely, their mechanical strength, biodegradation kinetics, topography and cell adhesion characteristics can be modified and reproduced with low batch to batch variation as opposed to natural hydrogels although latest advancements in natural hydrogel chemistries are alleviating this issue

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[71,74–76]. However, synthetic hydrogels can only support and not promote cellular function as they require chemical functionalization to integrate bioactivity to their structure [6,16,75]. The two most commonly used synthetic polymers in bioinks are Pluronic® and poly- (ethylene glycol) (PEG). The first lacks mechanical stability and is generally used as a supporting material while PEG is hydrophilic and bioinert, thus requires blending with other natural polymers or functionalization [17].

2.3.2 Decellularized Matrix-Based Bioinks

Each tissue has an extracellular matrix with a unique composition and topology generated during tissue development that is difficult to reproduce with single material bioinks. Decellularized extracellular matrix-based (dECM-based) bioinks are produced by using chemical, physical, or enzymatical processes to remove cells from the considered tissues while preserving their ECM. The dECM is then grounded into a powder and mixed into a cyto-friendly buffer solution to produce the bioink. A polymer can also be added to tune properties of the bioink such as its solubility or viscosity [6,17,21]. Kim and colleagues recently produced a dECM powder-based bioink by loading the dECM micro-particles into a gelatin mixture [77]. It resulted in a bioink with an elastic modulus increased up to nine times compared to conventional dECM bio-ink, a 93% cell viability in micro-patterns, and an improved printability. dECM bioinks also promote cellular function by simulating the natural micro-environment of tissues. In 2014, Pati *et al.* developed several dECM bioinks to print structures that mimic the specific natural environments of different tissues [78]. Their team used various dECM-based bioinks to produce scaffolds which displayed high cell viability, cell line-specific gene expression and supported ECM formation. Autologous ECM-based bioinks are highly biocompatible and present minor risks of host immune reactions. However, harvesting graft material rises concerns due to the requirement for an additional surgery and subsequent loco-regional morbidity, in addition to the limited availability of tissue. Allografts and xenografts are viable alternatives, but present higher risks of host immune reactions and disease transmission, especially for allograft-based

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materials [79]. Also, these bioinks have several drawbacks: they are expensive because of their restricted availability, limited to tissue type availability, and the decellularization processes are time-consuming requiring precise quantification of the DNA and the ECM components [6,21]. Additionally, given that each tissue has an ECM with unique composition and topology, dECM bioinks produced from different tissues will display different characteristics. Recently, Han *et al.* characterized the composition of dECMs from different tissues and evaluated the effect of unique compositional variation in each dECM component on mesenchymal stem cell behaviour in a tissue-specific context [80]. The different dECMs shared some common elements, but each one also showed a unique set of tissue-specific components and compositional variation, which might be linked to tissue-reflective behaviours. For usage as hard tissue substitutes, like bone and cartilage implants, ceramic materials or composite scaffolds might be required to produce stable constructs with suitable mechanical properties [81]. In their study, Parmaksiz and colleagues developed a multi-layered osteogenic tissue scaffold by assembling decellularized bovine small intestinal submucosa layers with synthetic hydroxyapatite microparticles and poly(ε-caprolactone) (PCL) binder [82]. The proposed scaffolds displayed compressive force values within the range of human cancellous bone while exhibiting osteoinductive properties.

2.3.3 Multi-Component Bioinks

Using single-component bioinks is ultimately of limited value in bioprinting due to two main reasons. First, as mentioned before, the properties required for optimal printability and print fidelity are at odds with those that enhance cell viability and function. Natural and synthetic polymeric hydrogels well describe that phenomenon: natural hydrogels generally have weak mechanical properties whereas synthetic hydrogels lack bioactivity. Secondly, natural biological tissues are composite materials possessing anisotropic properties that rely on their components, take for example the meniscus [83], and arrangements that single-material bioinks can hardly reproduce

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[70]. Using multi-material bioinks then seems like the obvious choice as a solution to address these issues. In this regard, hybrid bioinks that utilize both natural and synthetic biomaterials quickly emerged as a way of improving bioactivity and/or mechanical strength of bioinks. A great number of multi-material strategies have then been developed to strengthen hydrogels while retaining favourable biological properties for bioprinting applications.

2.3.3.1 Nanocomposites

As mentioned, natural tissues themselves are organic–inorganic hybrid nanocomposites comprising mostly of mechanically weak natural polymers, and minerals acting as their reinforcement units. Bones for example, are nano-composites materials with extracellular matrices consisting of minerals (65%, mostly hydroxyapatite), organic materials (25%), water (10%), and lipids (1%) [84]. Nanocomposite bioinks aim to mimic native tissues by crosslinking polymers with inorganic or organic nanofillers such as metals, polymers or ceramics, to alter the mechanical, physical and chemical properties of the bioinks and improve their structural and functional properties [85–87]. Depending on the nanoparticles considered, numerous properties including bioactivity, controlled drug delivery, stimuli-responsiveness, self-healing, remote-controlling, and specific rheologic behaviour can be incorporated into the hydrogels [16,70]. Filler geometry can also play a role in altering mechanical strengths of nanocomposites [88]. Comparative studies between bioinks loaded with different amounts of nanoparticles demonstrated that they could improve the bioinks mechanical properties. Recently, Lee and colleagues assessed the incorporation of aminopropyl-modified silica nanoparticles into an oxidized alginate bioink [89]. The particles were linked by reversible imine bonds, allowing the creation of a dynamic polymer with thixotropic and self-healing properties. Through incorporating 2 wt% of particles into the bioink, they reached a peak yield stress of 71 Pa compared to 13.8 Pa without nanoparticles (415 % increase). This peak at 2% filler loading can be explained by an optimised dispersion, after which defects appear in the composites due to poor matrix-filler interactions via the presence of agglomerated nanoparticles, leading to reduced tensile properties [90]. This

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composite bioink was then successfully printed by an extrusion technique into complex structures, such as centimetre-scale porous constructs and an ear structure with an overhanging geometry, with high cell viability (≃80%). Incorporating nanoparticles is also linked with reduced shrinkage and swelling of printed constructs during crosslinking, further improving print fidelity and mechanical strength to avoid structure collapse post print [91]. Rheological investigations also demonstrated that increasing the hydrogel's elastic modulus through nanoparticle-mediated interactions could exceed the maximum modulus obtained of purely chemically crosslinked gels [92]. Other studies pointed that the incorporation of inorganic nanoparticles such as gold nanomaterials, nanosilicates, etc. is linked with improved cell adhesion, organization, spreading and/or differentiation within printed structures [87,93–95]. Nanocomposites clearly offer the potential to improve both the mechanical and biological performance of bioinks.

2.3.3.2 Interpenetrating Polymer Networks

Another approach to multi-component bioinks is the use of hydrogel blends that use several polymers, natural and synthetic, to produce bioinks with tuneable mechanical and biological properties by compensating the weaknesses of each individual component [72]. Such blends include natural-natural, hybrid natural-synthetic and synthetic-synthetic polymer hydrogels.

Interpenetrating Polymer Networks (IPNs) can be defined as "unique blends of crosslinked polymers" [96]. They consist of multiple polymers held together by ionic and/or covalent crosslinks. They are at least partially mutually entangled together but not covalently bonded to each other so that they are not able to separate from each other without breaking their own crosslinked network [97]. Consequently, resulting hydrogels can display properties such as electrical conductivity that at least one of their constituents possess [98]. The different networks can be crosslinked consecutively (Sequential IPNs) or simultaneously (Simultaneous IPNs) with independent, non-interfering polymerization chemistries that are used for polymers to only crosslink with themselves [96]. IPN hydrogels are denser than conventional hydrogels, with stiffer,

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tougher mechanical properties and better drug delivery potential [71,99]. Semi-IPNs are a subclass of IPNs in which at least one polymer that is not crosslinked, to enhance miscibility [100]. They can also be used to create bioinks with suitable rheological properties for printing and fabrication of stable constructs with mechanical properties matching those of natural tissues [101]. Regarding their structures, IPNs can be divided into Double Networks (DNs) and Ionic-Covalent Entanglement (ICE) networks. DNs comprise two networks crosslinked through covalent bonds. They can display extremely high mechanical strength and toughness, greater than those of single-component networks of either of its constituent polymers, and comparable to those of cartilages and rubbers [16,102,103]. The entirely covalently crosslinked nature of DNs provides them with high bond energies and excellent mechanical properties. Namely, DNs can remain elastic under important deformations (up to 50%), be nearly strain rate-independent, and be less vulnerable to environmental parameters such as temperature, ions, or pH. However, their polymerization is time consuming and covalent bonds can only be irreversibly broken, making DNs prone to fatigue-induced failures [9]. ICE networks on the other hand, are formed with ionically crosslinked polymer networks that serve to dissipate energy from an applied load with covalently crosslinked polymer networks that maintains the elasticity of the hydrogel [21]. They are more vulnerable to environmental parameters and strain rate-dependent, but their ionically crosslinked networks allow for retention of mechanical properties [9]. ICE hydrogels have been successfully printed via simultaneous extrusion and photopolymerization to produce tough scaffolds with complex shapes at relatively large scales [102,104]. The structural differences between these different kinds of interpenetrating polymer networks are shown in the Figure 5.

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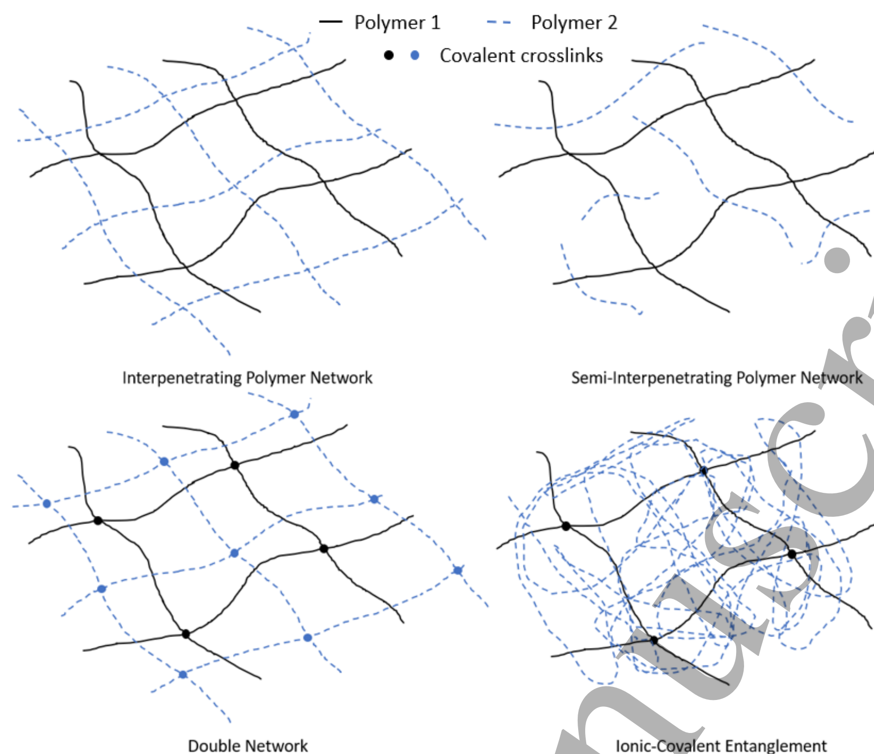

Fig. 5: Structural difference between the different kinds of polymer networks.

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2.3.3.3 Supramolecular Bioinks

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Supramolecular polymers are hydrophilic 3D networks composed of short functionalized repeating units self-assembled by reversible non-covalent interactions between functional end-groups to form large entanglements (Fig. 6). Supramolecular polymers integrate the reversibility of physically crosslinked networks along with the customizability provided by covalent bonds [105]. Subsequent bioinks can then be tuned to have similar mechanical properties than targeted biological tissues, reversible gelation properties through stimuli responsiveness, stress-relaxation, strain-stiffening, self-healing ability and shear-thinning behaviour [22,106–111]. Rodell *et al.* were able to tune the rheological properties of their supramolecular bioinks by modifying the polymer functionalization [112,113]. Unlike covalent bonds, these interactions can be reversibly broken, which enables supramolecular hydrogels to dissipate large quantities of energy under high stress, which creates tough materials capable of surviving multiple repeated deformations while maintaining mechanical integrity through self-healing, ideal for tissue engineering purposes [16,106,114,115]. This

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reversibility also gives supramolecular hydrogels interesting viscoelastic behaviours which enhance their printability: they demonstrate elastic properties below their yield point, behave like viscous liquids after yielding, and reform into an elastic solid after shear stress is removed [9]. Such bioinks have been successfully printed while exhibiting low viscosity under mechanical deformation and quick gelation post-printing to prevent supplementary flow, which results in high structural fidelity and integrity of biocompatible structures [3,111,113,116,117]. Supramolecular design also allows for enhanced drug delivery and targeting, giving the opportunity to combine multiple drugs, engineer specific stimuli-responsive and molecularly discrete releases [118–120].

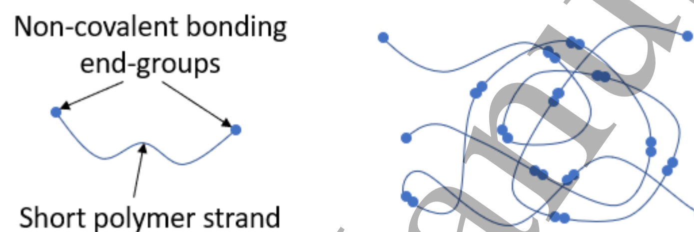

Fig. 6: Schematic overview of the supramolecular design.

However, as supramolecular hydrogels rely on non-covalent bonds, they have weak mechanical properties compared to other types of hydrogels. To assess this issue, numerous studies have assessed the combination of supramolecular with different mechanical reinforcement strategies such as covalent crosslinking. Gao *et al.* for instance, produced a chemically crosslinked GelMA hydrogel with supramolecular poly(*N*-acryloyl 2-glycine) (PACG) reinforcement [121]. Again, variations in concentrations of the polymers resulted in variations in mechanical properties of subsequent bioinks. Measurements demonstrated that the GelMA hydrogel had been considerably strengthened and stiffened by the dynamic hydrogen bonds formed by the PACG. The subsequent hydrogel displayed a high compressive strength (up to 12.4 MPa as compared to ≈200 kPa for conventional GelMA) and compressive modulus (up to 837 kPa as compared to ≈100 kPa). This novel hydrogel has been successfully printed into a biodegradable bilayer biohybrid gradient hydrogel scaffold [122] that supported cartilage and subchondral bone repair simultaneously in rat knee osteochondral defect

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and can potentially be applied in treatments of other load-bearing tissue defects. However, cells could not be directly printed within this hydrogel because of its small content of molecular ACG monomer. Thus, this hydrogel does not comply with the definition of bioink presented before. However, this strategy may still be extended to improve the mechanical strength of other natural biomacromolecule hydrogels.

2.3.4 Hydrogel Microspheres-Based Bioinks

Using hydrogel microspheres in bioinks is another trend in bioink development. These microparticles have been extensively, yet exclusively explored for several decades as drug delivery vehicles and large-scale cell expansion carriers due to their large surface-to-volume-ratios, which is an interesting feature for cell culturing applications [123]. Such particles have then been studied as fillers in bioinks, to further improve their biological performances. More recently, gels comprising of micro- & nanospheres have been developed into bioinks due to their interesting biological and mechanical properties.

2.3.4.1 Microcarriers

Microcarriers are microparticulate structures that have been extensively used to improve hydrogels cell loading capacity and viability. Microbeads, which are spherical solid microparticles composed of a porous matrix, and microcapsules, that are covered with a semi-permeable membrane, can all be referred to as "microcarriers" or "microspheres" [124]. They can be used alone for bioassembly strategies or combined for bioprinting [125]. In this latter case, microcarriers are suspended into hydrogels to act as supportive structures for cell growth and expansion of adherent-dependent cells [126]. They consist of natural or synthetic polymer spheroids with interconnecting pores creating structures with large surface to volume ratios, vastly improving cell culturing processes [126,127]. One gram of microcarriers can provide a surface area equivalent to fifteen 75cm² culture flasks [127]. Moreover, microcarriers contain the cells at the implantation site, protect them from host immune response, degradation and shear stress, while also allowing for excellent transport of oxygen, nutrients, and metabolic waste [124,128]. As aforementioned,

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microcarriers have been primarily used as drug delivery vehicles and large-scale cell expansion carriers, but recent studies proved they can also act as reinforcing units in bioinks, creating microscaffold-based bioink with superior mechanical properties [123,129]. Levato and colleagues demonstrated that microcarriers allow for phenotypic control of the seeded cells by modifying the compressive modulus of bioprinted structures, supporting differentiation of stem cells into desired lineages, and preserving phenotypic stability at high cell concentrations [125]. Their high microcarrier concentration bioink displayed good printability and the printed structures showed high cell density, viability, adhesion, and osteogenic differentiation compared to conventional cell-loaded hydrogels.

2.3.4.2 Micro- & Nano-gels

In some cases, those microparticles are densely packed to create microgel bioinks, or their nano-sized counterparts nanogels, sometimes referred to as granular hydrogels. They are comprised of crosslinked spherical hydrogel particles of typically 1-350 µm and 20-250 nm scale dimensions respectively. They have excellent biocompatibility, high water content, tuneable sizes, large surface area for multivalent bioconjugation, and abundant space to accommodate bioactive molecules such as drugs and live cells, making them very attractive for delivery [130–132]. Nanogels also have tuneable particle size, shape and sensitivity to external stimuli, allowing for controlled release properties [133,134]. In addition, different properties of nanogels can be tailored by surface modification, such as their multifunctionality and their circulation time [135]. Differently from microcarriers, micro-/nano-gels are jammed systems composed of densely packed microparticles held together by physical interactions. Such systems can deform elastically with no movement of individual particles, until the applied force overcomes the packing force determined by their internal crosslink density and movement, independently from the particles size or polymer used [136,137]. Above this threshold, the dynamics behind the rearrangement of particles and flow properties depend on the interparticle friction [138]. Bioinks produced from such systems display shear-thinning behaviours independently from the polymer used. Such property allows to use any hydrogel that

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can be processed into nano- or micro-gels [136]. Porosity and internal microstructure of the fabricated scaffolds can also be controlled independently from material used. Granular hydrogels possess three-dimensional, inter-connected, porous networks through which cells may freely migrate and mass transport occurs [139]. The size-scale of the hydrogel microspheres directly influences the size-scale of the pores they form: for example, a microgel composed of micron-sized particles possess micron-sized pores, resulting in a better cell spreading compared to nanogels because of the micron size of most cells [140–142]. Additionally, the curved surfaces of microspheres have been shown to influence cell behaviour, such as cell migration and differentiation [143]. The independence between these properties and the materials used also allows to produce granular hydrogels with heterogenous properties by using microspheres of different compositions, properties and sometimes encapsulated cells [142,144,145]. The structural difference between microcarrier-laden and granular hydrogels is shown on the Figure 7 below.

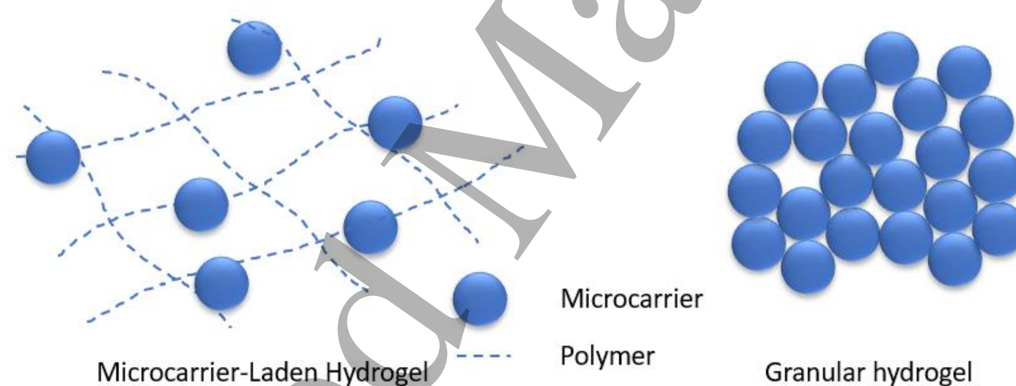

Fig. 7: Structural difference between microcarrier-laden granular hydrogels. In the first case, microcarriers are suspended into a polymer matrix which provides most of the mechanical properties, whereas granular hydrogels rely on particle-particle interactions of densely packed systems.

2.3.5 Hybrid 3D Bioprinting Strategies

Hybrid strategies in bioprinting are different than the other methods presented above. Also referred to as thermoplastic reinforcement, a thermoplastic frame is printed as the structural component to provide an organized mechanically stable scaffold, while a cell-seeded hydrogel is co-printed to compensate the lack of bioactivity of the thermoplastic (Fig. 8). These strategies closely mimic natural tissues

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which are generally composed of rigid acellular structural components, surrounded by softer matrices loaded with varying quantities of cells [146].

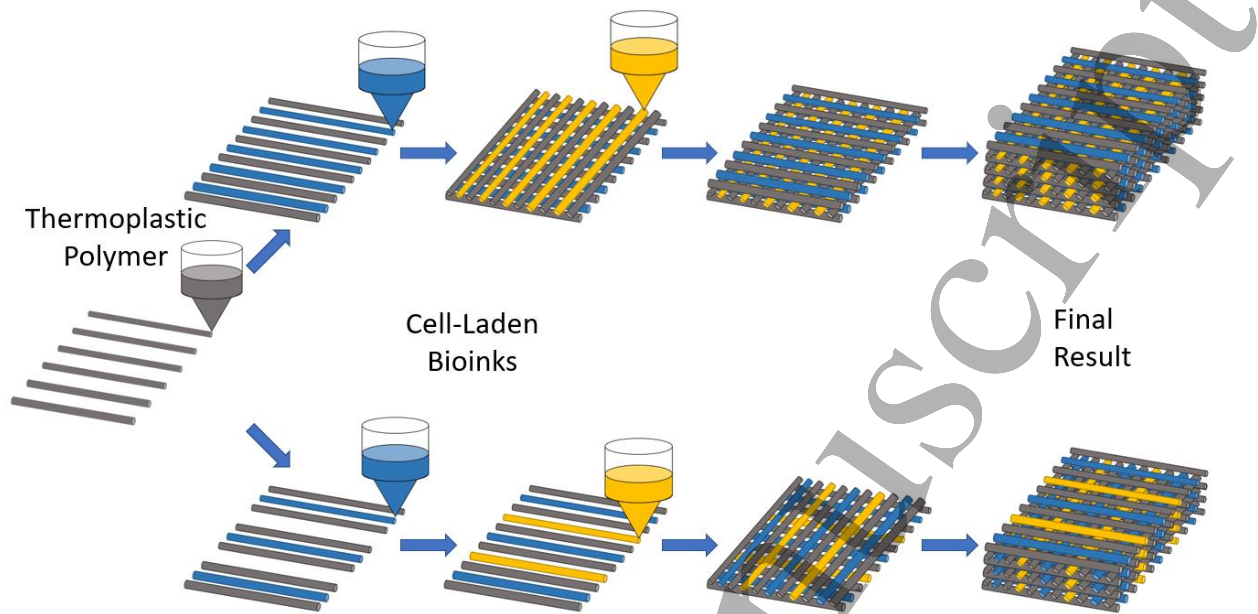

Fig. 8: Schematic overview of hybrid bioprinting processes: layers of thermoplastic polymer and cell-laden hydrogels are stacked on top of each other to create hybrid constructs. Different hydrogels can be alternatively stacked (top panel) or printed within the same layers (bottom panel).

Several studies demonstrated that the architecture of the thermoplastic scaffolds greatly affects the mechanical properties of the fabricated constructs, which allows them to be tuned to mimic those of natural tissues [146–151]. For example, Schipani *et al.* recently reinforced alginate – GelMA IPNs with PCL networks printed using fused deposition modelling to develop biomechanically competent composite constructs for cartilage tissue engineering [148]. Two different architectures of the PCL reinforcement were tested ("Double Offset PCL" vs. "Aligned PCL") to assess their influence on the mechanical properties of the resulting scaffolds. These architectures led to different mechanical properties values, and one of them led to significantly superior values compared to those of its individual components, and even their sums, and were comparable to those of native articular cartilage. Other studies demonstrated that the co-printing approach could also influence the mechanical properties of the final construct: layer-by-layer bioink deposition can for example interfere with the adhesion of PCL fibres, significantly reducing the mechanical

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properties of the subsequent construct [152]. PCL is generally used for such techniques due to its biocompatibility and relatively low melting temperature (60 °C) compared to other thermoplastics. Subsequently, co-printing PCL presents lower risks of inducing thermal injury to cells, which allows for better cell viability post-printing [9,151]. However, its long degradation time (more than 2 years [153]) may limit its use because residual filaments can act as a barrier to tissue formation [154]. Alternatives such as the chemical modifications of the PCL or the use of poly(lactic-*co*-glycolic acid) (PLGA) allow for similar mechanical properties with shorter degradation times [155,156]. Such techniques have been widely used in cartilage and bone tissue engineering for their high mechanical performances [154]. Hybrid 3D Bioprinting appears as a very promising approach to reproduce natural tissues and their organic–inorganic hybrid composite natures. Hybrid 3D Bioprinting is an effective way create highly organized composite structures with a great freedom of design, allowing to easily modify architectural parameters and play on the properties of resulting constructs. However, traditional hybrid 3D Bioprinting has intrinsic issues that limit its performance. The acellular thermoplastic frame may account for a large portion of the volume of the constructs, which would greatly reduce their cell loading capacities. In addition, cells in contact to the thermoplastic frame may be subjected to stimuli that will not direct their differentiations into the desired phenotypes. Finding the appropriate combination of acellular components and cell-loaded bioinks is a challenging task, reminiscent of the biofabrication window paradigm and the difficulty of creating bioinks with appropriate mechanical and biological properties.

3 Cutting-Edge Progress in Bioink Development

Technological progress of 3D Bioprinting and its related fields constantly allows for new technologies to be developed. Recently, cutting-edge advances in additive manufacturing and polymer chemistry permitted to improve the resolution and properties of printed bioinks. This section gives an in-sight of the most promising technologies recently developed.

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3.1 3D Bioprinting Technologies Development

3D Bioprinting dates back from 1988 when Klebe modified a standard Hewlett-Packard (HP) inkjet printer to deposit cells by cytoscribing [157]. Since then, many 3D Printing technologies have been adapted to be used in biomedical applications. Generally, 3D Bioprinting modalities can be divided into 3 main categories: extrusion-, droplet- and energy-based technologies. Extrusion-based modalities use mechanical force to extrude a continuous stream of bioink from a nozzle onto a printing platform or into a liquid medium. The force driving the flow can be issued from pneumatic pressure, from the use of a piston (or plunger) or a rotating screw [158–162]. Droplet deposition technologies lay discrete volumes of material onto a deposition surface using several droplet-forming techniques. These latter techniques rely on physical phenomena and/or mechanical devices to generate and eject the droplet [163,164]. Energy-based techniques rely on focusing an energy source (high energy light or laser) at the printing surface to solidify or stabilize bioinks through light or generated heat. These modalities can be divided into stereolithography, in which a beam of light scans the entire material layer point-by-point [165,166], and projection-based techniques which expose the whole layer to a single projection [167–169].

The easy access and extensive knowledge on the different bioprinting modalities currently available make them very appealing to many laboratories in biomedical research. They are becoming more accessible and less expensive every day, while their versatility continues to attract a broader range of researchers who are implementing them in their respective fields. Advances in other scientific disciplines allow for the transition of new technologies in bioprinting. Hence, novel bioprinting modalities continue to appear thanks to constant technological progress. Other fields of research can also serve to improve currently available bioprinting techniques. Microfluidics for example, which is the study of small volumes of fluids confined in micro-channels, is used to engineer and improve printing hardware of commonly used

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modalities, permitting to overcome the challenges they face while expanding their applications.

3.1.1 Microfluidics-Enhanced 3D Bioprinting

Microfluidics is a growing area of research which studies the behaviour, precise control, and manipulation of fluids geometrically constrained at the nano-/micro-scale, subjected to greater surface forces than volumetric ones. The recent development of this field concorded with the emergence of barriers that bioprinting faces when downsizing to micro-scale resolutions. Namely, the lack of precisely controlled multi-material deposition, cell damage during printing processes and clogging are common issues that limit bioprinting performances at finer resolutions. Thus, microfluidics systems promptly found their way to further improve bioprinting strategies [170–172]. Microfluidics-enhanced bioprinting modalities aim to improve bioink and cell handling to address these issues. Recently, many studies used microfluidics techniques in tissue and organ modelling [173–176], tissue engineering [177–179] and in drug synthesis, screening & testing [173,180,181]. In 2016, Snyder *et al.* developed a microfluidic synchronized multi-material printing nozzle with several inlets and only one outlet for enhanced multi-material bioprinting [182]. This printing nozzle allowed for the lamellar flow of heterogeneous filaments made from several materials. Their team was able to encapsulate multiple cell types into 200 nL droplets to model human liver cells for pharmacokinetic research, and they were also able to extrude hetero-cellular 3D scaffolds. More recently, Serex *et al.* developed smart print heads which improved dispensing capacities in extrusion-based bioprinting [183]. Their multi-inlets nozzles were engineered to allow fast material switching, mixing of reactive components before their dispensing at varying ratios, using hydrodynamic focusing mechanics to precisely control material thickness, and crossflow filtration to concentrate particles or cells in deposited material. This study showed the great potential microfluidics hold to improve printing resolutions and the range of materials used.

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Microfluidics-enhanced bioprinting has also been recently used to improve co-axial printing [178,179,184]. This latter technique, also called core-shell bioprinting, allows to extrude bioinks at the core of the nozzle, surrounded by a crosslinking solution, or inversely to produce hollow bioink fibres. Co-axial bioprinting can also allow to use lower viscosity bioinks which crosslink in the nozzle only, greatly reducing risks of clogging [171]. Recently, Yang and Bartolo engineered a co-axial printing nozzle using microfluidics to improve the uniformity and balance of the fluids flows inside, guaranteeing a higher alignment of polymer chains during extrusion (Fig. 9) [185]. These studies show the great potential of microfluidics to improve commonly used 3D Bioprinting modalities. Re-engineering printing hardware using acquired knowledge from other scientific fields could allow to overcome many of the limitations faced in biofabrication. Microfluidic-enhanced multimaterial printing heads bring expectations of using novel material combinations in bioprinting, while novel co-axial printing heads show great potential for printing vasculatures.

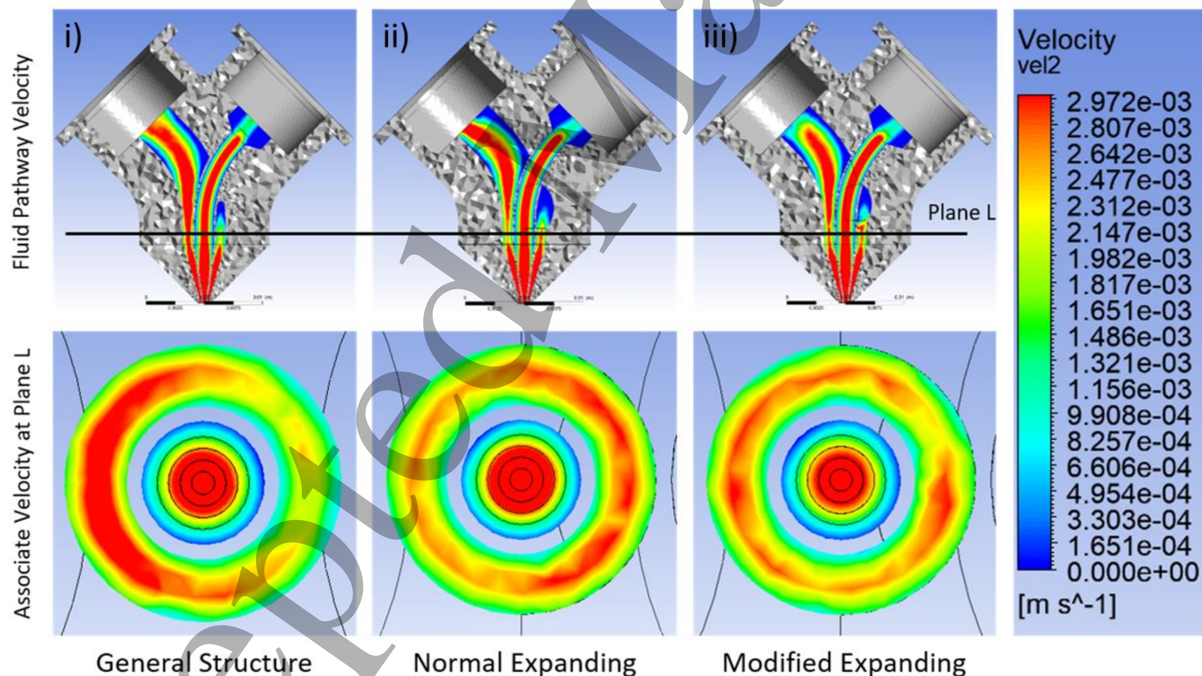

Fig. 9 Fluid pathway and associated fluid velocity profiles for (i) compression co-axial nozzle without expanding chamber; (ii) with a large expanding chamber nozzle; (iii) with a small expanding chamber. Reprinted from [185] with permission of Dr. Jiong Yang, Copyright © 2020 Infinite Science Publishing, licensed under CC BY 4.0 (http://creativecommons.org/licenses/by/4.0).

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3.1.2 Electrohydrodynamic 3D Bioprinting

Electrospinning is a versatile method to produce ultra-thin fibres with diameters ranging from the nano- to the micro-metric scale, from various materials. This method has already been used in biofabrication for applications which do not require to produce precise patterns. Since early 2000s, great efforts have been into developing a technique which allows for the controlled deposition of electrospun fibres. In 2011, Brown *et al.* were able to combine melt electrospinning and layer-by-layer additive manufacturing to produce 3D scaffolds from PCL with uniform 10 µm fibre diameter in controlled and automated manner [186]. This technique greatly improved fibre resolution to the cell-size scale. Since then, melt- and solution-based electrohydrodynamic bioprinting technologies have been extensively developed to produce high resolution micro/nanoscale scaffolds and living tissue constructs [187]. In 2016, Yeo *et al.* proposed an electric field-assisted extrusion cell printing technique in which the electric field stabilize the extruded struts of bioink to reduce cell damage due to shear stress during extrusion [188]. This technique allowed for rapid production of cell-laden structures with a 300 µm resolution and high initial cell viability (87%). More recently, He *et al.* further improved this technology by fine tuning its parameters, and were able to print alginate filaments with a diameter of 80 µm with higher cell viability (95%) as seen in Fig. 10 [189]. However, the height of their scaffolds was limited to 145 µm due to crosslinking failure. The same team then used a co-axial printing head to incorporate calcium chloride, inducing instant crosslinking [190]. This technique allowed to print 80 µm diameter bioink filaments with high cell viability (>90%) into 3D living constructs with a maximum height of 1465 µm.

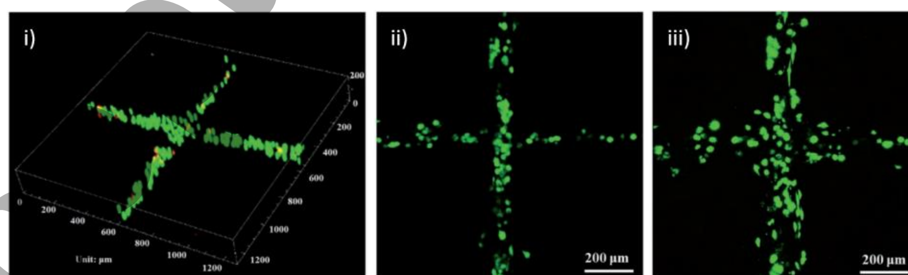

Fig. 10 Electro-hydrodynamic printing of multiple layers of cellular structures. (i) 3D fluorescent profile of electro-hydrodynamically printed cell-alginate hydrogel with ten layers. (ii, iii) Cell viability and morphology when cultured for 1 and 7 d in vitro. Reprinted with permission from [189]. Copyright © 2017 John Wiley & Sons, Inc.

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Electrohydrodynamic printing can also be used to produce hybrid scaffolds with nano-/microscale thermoplastic reinforcements. In 2017, Bas *et al.* used melt electrospinning writing (MEW) to produce medical grade poly(ε-caprolactone) (mPCL) microscale fibrous networks used to reinforce poly(ethylene glycol)/Heparin hydrogels [191]. The resulting constructs displayed compressive moduli up to 42 times higher than those of not reinforced scaffolds, comparable to the compressive modulus of natural human articular cartilage. Moreover, the mPCL scaffolds only accounted for 12% of the volume of the final constructs. These composite constructs displayed viscoelasticity, mechanical nonlinearity and anisotropy while also exhibiting great biological properties. These structures supported high chondrocyte viability and their differentiation under compressive strains comparable to those exerted during natural tissue function. However, the fabrication of the scaffolds was time-consuming. These constructs resulted from a two-step process, involving the printing of the thermoplastic networks, and their subsequent infiltration by PEG/Hep hydrogels. This approach greatly limits the freedom of design for the microscale reinforcement structure, the use of multiple materials and of cell types. In 2018, de Ruijter and colleagues combined the extrusion printing of cell-embedded bioinks and the MEW of sub-micrometre fibres into a single-step biofabrication process [192]. This technique allowed for the first time the 3D deposition of MEW fibres within precisely organized bioprinted structures (Fig. 11). Very recently, the same group produced a personalised osteochondral implant in a similar fashion [193]. The condyle-shaped implant was composed of a chondroprogenitor cells-laden GelMA hydrogel supplemented with MEW PCL fibres. During 28 days of *in vitro* culture, this construct maintained its geometry, displayed enhanced compressive properties, and supported abundant cartilage-like matrix formation. Electrohydrodynamic 3D Bioprinting is a successful approach to produce highly organized 3D composite living constructs with fine resolutions and close similarities to native biological tissues in clinically relevant applications.

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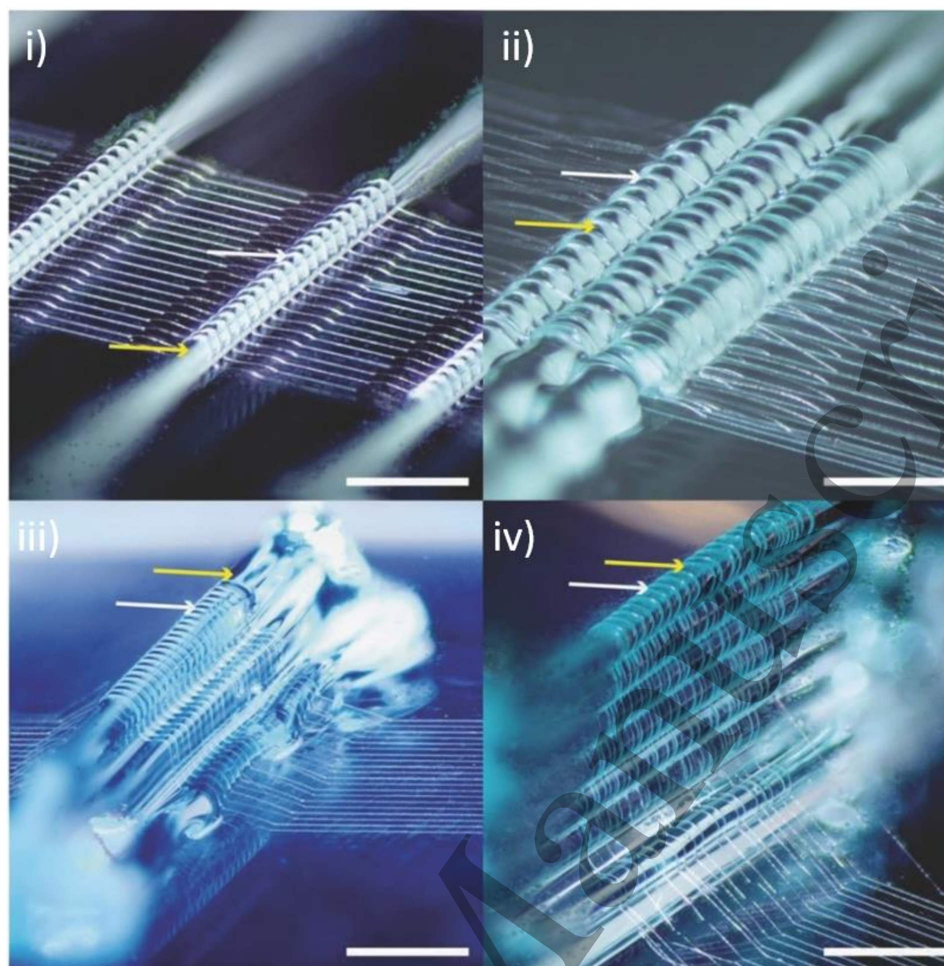

Fig. 11: Pluronic hydrogel (40% w/v, yellow arrows) is used to guide de direction of MEW PCL fibres (white arrows). MEW fibres are printed over: (i) a single strand of Pluronic, or (ii) interlocked within hydrogel strands. This allows for (iii) more complex fibre architectures and (iv) out-of-plane fibre deposition. Scale bar = 500 µm. Reprinted with permission from [192]. *Copyright © 2018 John Wiley & Sons, Inc.*

3.1.3 Voxelated 3D Bioprinting

Similarly to pictures displayed on screens which can be broken individual pixels carrying specific colorimetric characteristics, 3D structures can be broken down to matrices of voxels, which are finite-volume-elements transporting the characteristics of the structure (material composition, mechanical and physical properties, etc.) depending on its spatial localization. Voxelated, or bitmap, 3D Printing is an emerging modality which relies on the printing of discrete volumes of distinct materials to build up structures, in opposition to conventional CAD 3D Printing which relies on the continuous distribution of material along determined geometries [194–196]. This

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approach, where the materials have not yet been combined with cells, serves to precisely modify local properties of structures on the scale of the printed voxels. Ideally, the volume of these voxels is equal to D^3, with D being the diameter of the nozzle. This technique however presents a major drawback: to preserve the resolution of a construct when its linear scale is increased by a factor L, the number of voxels and the subsequent printing time are increased by L^3. Thus, voxelated printing has limited performance for large structures fabricated using materials with short printing windows. Recently, Skylar-Scott *et al.* designed a multimaterial multinozzle 3D printer to rapidly print voxelated structures at large scales, capable of depositing 4 different materials through 16 nozzles [194]. This technique allows to greatly reduce the printing time, but is limited to produce 3D structures which complexities are limited to periodic layouts since each nozzle cannot switch material independently. So far, voxelated modalities has not been used in bioprinting applications yet. But this technique shows great promises to precisely deposit multiple biomaterials, drugs or cells, to create complex scaffolds with a precise microstructure.

3.1.4 In Situ and In Vivo 3D Bioprinting

3.1.4.1 In Situ 3D Bioprinting

The rapid expansion of computer-assisted medical intervention attracted a broad range of researchers to develop tools to accelerate new tissue implantation and improve patient outcomes. Technological progress in medical imaging allowed for the production of patient-specific scaffolds from detailed topographical analysis of defect sites [197]. In the last few years, expectations of efficient *in situ* cell-laden tissue deposition were brought closer by improved bioprinting technologies which could operate at mild conditions. In 2015, Catros *et al.* proposed a novel approach of biomaterial layer-by-layer deposition using laser-assisted bioprinting (LAB) of nanohydroxyapatite in mice calvaria defects [198]. They successfully printed *in situ* 30-layer scaffolds which supported bone regeneration after 4 weeks in the defect areas. In 2017, the same team used LAB for the *in situ* deposition of MSCs-embedded

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collagen/nanohydroxyapatite bioinks in a similar application [199]. *In vivo* assessment demonstrated that printed MSCs could proliferate for 42 days post implantation and improve the regeneration of the calvaria defects (Fig. 12). This technique was since improved to induce pre-vascularization of critical bone defects, and further enhance bone regeneration [200].

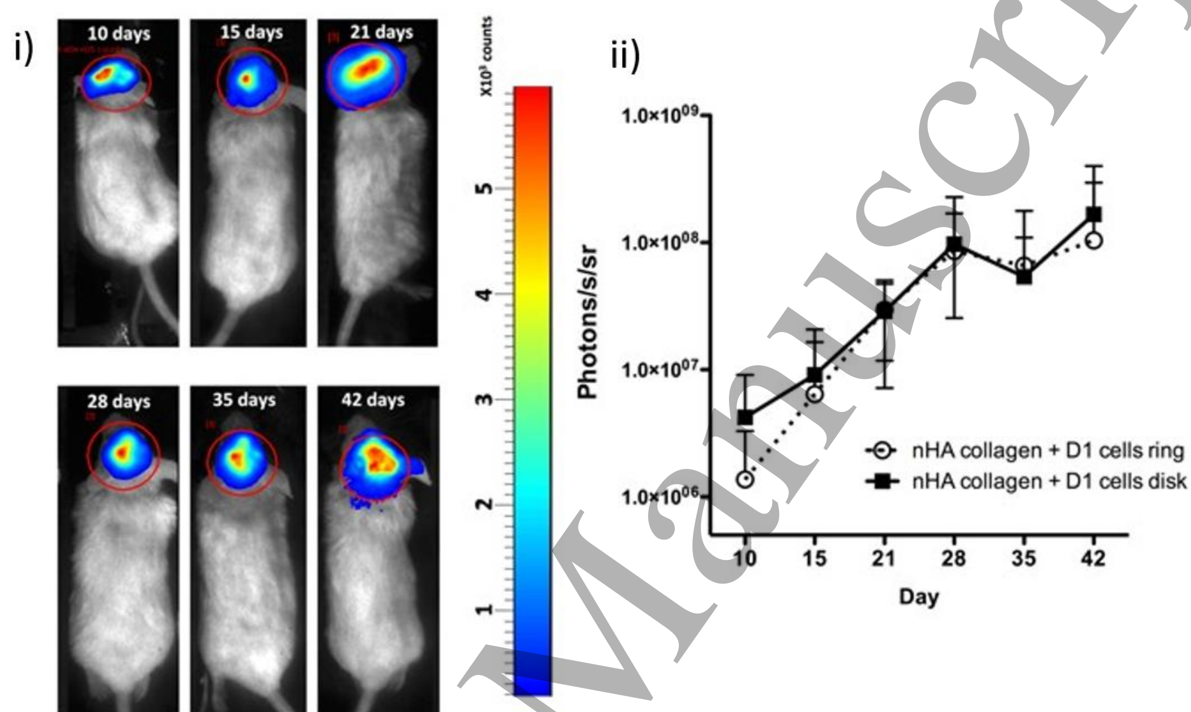

Fig. 12: (i) Representative luminescence imaging of luciferase positive D1 cells in a ring geometry at 10, 15, 21, 28, 35 and 42 days post printing, in a mice calvaria model. (ii) Quantification of the luciferase signal of luciferase positive D1 cells in a ring and disk geometry in a mice calvaria model (Average ± SD, n = 5). Reprinted with permission from [199]. Copyright © 2017 Virginie Keriquel et al., licensed under CC BY 4.0 (http://creativecommons.org/licenses/by/4.0).

3.1.4.2 In Vivo 3D Bioprinting

Very recently, the trend of minimally invasive and non-invasive *in vivo* procedures took a major step forward with its development in bioprinting techniques. In 2020, the team of Zhao *et al.* established the proof of concept of a novel *in situ in vivo* bioprinting process using microrobots, as a first step towards the development of a novel bioprinting strategy [201]. They developed a minimally invasive micro bioprinting platform to be placed at the extremity of an endoscope. This platform folds itself when entering the body, then unfolds before beginning the bioprinting operation. They first tested the feasibility of their system by printing two-layer tissue

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scaffolds into biological models of human stomach, then they assessed the bioprinting performance in cell culture dishes. Printed cells remained viable for 10 days and displayed steady proliferation. This study showed the great potential of their novel bioprinting method to carry out tissue repair inside the body.

A few months earlier, two separate teams published their research on relatively similar *in vivo* light-induced 3D Bioprinting techniques (Fig. 13). Chen *et al.* used digital near-infrared (NIR) photopolymerization (DNP) to noninvasively print *in vivo* 3D biological tissues [202]. Their approach relied on subcutaneously injecting a photo-crosslinkable bioink *in situ*. This bioink was then printed by *ex vivo* irradiation of patterned NIR into customized tissue constructs. They successfully printed in mice personalized ear-like tissue constructs which supported chondrification, and cell-laden conformal scaffolds for muscle tissue healing. To achieve these technical feats, they developed an initiator which relies on the conjugated effects of an up-conversion nanoparticle (UNCP) coated with UV/blue-light photo-initiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP). Succinctly, the NIR irradiation of the UCNPs resulted in the emission of UV photons by up-conversion. These photons were then absorbed by the LAP coating to initiate photopolymerization, which allowed to avoid the detrimental effect that UV induce onto cells. Less than a month later, Urciuolo *et al.* published their study on a similar process using two-photon crosslinking to precisely produce constructs across and within tissues in different target organs in live animals, along with real-time imaging [203]. Their team used laser with 850 nm wavelengths to allow for deep tissue penetration and functionalized their hydrogels with coumarin derivatives as NIR photosensitive crosslinking moieties. Their strategy, called intravital 3D Bioprinting, allowed for the fabrication of tissues such as the dermis, skeletal muscles, and brain. Although these techniques are highly perfectible, they are formidable breakthroughs in the field of 3D Bioprinting. These studies bring closer the expectations of non-invasive procedures to correct tissue defects. Bypassing invasive tissue replacement surgeries to closely mimic endogenous tissue repair would avoid damages to surrounding tissues and scar formation, and could greatly improve patient outcomes and rehabilitations, while simplifying and accelerating the treatment

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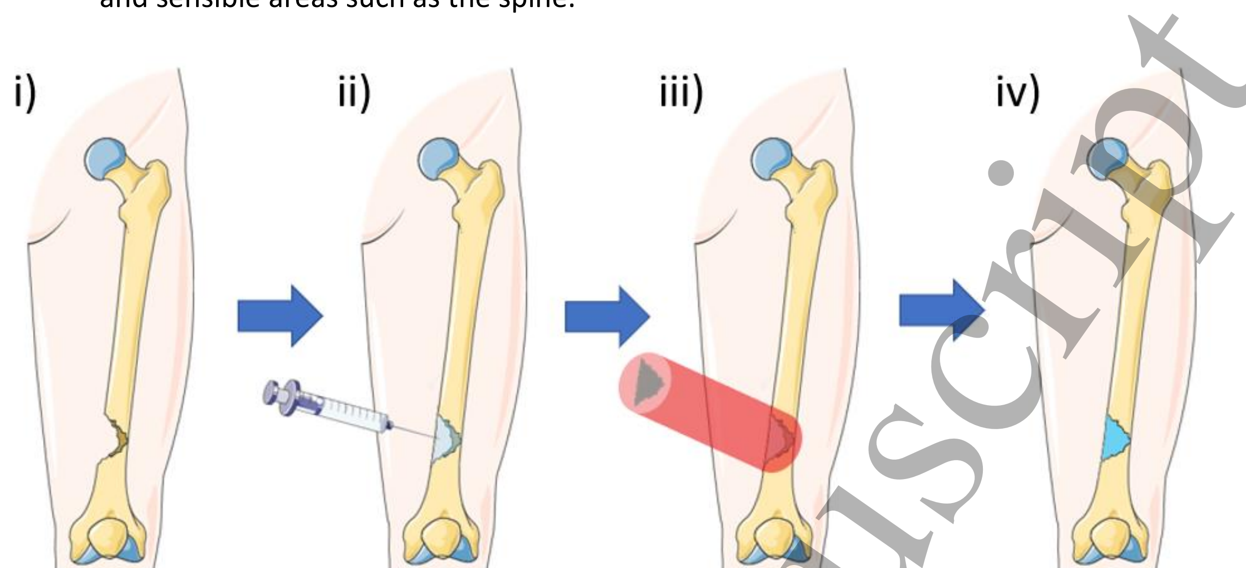

Fig. 13: Principle of noninvasive in vivo NIR light-induced bioprinting. (i) A tissue defect necessiting a graft is detected. (ii) A photocrosslinkable bioink is injected in the defect area. (iii) Ex vivo NIR light irradiation is used to locally crosslink the bioink and create the tissue substitute. (iv) The fabricated construct remains in the body and acts as any bioprinted implant. This figure was created using images from Servier Medical Art Commons Attribution 3.0 Unported License.(https://smart.servier.com/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

3.2 Next Generation of Bioinks

Similarly to bioprinting processes, bioink chemistry recently met considerable improvements initiated by the technical development of polymer chemistries. Herein, we review some very promising approaches to further improve bioinks, rising expectations of further progressing in the biofabrication window.

3.2.1 Combinatorial Bioinks

Combinatorial bioinks rely on the dual effect of combined non-mutually exclusive bioink reinforcement strategies previously described. Nanoparticles for example, can be easily integrated within polymeric network while having direct outcomes on resulting hydrogels properties. Recently, IPNs have been reinforced with

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fibres to produce composites and further enhance their mechanical properties [9,204]. In 2018, Chimene and colleagues developed a bioink that combines nanocomposite and ionic covalent entanglement (ICE) reinforcing strategies into a nanoengineered ionic-covalent entanglement (NICE) (Fig. 14i) [205]. Their bioinks were successfully printed into personalized cell-laden scaffolds with high structural fidelity and mechanical stiffness, with great potential in tissue engineering applications. These NICE bioinks were produced from bioactive GelMA with kappa-carrageenan (κCA) and reinforced with nanosilicates (Laponite). Subsequent bioinks displayed shear-thinning behaviours, and fabricated structures became tough and elastic after crosslinking. Stress and frequency sweep results showed that NICE reinforcement (14.2± 1.4 kPa) resulted in a storage modulus four-fold higher than GelMA (3.1±0.3 kPa) and two-fold higher than GelMA/κCA ICE (7.4±0.9 kPa). Compression tests also demonstrated that the NICE reinforced hydrogel was more stable after multiple cycles than the GelMA/κCA ICE. Furthermore, embedded cells maintained high viability (>90% immediately after 3D Bioprinting), attachment, and spreading throughout a 120-day period. They also assessed the effects of different formulas of NICE with various component ratios on mechanical properties (Fig. 14ii) and printability [206]. They concluded that varying the component ratios of such IPNs has the potential to tailor mechanical performance and printability in tandem.

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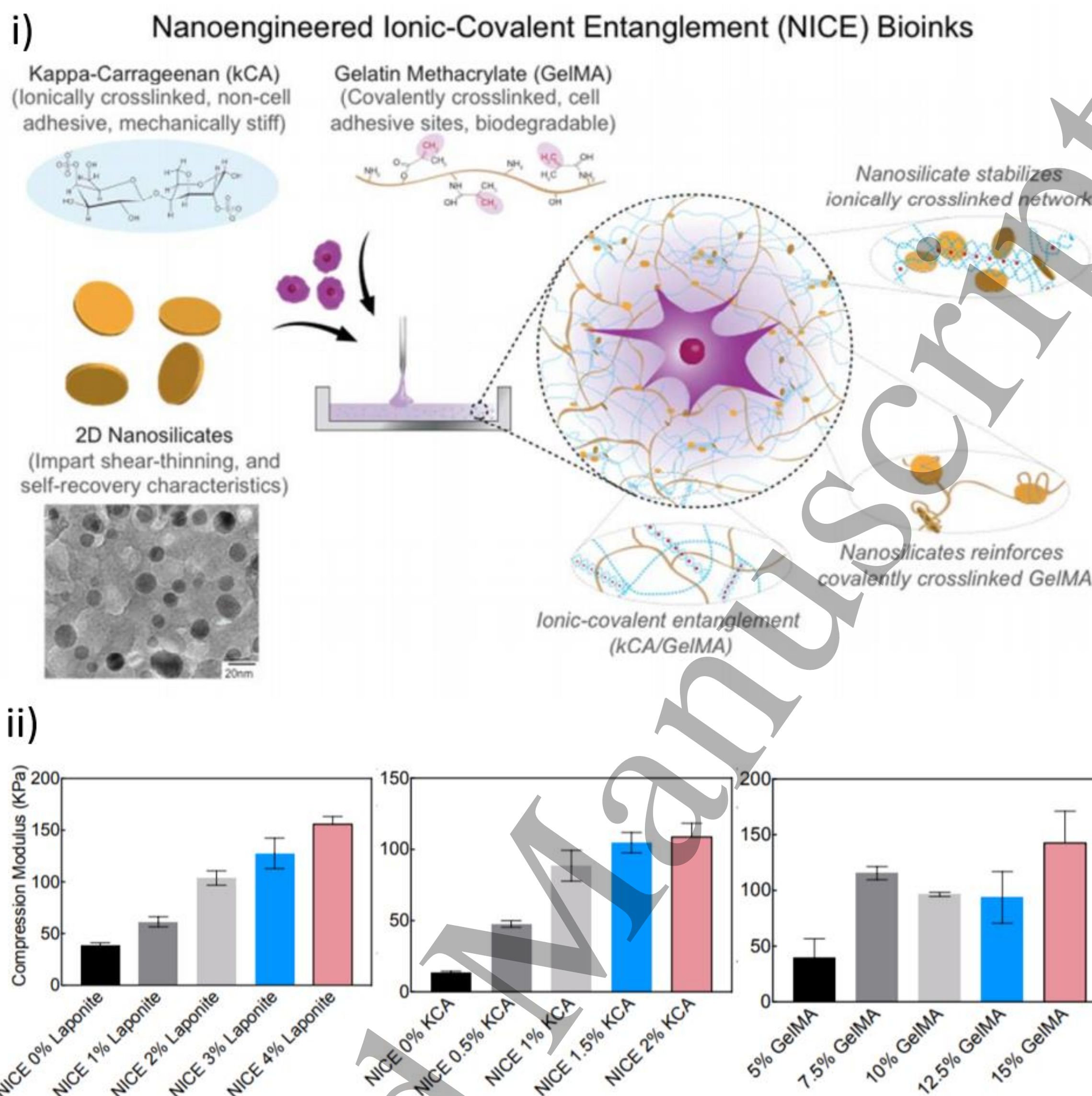

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Fig. 14: NICE bioinks combine nanocomposite reinforcement and ionic-covalent entanglement reinforcement mechanisms to create a bioink that is tough, elastic, and highly printable. (i) NICE bioinks use nanosilicates to reinforce an ionic-covalent entanglement hydrogel made from GelMA and κCA, creating a dually reinforced hydrogel network. These interactions allow the NICE bioink to behave as a solid at low shear stresses and improve shear thinning characteristics during bioprinting. After crosslinking, ICE and nanosilicate reinforcement synergistically improve mechanical strength. TEM imaging of two-dimensional nanosilicate particles showing uniform morphology. Reprinted with permission from [205]. *Copyright © 2018 American Chemical Society. (ii) Mechanical properties of different formulas of NICE reinforced bioinks. Reprinted with permission from* [206]. *Copyright © 2018 Society For Biomaterials.*

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Similarly, in 2017, Zhai *et al.* supplemented a poly-(N-acryloyl glycinamide) (PNAGA) supramolecular hydrogel with nanoclay (Laponite XLG) nanoparticles [207]. The resulting hydrogel displayed high strength from dual amide hydrogen bonding and nanoclay-polymer chain interactions, while the release of Mg^{2+} and Si^{4+} from the

Laponite was shown to promote the osteogenic differentiation of rat osteoblasts cells. Scaffolds produced from this supramolecular nanocomposite were implanted in tibia defects of rats and displayed considerable new bone formation compared to non-treated defects during the first 8 weeks.

In 2018, the same group produced a supramolecular double network hydrogel based PNAGA and sodium carboxymethyl cellulose [208]. Both polymers have separate crosslinking mechanism: PNAGA used hydrogen bonding crosslinking, whereas sodium carboxymethyl cellulose is crosslinked through Fe^{3+} coordination. The resulting hydrogel was entirely physically crosslinked, exhibiting high compressive mechanical properties (7.3 MPa at 80% strain without fracture), high stiffness, toughness, and tear energy. It also exhibited resistance to fatigue and self-recoverability due to its reversible physical crosslinked nature. Interestingly, this hydrogel could also repair fatal damage after adequate treatment, leading to full recovery of its mechanical properties. This hydrogel has great potential to be used as a load-bearing soft tissue substitute.

3.2.2 Engineered Living Materials as Bioinks

In addition to encapsulating cells into bioinks, various studies worked on incorporating functional microbes as biocatalysts. Engineered Living Materials (ELMs) are composed of living cells that form or assemble the material itself, or modulate its functional performance in some manner [209]. They are designed to recreate the dynamic features of biological tissues by using the biosynthetic potential of living organisms. ELMs are meant to display properties such as self-regeneration, self-healing, environmental responsiveness, and homeostasis [210,211]. Most studies utilize diluted cell suspensions added to bioinks. These bioinks have low cell loading capacities which limits their efficiencies. Recently, Qian *et al.* developed a new kind of bioink system that employs freeze-dried live cells as both biocatalysts and fillers, resulting in a high-performance bioink with unprecedented cell loading density (42.8 wt % or 75 vol %) [212]. Nanocellulose was also mixed in the bioinks as a secondary

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filler to provide shear-thinning behaviour and the ability to fine tune the cell density and intercellular distance, resulting in an optimal control over bioink rheology for 3D Bioprinting purposes. Self-supporting constructs could be fabricated with a fine resolution (filament thickness down to 100μm), providing a high and tuneable cell density. The encapsulated cells showed long-term viability up to 4 months (comparable to the viability of cultured cells) and could redistribute themselves via local proliferation. Baker's yeast (Saccharomyces cerevisiae) was used as an example, but this technique is believed to be compatible with other kinds of cells, enzymes, and catalytic microbes to address a broad range of bioprocesses. Its use towards biomedical applications has yet to be determined, but it appears as a promising approach to produce mechanically viable living constructs with very high cell loading capacities.

3.2.3 Transformable Bioinks for 4D Bioprinting

4D Bioprinting is an emerging field defined as an extension of traditional 3D Bioprinting by incorporating "time" as a 4th dimension. This strategy aims to produce structures that can change their shape and/or functions post-printing for practical purposes. Structures comprising complex features, such as overhangs, are difficultly achieved by common 3D Bioprinting techniques, and generally require support structures or sacrificial material to be constructed. 4D Bioprinting utilizes transformable bioinks that allow to obtain structures that cannot be originally printed or implanted as such. Such constructs have already found applications in *in vitro* tissue modelling, cell culturing, and tissue engineering by mimicking the geometry of tissues/organ and/or their functions [213–219]. Ultimately, transformable bioinks could provide a minimally invasive implantation routes for complex structures.

Achieved transformations can be categorized on two levels. First, the evolution can occur spontaneously or be triggered by external stimuli. This latter option is generally preferred as it allows for more precision and control over the process. Secondly, the transformations can be categorized as one-way, two-way, or multi-way.

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If we consider structural evolutions, one-way shape morphing structures change their shapes only once in their lifetime, without possibility to recover to their original shape by themselves. It is different from normal deformations or degradations in the way that these transformations are predicted and designed as functional features of the constructs. Two-way shape morphing structures can recover to their original geometries and repeat the cycle of morphing-recovering. The recovery can be due to the removal of the stimulus that triggered the first transformation, or by applying another stimulus. Multi-way shape morphing structures can transform and recover between several preprogramed shapes. Thus, they require multiple transformable materials, triggered by distinct stimuli [213].

Various stimuli are currently used to trigger the evolutions of transformable bioinks. Early research worked on moisture-responsive materials which could absorb liquids such as water or ethanol and deform into desired shapes [214,220]. Miao and colleagues for example, produced two-way shape morphing flower structures that contracted when submerged in water and bloomed when submerged in ethanol (Fig. 15) [214]. Other stimuli such as temperature [221], light [222], electric [223] and magnetic [224] fields, mechanical force [225], chemical (pH [226], ionic concentration [227]) and biological cues [228] have then also been used. For biomedical purposes, these stimuli must be biocompatible, as well as the materials they transform. These smart materials must also comply to all the obligations required for 3D bioinks previously stated. However, several techniques allow to use non-biocompatible smart materials in cell-laden constructs by minimising the contact between cytotoxic smart materials and cells. A possible strategy is to print a 4D non-biocompatible structure along with a 3D biocompatible one. This first structure will deform accordingly to a stimuli, while the second structure will act as a reservoir for the cells, indirectly transformed. Following the same principle, Luo *et al.* printed a cell-laden shape-morphing biphasic structure of alginate/polydopamine and alginate/gelatin methacryloyl hydrogels [222]. The first polymer allowed the printed structures to be deformed by near-infrared irradiation while the second one allowed to encapsulate cells. Their study also highlighted the effect of varying stimulating, printing and

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geometrical parameters on the deformations. By changing the concentrations of the materials, the designed patterns in the scaffolds, the laser power and the irradiation time, their team successfully transformed the same original shape (a bilayered scaffold) into different structures (tubes and saddle-like constructs).

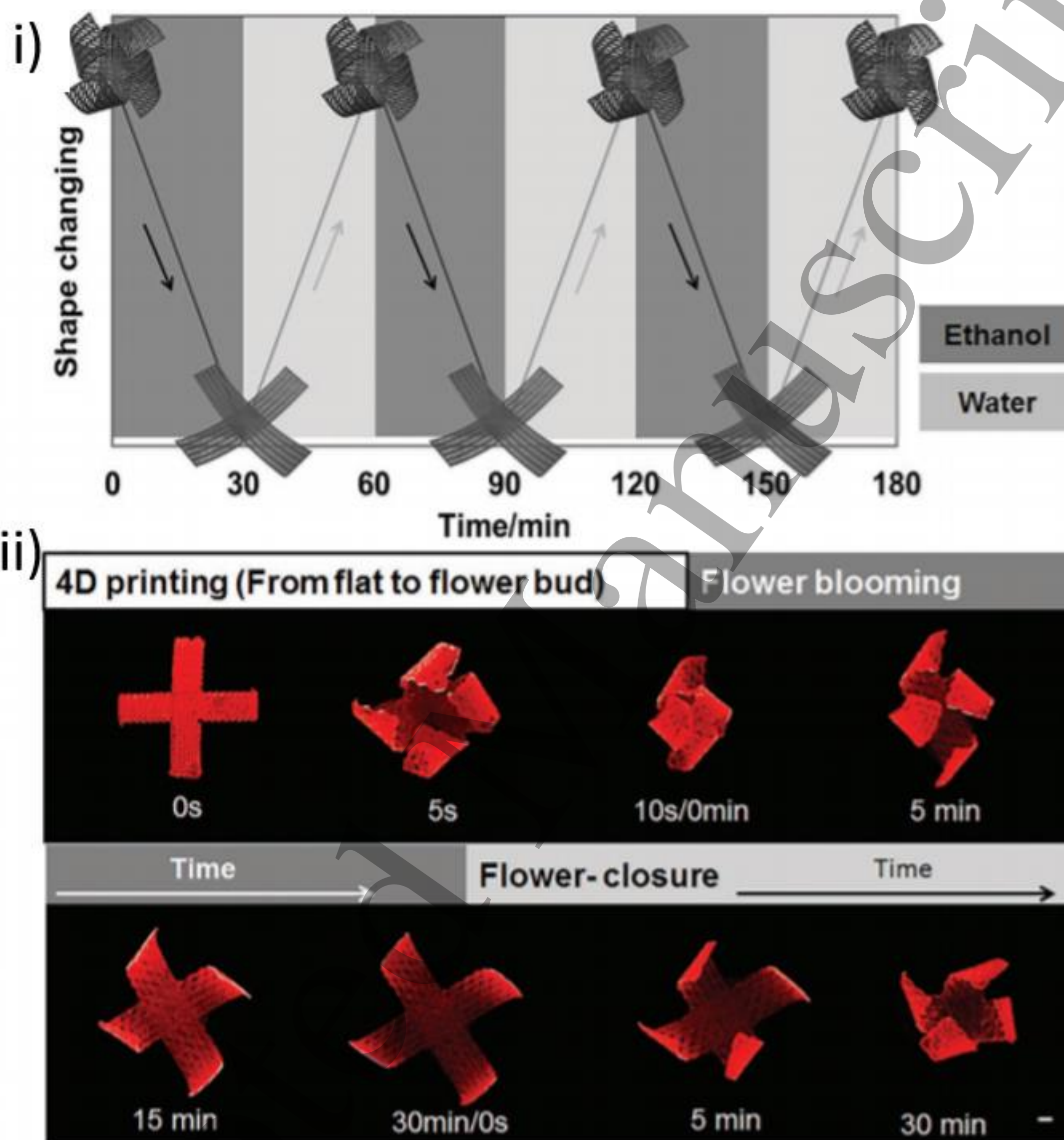

Fig. 15: Example of a two-way shape morphing structure. (i) The principle of the reversible and repeatable dynamic process of a 4D printed flower structure which blooms when submerged in ethanol and closes when submerged in water. (ii) Pictures of the process: at 0 s, before the structure undergoes shape morphing; at 5 s, after 4D shape change as it was immersed in ethanol immediately; 0–30 min, the flower structure gradually flattened as it was soaked in ethanol; flower closure (0–30 min), the flattened structure gradually recovered its flower shape after it was immersed in water. Scale bar, 2 mm. Reprinted with permission from [214]. *Copyright © 2018 John Wiley & Sons, Inc.*

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4D bioprinting has a considerable potential for biomedical purposes, but a lot of improvements are still required. Just as the transition from 3D Printing to Bioprinting, not all materials used in 4D Printing can be used in 4D Bioprinting. A whole new set of biomaterials is indeed required to produce these new transformable bioinks. This technique is also still very expensive and can be challenging to implement. Computational models are required to assist in designing 4D constructs, but sophisticated & reliable models are high-priced and difficult to make. Current studies mainly rely on empirical data and the experimental pursuit for the desired transformation. Furthermore, existing shape-morphing processes of 4D printed structures are still very simple and cannot yet meet the requirements for complex structures in clinical applications.

Recently, numerous studies have also focused on temporally incorporating materials with highly complex self-assembly/disassembly mechanisms based on out-of-equilibrium dynamics. These mechanisms include enzymatic reactions [229–234], chemically driven [235–239], redox reactive species [240,241], light-induced [242], and magnetic field-induced [243], and could potentially allow to closely mimic natural tissue generation. However, such strategies still have a long road ahead before making their way into biofabrication and bioprinting.

4 Clinical Translation and Future Perspectives

To date, 3D Printing has met great success in biomedical applications to produce personalized orthopaedic protheses, maxillofacial implants, anatomical models and other acellular devices [244–247]. However, despite the extensive development of 3D Bioprinting which allowed researchers to produce living constructs with closer similarities to natural tissues, finer resolutions and higher cell viabilities, this technology still faces considerable challenges for its clinical translation. We herein review the challenges 3D Bioprinting must face, and the opportunities it is given to become a standard procedure in future treatments.

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4.1 Current Challenges of 3D Bioprinting

The current trend of biofabrication is to keep increasing the complexity of produced constructs to better mimic natural tissues and organs. This complexity relies on the hierarchical organization of fabricated constructs at various scales, with suitable mechanical properties, geometries, and functions. Integrating the adequate environment required for the survival and the differentiation of stem cell into the desired phenotypes is an essential prerequisite to achieve relevant tissue constructs. This challenge is being approached by the multiscale, multimaterial fabrication of highly organized structures. 3D Bioprinting is a promising tool to achieve such constructs, but different bioprinting modalities are generally required to achieve the optimal printing of various materials at their relevant scales. Combining different bioprinting modalities into a single construct can be challenging as they may require distinctive hardware, printing conditions, etc.

Printing highly complex structures at large scales, or switching between several deposition modalities for example, also increase the fabrication time, which can be detrimental to cell viability [248]. During the printing process, cells are exposed to an environment which may not be cytocompatible, resulting in potential cell death. Increasing printing speeds can reduce this exposure time, but it may increase shear stresses to which the printed cells are subjected. However, the printing environment can also be tuned to mirror the conditions found in an incubator to allow higher cell viability throughout printing [249].

One of the major challenges in producing relevant 3D living structures remains in the vascularization of the constructs [250,251]. Vasculatures are essential to maintain the viability and functionality of tissues and organs by allowing for the delivery of nutrients and oxygen, and the removal of metabolic waste. Non-vascularised tissues encountering insufficient nutriment and oxygen ingress are subjected to incomplete tissue formation or necrosis [252]. *In vivo*, most living cells lie within 100-200 µm from at least a single capillary from vascular network,

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corresponding to the diffusion limit of oxygen [253,254]. Bioprinting vascular networks faces challenges in terms of printing resolution, printing speed, structural and functional integrity [250,251,255,256]. Capillaries, for example, may be as small as 3 µm in diameter [257], a resolution so far only achieved by two-photo lithography, a high-end light-based bioprinting technology limited to laboratory applications due to its slow and highly expensive functioning [255,256,258,259]. Besides, high-resolution printing usually requires low viscosity biomaterials which generally do not possess the structural integrity required, making them prone to collapsing [260,261]. Additionally to vasculatures, clinically relevant 3D bioprinted constructs also still lack nervous and lymphatic components, which are already integrated into organs- and tumours-on-chips for *in vitro* modelling [262–264].

Most of currently bioprinted tissues also contain one or two cell types only, missing many functional and supporting cell types required for the natural behaviour of biological tissues. The bioinks in which they are loaded must be able to guarantee their survival during printing and implantation, as well as their growth, differentiation, migration, and organization into relevant biological structures. Usually, cells are encapsulated within soft hydrogels, which are combined with strong synthetic materials to create multimaterial bioinks. Such approach implies that cells may not be exposed to the appropriate cues to direct their differentiation into the desired phenotypes.

Finally, the regulatory approval procedure for 3D bioprinted constructs must be addressed. Although these structures are produced from similar materials than many currently approved implants, bioprinted constructs must undergo a thorough assessment of their properties and characteristics before being possibly used in clinical applications. These evaluations are time consuming and lead to considerable delays before the applications of 3D bioprinted constructs. So far, only a few bioprinted implants have been used. Efforts must be made to improve the current situation and accelerate the regulatory approval of these structures.

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4.2 Future Perspectives

As presented, 3D Bioprinting still faces many challenges to produce clinically relevant living constructs. Technological progress in fields such as polymer chemistry and additive manufacturing is essential to keep improving bioinks to better comply with application-specific requirements for enhanced printability and biocompatibility. Different from these challenges, several trends can be distinguished from current literature, giving an in-sight into the future of the field.

Bioprinting hardware is still rather expensive compared to other technologies. Current research generally focuses on producing bioinks from easily accessible materials to reduce costs. Generic and/or universal bioinks should provide a short-term solution but limits the personalized dimension of this technology. Marine-based materials spark vivid interest due to their availability and low costs. Our group has been working on extracting collagen from Atlantic codfish swim bladders and skin, which are direct by-products of its processing into food products [265,266]. These by-products are generally being directed to animal feed, thus extracting collagen from them represents a valuable strategy for their revalorization.

Most of current studies have also been focusing on reproducing accurate tissue substitutes, leading to the high complexities of tissue bioprinted constructs as aforementioned. So far, this biomimicry strategy has only resulted in close approximations of natural tissues, and it is uncertain whether the high costs and difficulties encountered are worth the outcomes. A few studies have been drifting away from that strategy, prioritizing the functional aspect of their constructs over their similarities to natural tissues. Keriquel *et al.* for example worked on different cell printing patterns and demonstrated that specific cellular arrangements could enhance bone tissue regeneration [199]. The influence that printing parameters have on cells must be further explored for this new functionality-oriented biofabrication strategy to produce more efficient constructs.

The bioprinting community also widely agrees on needing more standards and guidelines. None of these currently exist regarding the technologies, materials or

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processes used. Evaluative standards and metrics for quality control could also help comparing studies between themselves more accurately. Mechanical properties can be assessed with standardized methods which provide relatable measurements, but the evaluation of cell viability for example, differ from one study to another, regarding the cells employed, the culture media, etc. Standardization of the field would most certainly accelerate its development, regulatory approvals, and clinical translations.

Finally, most of studies have taken place *in vitro* so far. Naturally, bioprinted constructs must be tested *in vivo* in small and large animal models for an accurate screening of their performance and to validate their success. The long-term *in vivo* assessment of bioprinted constructs also allows to ensure that these structures survive, integrate and remodel in the body in desired manners.

5 Conclusions

3D Bioprinting performances greatly relies on the bioinks ability to produce stable high-resolution constructs while maintaining cell viability during and after fabrication. Bioink properties are heavily influenced by material choice and printing parameters. Therefore, they can be tailored to match patient specific and tissue specific requirements. Two main classes of materials are used to produce bioinks: natural and synthetic polymers. Each class has its own benefits over the other and can be used alone or in blends to provide enhanced performance for the preferred bioprinting methodology and for the intended application. Properties influencing material selection for bioinks include biological, printability, structural integrity, mechanical, and biodegradation. Performances of single-component systems are limited since properties required for optimal printability and print fidelity are at odds with those that enhance cell viability and function. However, the development of bioprinting modalities and the emergence of a new generation of bioinks offers a solution. Novel bioinks are formulated form several materials at different scales, and are being printed at higher resolutions to allow limitations of the past to be overcome. The development of these innovative bioinks, brings the biomedical engineering

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community closer to the clinical expectations of fabricated constructs of all-encompassing ideal properties capable of replicating native tissues, whilst further enhancing regeneration and therapeutic outcomes.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

Conception and design of the manuscript: G.D., J.C, J.M.O. Drafting the manuscript: G.D., J.C, J.M.O. Critical revision of the manuscript: G.D., J.C, J.M.O., M.N.C. Final approval: G.D., J.C, J.M.O., M.N.C., J.S.C, R.L.R.

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