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Natural Origin Biomaterials for 4D Bioprinting Tissue-like Constructs

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Leveraging 4D biofabrication for engineering biomimetic living constructs is rapidly emerging as a valuable strategy for recapitulating native tissue dynamics, via on-demand stimuli, or in a naturally evolving mode. Carefully selecting smart materials with suitable responsiveness and cell supporting functionalities is crucial to take full operational advantage of this next-generation technology. Recent endeavors combining naturally available polymers or hybrid smart materials improved the potential to manufacture volumetrically defined, cell-rich constructs that may display stimuli-responsive properties, shape memory/shape morphing features and/or dynamic motion in time. In this review, we highlight natural origin biomaterials and the stimuli that can be exploited for granting dynamic morphological features and functionalities post-printing. A broad overview of recent reports focusing on 4D bioprinted constructs for tissue engineering and regenerative medicine is also provided and critically discussed in light of current challenges, as well as foreseeable advances. We envision that upon assurance of key regulatory demands, such technology will become translatable to numerous biomedical applications that require fabrication of constructs with dynamic functionality.

1. Introduction

Up-to-date, extensive research has been conducted within the fields of tissue engineering and regenerative medicine (TERM), with the intent of developing artificial biological elements capable of replacing, restoring, maintaining or improving biological functions of damaged tissues or organs.[1] Even though there have been many advancements in this field, researchers are still striving to fully replicate the natural cellular heterogeneity and dynamic biofunctionality of living tissues in biomaterial-based platforms.[2],[3] Among the several techniques available for tackling these challenges, additive manufacturing (AM), allied with the use of computer-aided design (CAD) specialized software, provide a user-defined, reliable and reproducible methodology to fabricate complex 3D printed structures with biomorphic features.[4] Particularly, 3D bioprinting has been rapidly emerging as a key AM technique for biomedical applications, due to its versatility, ability to produce evermore complex 3D architectures and the possibility to fabricate cell-embedded structures on the fly, by mixing living cells with biomaterial inks.[5]

The concept of 3D printing was initially introduced in the 1980s,[6] and its underlying mechanism is based on the controlled and consecutive deposition of several layers of a given material, to obtain a 3D object with a well-defined structure. Within the scope of Tissue Engineering, this AM approach gained visibility when biocompatible materials in combination with cells and bioactive molecules started to be employed as bioinks, 3D constructs with an accurate control over their architecture, thus giving origin to the term 3D bioprinting.[7] However, there has been some ambiguity in the literature when it comes to the terms related to 3D printing applied to the biomedical sciences,[1] most likely due to the rapid emerging of this technology and the fast-growing number of studies being published in recent years.[8] In fact, while some authors recognize the term “bioprinting” to encompass any printed construct that is either (i) suitable for biomedical applications; (ii) biocompatible and viable for human

transplant; or (iii) loaded with living cells within its structure,[9] it is our understanding that a more accurate definition of the term "bioprinting" is one where it refers only to constructs that have been printed from bioinks containing both the biomaterial precursor (usually a polymer or polymer mixture) and living cells. Additionally, these bioinks may also include other biomolecules, such as growth factors.[7] From this perspective, another distinction can be made between the term bioink, defined previously, and biomaterial ink, which consist of a biomaterial used for the controlled deposition of 3D constructs with precise spatial arrangements which does not include embedded cells in its composition.[10],[11] 3D constructs printed from biomaterial inks can then be seeded with cells post-printing and used for several biomedical applications.[11]

3D bioprinting may also enable the manufacture of individually designed constructs customized for each patient, providing a new ground for advancing personalized medicine.[12] This is generally materialized, by combining imaging techniques, such as magnetic resonance imaging (MRI) and computed tomography (CT), to generate patient-specific data that is converted to digital CAD models and then 3D bioprinted as personalized living constructs.[13] Building on this, the possibility to combine patient's own cells with bioactive biomaterial inks, further expands the potential of this customized approach, while preventing or mitigating constructs rejection upon implantation.[14] Adding to this versatility, recent advances in 3D bioprinting techniques exploiting suspension bioprinting have also enabled the fabrication of truly freeform constructs with more detailed biomorphic features that can be highly valuable for specific biomedical applications.[15]

Despite highly complex geometries can be generated by employing 3D bioprinting methods, such technique still lacks the ability to mimic the active modifications that native tissues experience as a result of their interactions with their naturally dynamic surrounding environment.[16] In the human body, natural tissues' microenvironment not only provides support for their development, but also entangles a series of different biochemical and

biophysical signals which regulate their biological functions. Considerations regarding the resemblance of artificial bioconstructs with living tissues where they will be implanted is becoming a primary concern in AM, alongside with the biocompatibility, biodegradability and mechanical properties of the developed biomaterials.[2] With a view to better recapitulate the features of each living tissue, it is important to proceed carefully within the selection of the biomaterials that will be used for producing 3D constructs, acknowledging that underlying bioactive properties and interactions with cells/host tissues will strongly affect the overall biological performance. This selection is highly dependent on the nature of the targeted tissue and on the specific physicochemical, biomolecular, topographical and mechanical properties that are required to restore full functionality.[17] For an optimal outcome of the engineered biomaterial ink, the choice of embedded cells is also a fundamental aspect to be considered, as natural tissues are highly heterogeneous, increasing the degree of complexity for their *in vitro* replication via AM techniques. The possibility to seamlessly combine different biomaterials and cell types in a 3D bioprinted construct allows researchers to closely resemble some of the anisotropic features of native tissues.[2] Moreover, cellular spatial distribution, either in single or multicellular 3D aggregates, has shown to considerably influence numerous intra-/intercellular processes occurring in constructs during *in vitro* maturation or upon *in vivo* implantation, and must also be considered in the design stages.[18],[2] Fortunately, both 3D and 4D bioprinting techniques allow a suitable control over this parameter, thus granting the possibility to produce heterogenous scaffolds, comprised of several biomaterials and cell types, with a more controlled spatial arrangement for each final application when compared to other available technologies[19] (e.g., electrospinning, solution casting, particulate leaching and micromolding, in which cell distribution is generally random[20],[21]). Additionally, it is extremely important for future engineered biomimetic scaffolds to be able to grasp the intrinsic dynamism of the supporting extracellular matrix (ECM), moving away from the

traditional three-dimensional paradigm of bioprinted constructs as inanimate structures, and towards a time spanned 4D approach.

Within the AM spectrum, 4D printing and 4D bioprinting, i.e. manufacturing approaches based on the 3D bioprinting of stimuli-responsive constructs, are building momentum owing to their potential for providing smart materials to operate as autonomous soft robotics/actuators,[22],[23],[24] for designing intricate drug delivery systems,[25],[26] for improving adhesion upon implantation[27] and/or for generating 4D cell-laden constructs mimicking living tissues dynamics. In 4D bioprinting, manufactured constructs are built from functional biomaterial inks that undergo a shape or functionality change over time, upon exposure to certain stimuli, or derived from the progressive tissue maturation due to naturally occurring cellular processes (e.g., cell organization, intra/inter communication and/or *de novo* matrix deposition), that take place along time – fourth dimension.[28] Post-printing modifications can be programmed beforehand, so that when constructs are exposed to certain stimuli, they evolve in a predictable manner, towards a specific goal.[16] One important aspect to point out is that the controlled degradation of 3D printed biomaterials should not be mistaken by 4D functionality, and therefore should not be considered within the scope of 4D bioprinting.[29] This means that a more accurate reproduction of the native target tissues and its dynamic interactions with cells, may be partially achieved with 4D bioprinting. This plays a key role in achieving promising results regarding the control over cellular processes and over the final therapeutic outcome.[13] For instance, the control over hydrogels stiffness, by employing hydrogels with stimuli-dependent viscoelastic behavior, allows to guide mesenchymal stem cells differentiation toward specific phenotypes.[30],[31] This feature can, ultimately, translate in an enhancement of both *in vitro* and *in vivo* performance of the produced constructs.[32] Up-to-date, the dynamic features of 4D bioprinting have granted exceptional progress in several areas within the tissue engineering domain, namely tissue vascularization,[33] cardiac,[34] bone,[35] muscle[36] and neural[22] tissue engineering and stents[37] production.

While there are still many challenges to be overcome, the 4D bioprinting technique has the potential to change the paradigm of tissue engineering, by being able to input a level of dynamism and response to stimuli currently unattainable by more conventional biofabrication techniques.[9],[29]

To materialize such dynamic constructs with programmable and controlled shape or functionality changes, smart polymeric materials of natural, synthetic or hybrid origin, processed into stimuli-responsive and shape memory hydrogels have become the main source of biocompatible bioinks for 4D bioprinting.[2],[38] Such kind of materials are able to change their properties upon the variation of external variables in a highly non-linear manner.[39] Here we present a brief overview of the 4D bioprinting technology and its operational features, its usefulness to move towards the biofabrication of biomimetic tissues, as well as provide a broad overview on the myriad of biomaterials and stimuli that can be exploited. A particular focus is given to seminal studies that explore the potential applications of 4D bioprinting within the scope of tissue engineering. Systems containing natural-based polymers will be focused as they are often assumed as materials with adequate bioinstructive and structural properties for a variety of biomedical applications.[40] Finally, the most pressing challenges to be overcome, as well as future perspectives for this emerging technology are outlined and critically discussed in light of envisioned future advances.

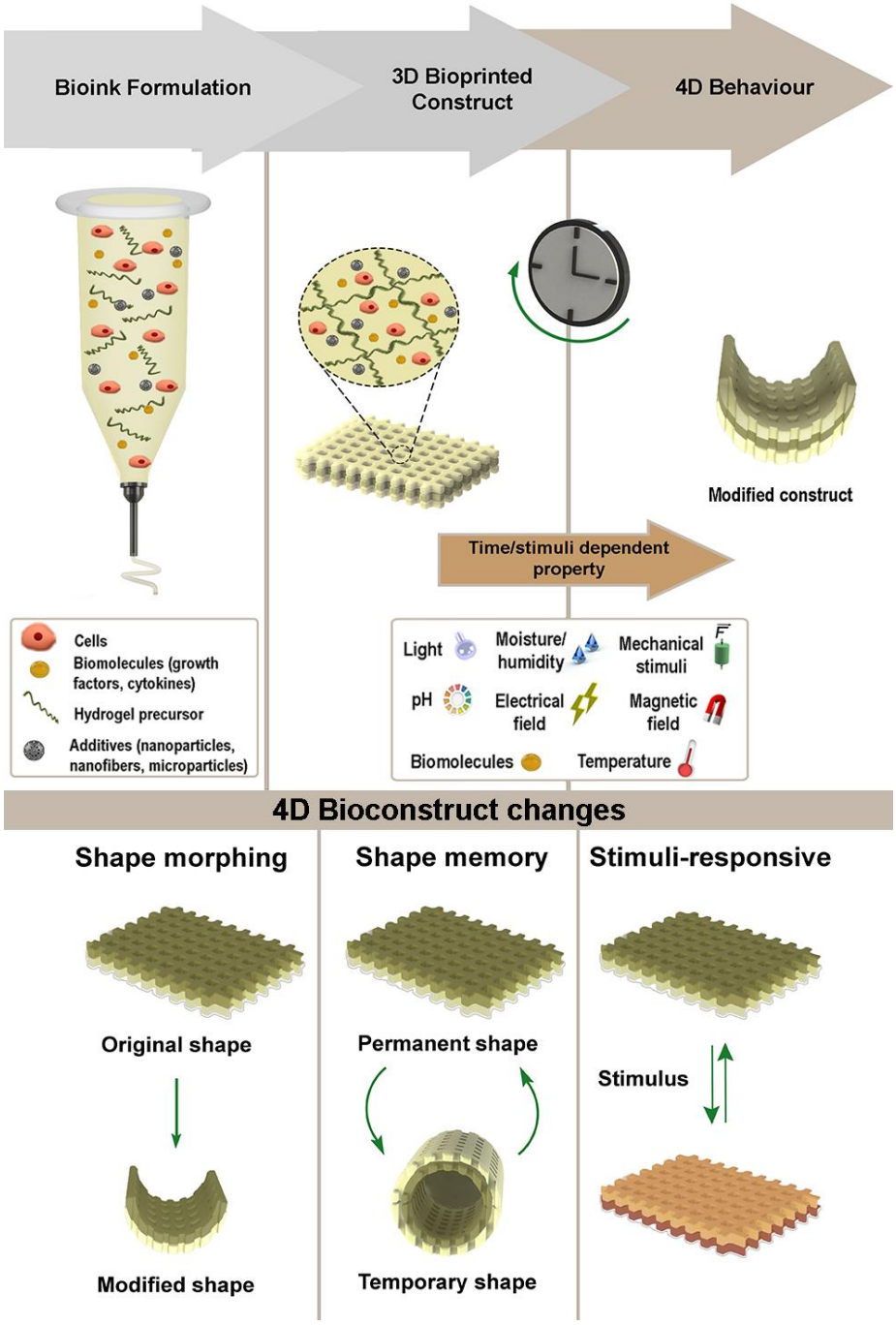

Figure 1. Representation of the 4D bioprinting process: bioink formulation and time/stimuli dependent 4D behavior (top); examples of possible changes that can occur in 4D bioprinted constructs (bottom).

2. Biomaterials for 4D Bioprinting

The development of 4D bioconstructs implies the use of stimuli-responsive materials as a design rule, for enabling researchers to pre-program a particular shape or enable a

functionality change, depending on the envisioned final biomedical application.[7] Besides stimuli-responsiveness, for bioprinting purposes it is also crucial to take into consideration the physicochemical and rheological properties of the bioink formulation, to assure printability without compromising cell viability during the printing process,[13] as well as guarantee the maintenance of bioprinted 3D constructs structural stability in the desired time-frame post-printing.[2] When working within the scope of biomedical related applications, these materials must meet important additional requirements, including: (i) biocompatibility, (ii) non-immunogenicity, (iii) mechanical robustness and, in some cases, (iv) biodegradability,[16] as well as being able to be processed and perform shape change under cell compatible conditions.[41]

The realm of biomaterials that are currently being explored as precursors for fabricating 4D bioprinted structures are mostly based on natural, synthetic or hybrid smart polymers,[7],[42] with an emphasis on the use of stimuli-responsive polymeric hydrogels,[38] which can also possess shape memory[4] and injectability[43] properties. Besides single-component materials, the use of multi-component polymeric bioinks and other additives (e.g., biomolecules, nanomaterials, etc) has become a valuable approach to further extent the library of biomimetic 4D constructs that can be materialized.[2][30][43] These topics will be addressed in further detail throughout this section and a particular focus will be given to natural origin materials owing to their wide availability, versatile chemical processability, bioactivity, general biocompatibility and biodegradability.

2.1. Smart polymeric materials

The use of polymeric materials for biomedical applications has been well-established over the years, not only regarding their biocompatible properties, but also owing to their similarities to

some components of natural tissues or their surrounding environment, and to their ability to be processed under relatively mild conditions.[7] In the context of 4D bioprinting, a group of smart polymers frequently used are shape memory polymers, which can retain memory of a deformed temporary shape, and then recover to their initial permanent shape when an external stimulus has been particularly explored.[44] Besides the conventional shape memory process, 4D printed smart polymers can also undergo other types of transformations, namely self-deformation, self-assembly and/or self-healing.[41] For example, shape morphing anisotropic polymeric materials exhibiting bending deformation (which can result from either the combination of two materials with different properties or the gradient/pattern-driven/nematic arrangements distributed in a single material[45]), are frequently employed to convey shape change over time to 3D bioprinted constructs.[7]

In recent years, both natural and synthetic stimuli-responsive polymers have been explored as potential bioinks for numerous 4D bioprinting applications, such as skeletal muscle[46] or bone tissue engineering[29], among several others. In this regard, proteinaceous materials and polymers derived from natural sources, which are generally regarded as biocompatible, have the advantage of possessing bioactive properties when compared to their more inert synthetic counterparts;[7][47][48][49] proteins obtained from human plasma, for example, provide a very interesting source of biomaterials in a personalized perspective.[50] Yet such materials generally present considerable batch-to-batch variability and less tailorable mechanical properties when in a pristine state, two parameters that must be taken into consideration during the design of bioinks for 4D bioprinting. Conversely, synthetic polymers provide a higher control over batch-to-batch variability, molecular weight dispersity, and a higher versatility over crosslinking mechanisms and mechanical properties.[51] However, these polymers are generally highly bioinert, lacking the presence of intrinsic cues to induce biological activity (e.g., cell adhesion molecules, morphogens binding, etc).[7] Several bioinks developed for 3D/4D bioprinting comprise natural polymers or a combination of natural and

synthetic polymers. Nevertheless, there have also been reports where synthetic polymers alone have been used with interesting results but mainly in 3D printing applications that do not include cells during the printing process, instead cells are generally seeded in scaffolds post printing – top-down engineering. For instance, Hendrikson *et al*. employed a synthetic thermo-responsive shape memory polymer, polyurethane, to fabricate 4D printed scaffolds to assess cellular behavior upon modification of the mechanical properties.[52] The researchers performed cell seeding after fixing the temporary shape of the scaffold, and observed that after recovery to its permanent shape, cells experienced morphological changes, while maintaining a high viability. These approaches hold particular potential for supporting the advancement of cutting-edge tissue regeneration applications, however the efficacy and yield of cell seeding in such top-down approaches remains to be optimal when compared to bioink formulations laden with cells on the fly.

In terms of natural origin smart polymers , the reports from Miao *et al*.[35],[53] are particularly interesting owing to its use of a relatively unexplored and renewable polymer – namely soybean oil epoxidized acrylate (SOEA) – to produce 4D smart scaffolds with shape memory features. This plant-derived oil polymer is responsive to temperature changes, enabling temporary shape fixation at very low temperatures (-18 °C) and permanent shape recovery at 37 °C (i.e., physiological temperature). Biocompatibility assessment of this material revealed a suitable attachment and proliferation of human bone marrow mesenchymal stem cells.[35]

Due to the beneficial properties of biopolymers, these materials are being extensively explored to formulate bioinks for both 3D/4D bioprinting. To build 4D bioconstructs, the presence of stimuli-responsive groups is generally required. In the event that polymer precursors do not exhibit responsive properties to external stimuli, or in case their responsive properties are not suitable for certain biomedical applications, it is often possible to use precision chemical tools for imprinting functional groups in the polymeric backbone and that confer stimuli responsive functionality in a user-programmed and application oriented

mode.[16] This strategy can also be applied to achieve other desirable properties,[16] such as solubility in water,[54] control over the degradation rate[55] and enhanced mechanical properties.[56] Gathering on this, some of the most explored natural polymers for 4D bioprinting including: alginate,[33],[57] collagen,[58] gelatin,[59],[34],[60] hyaluronic acid[18][33] and chitosan[61] are depicted in Figure 2. Within 3D bioprinting applications, alginate hydrogel bioinks are undoubtedly one of the most broadly researched natural biomaterials. The extremely rapid crosslinking when in contact with divalent ions (e.g., calcium, barium, magnesium, etc), suitable biocompatibility and rheological properties render it an excellent choice for assuring good printability without significantly compromising cell survival during and after the bioprinting process, making it a reference in this field.[62],[63] To be employed in 4D bioprinting, alginate can be further chemically modified using precision chemistry tools (e.g., zero length coupling,[64] grafting of click-chemistry moieties[65] and/or caged cell adhesion motifs,[66] etc) to induce stimuli-responsiveness onto the bioprinted material.[33] Moreover, the possibility to use alginate in combination with a variety of stimuli-responsive biomaterials has the potential to generate bioinks with pH,[67] temperature[68] and acoustic responsiveness,[69] thus attesting its enormous versatility for being used in 4D bioprinting applications. Collagen and gelatin possess an exceptional trait for ECM-biomimetic hydrogel bioinks formulation , since in essence they are natural-ECM derived biomaterials[70] – i.e., collagen proteins make up a significant portion of the natural ECM in mammalian tissues, and gelatin corresponds to the denatured form of collagen – which significantly improves cell attachment, proliferation and activity, making them particularly suitable for bioprinting applications and TERM.[7] This feature, allied with the inherent temperature[61] and pH[32] responsiveness of gelatin , makes it a particularly suitable precursor for 4D biomaterial inks formulation and dynamic constructs manufacture. Moreover, gelatin modified with methacrylic groups (GelMA) has been frequently employed as a bioink.[12][43][57] The use of GelMA for bioprinting provides efficient means to perform

covalent crosslinking of the bioconstruct generally initiated by UV light exposure. The photo-crosslinking process can be controlled to endow GelMA-based bioconstructs with 4D functionality, by controlling the crosslinking degree throughout the constructs, thus generating a differential crosslinking, making the resulting bioconstructs responsive to humidity/moisture due to the differential swelling degree, when in contact with a solution.[46] Similarly, hyaluronic acid biopolymers are also an asset for the fabrication of bioconstructs with close resemblance to the natural ECM, as they can also be found in several native tissues (in particular, in some connective tissues and cartilage), and are known to contribute for cell viability and to activate key cellular signaling pathways both in healthy and diseased tissues.[7],[71] While chitosan has been recognized as a natural polymer with very interesting properties for several biomedical applications[72], being easily modified using well established chemical routes[73], its use for cell-laden bioinks formulation has not been extensively explored, mainly due to the fact that chitosan is insoluble at physiological pH (~7.4) – instead, a solution of weak acids (e.g., acetic acid) is required to dissolve this biopolymer. At this pH, cellular viability is significantly affected (depending on the time of incubation in these conditions), making chitosan hydrogels unsuitable for long-term cell encapsulation. However, this limitation might be overcome by using multi-component materials bioinks or by chemically modifying chitosan polymeric backbone with certain functional groups to improve its properties including solubility.[13],[74]

As favorable as natural polymer-based materials are for biomedical applications, they also encompass some disadvantages. Even though hydrogels derived from synthetic polymers may mitigate some of these shortcomings, they are also more likely to contain cytotoxic components, such as unreacted monomers, initiators and crosslinkers, as well as generate toxic reaction by-products, which means that this type of polymers must be carefully selected in order not to compromise biocompatibility.[13] Poly(*N*-isopropylacrylamide) (PNIPAAm) and poly(ethylene glycol) (PEG) are examples of synthetic polymers that have been

frequently used in combination with natural polymers for biofabrication purposes, including bioprinting. PNIPAAm bioinks are often formulated together with biopolymers such as sodium alginate[75] or hyaluronic acid,[76] to convey temperature responsiveness onto them. On the other hand, the use of PEG derivative forms [namely poly(ethylene glycol) diacrylate (PEGDA) and poly(ethylene glycol) methacrylate (PEGMA)] has been known to improve mechanical properties of natural-based hydrogels.[2] Other polymers derived from renewable sources,[77] including polylactic acid,[37],[78] and other biodegradable polymers, such as polycaprolactone,[79],[80] have been widely used for 4D bioprinting.

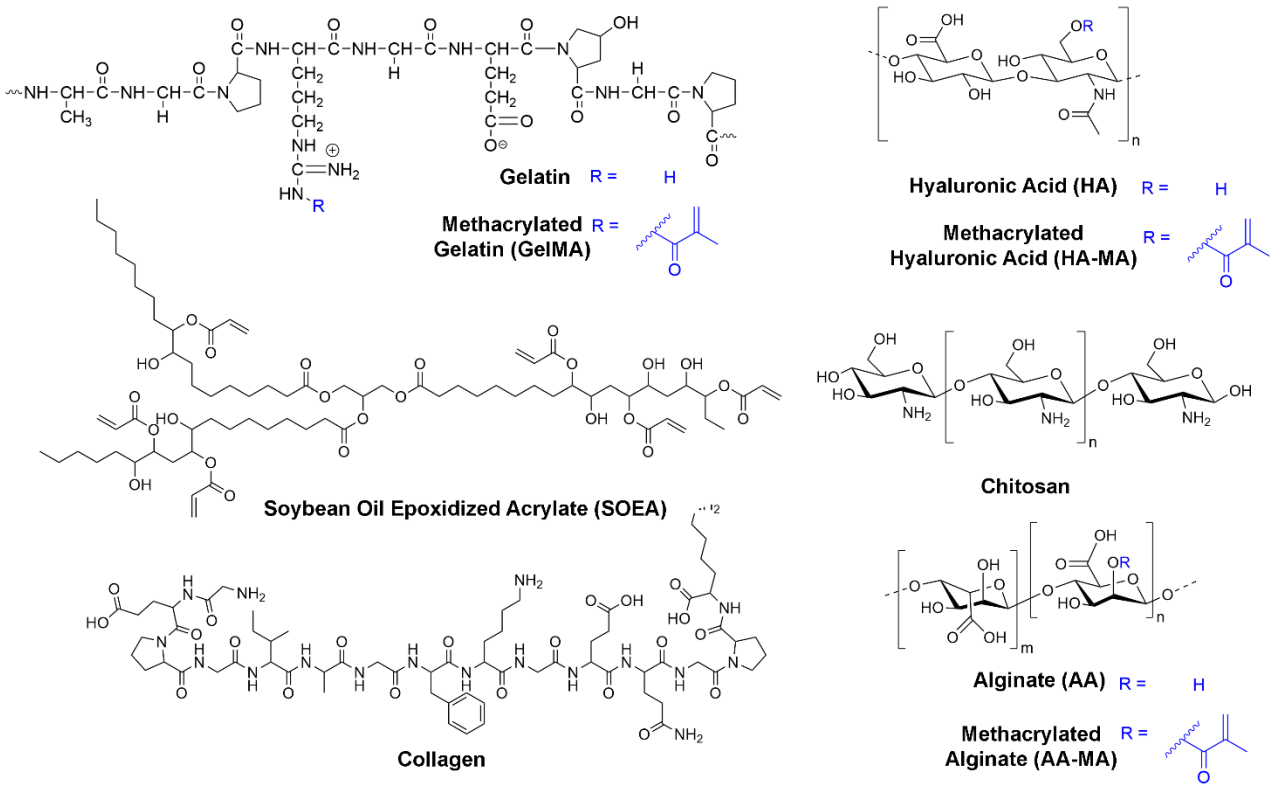

Figure 2. Chemical structures of natural and bio-based polymers which can be used in 4D bioprinting.

2.2. Combinatorial and hybrid smart materials

Analogously to what has been previously described for the smart polymeric materials, 4D bioinks can also be formulated by the combination of different natural biopolymers (i.e., polysaccharides, or proteinaceous materials, etc[81][82]) – combinatorial materials – or by the combination of natural and synthetic polymers to attain complementary properties[2] – hybrid materials – to overcome some limitations for more demanding biomedical applications (for instance, improved mechanical properties, for bone tissue engineering[83]). Such combinations are also highly valuable to better mimic the complexity, heterogeneity or topography of natural ECM.[30] A relatively straightforward practice used to tune constructs properties is to formulate multi-component bioinks, which can comprise two or more different polymers, two or more different cell types and a wide variety of biomolecules or other additive materials.[2] So far, there are already a significant number of combinatorial and hybrid polymer mixtures (including biopolymers-only, synthetic polymers-only or a mixture of both) which have been employed to develop 4D bioprinted constructs. Table 1 presents an overview of several polymers and additive materials that have been reported to produce bioconstructs with 4D functionality. Here, the biofabrication technique, type of stimuli employed, and envisioned final applications are also presented. For the most part the works presented in Table 1 are described in more detail throughout this review. An analysis on Table 1 shows that while several 4D bioconstructs include cells within the bioprinted material, and therefore in the bioink formulation, in some cases cell embedding is not required for the intended final application. In those instances, there is a higher degree of freedom in the bioink formulation, as the concern for cell viability only poses after the bioprinting process takes place. The production of composites, including the combination of hydrogels with additive materials to improve their performance, has also been the subject of several research works.[84] For example, nanomaterials, such as graphene[22] and iron nanoparticles,[58] represent a class of materials that have been added to bioinks to enhance electric and mechanical properties of bioconstructs intended to be explored for neural and muscle tissue engineering.

It has become increasingly important to investigate the possibilities to combine several materials for multi-component bioink formulations, considering that in general the use of different elements results in more versatile biomaterials, with improved biomimetic features. Besides combining different materials in the bioink formulation, different bioinks can also be alternately used to print different layers or different segments of materials that will make up the final construct. The recent development of multi-nozzle 3D printers facilitates the build-up of these heterogeneous scaffolds with distinct bioinks.[2]

Table 1. Examples of natural-based polymeric materials and/or combined with other materials/additives reported for 4D bioconstructs fabrication.

Polymers / Additives	Type of biomaterial	Biofabrication technique	Stimuli to induce 4D functionality	4D behavior	Cell type	Cell culture	Applications	Reference
Alginate/PDOPA [a] + Alginate/GelMA [b]	Combinatorial	3D bioprinting: Extrusion	Near-Infrared (NIR) Light	Shape morphing behavior	293 T cells	Cell-laden material	Various TE applications (including skin, cartilage and cardiac tissue)	[57]
Agarose / Type I collagen / Iron nanoparticles	Combinatorial	3D bioprinting: Drop-on-Demand (DoD)	Magnetic field	Stimuli-response (alignment)	hKAC [i]	Cell-laden material	Cartilage TE	[58]
SOEA [c]	Natural-based	3D printing: Stereolithography	Temperature	Shape memory behavior	hMSCs [j]	Cell seeding post-fabrication	Various TE applications	[35]
SOEA	Natural-based	3D printing: Photolithographic-Stereolithographic-Tandem Strategy (PSTS)	Moisture/ Humidity & Temperature	Shape morphing behavior & Shape memory behavior	hMSCs	Cell seeding post-fabrication	Cardiac TE	[53]
HA-MA [d] AA-MA [e]	Natural-based	3D bioprinting: Extrusion	Moisture / Humidity & CaCl2/EDTA	Shape morphing behavior & Shape memory behavior	mBMSCs [k]	Cell-laden material	Tissue vascularization	[33]
Gelatin + GelMA fibers	Natural-based	3D bioprinting: Extrusion	Moisture / Humidity	Shape morphing behavior	C2C12 cells	Cell-laden material	Muscle tissue engineering	[46]
Alginate/PNIPAAm [f]	Hybrid	3D printing: Extrusion	Temperature	Shape memory behavior	-	-	Smart valve	[68]
Gelatin/Chitosan	Combinatorial	3D printing: Extrusion	Temperature	Stimuli-response (patterning)	mBMSCs	Cell seeding post-fabrication	Tissue vascularization	[61]
SOEA/Graphene	Natural-based	3D printing: Stereolithography	Temperature	Shape memory behavior	hMSCs	Cell seeding post-fabrication	Nerve regeneration	[22]
GelMA/Iron oxide nanoparticles	Natural-based	3D bioprinting: Extrusion	Magnetic field	Stimuli-response (alignment)	C2C12 cells	Cell seeding post-fabrication & Cell-laden material	Muscle tissue engineering Soft robotics	[59]
PLA [g]	Natural-based	3D printing: Extrusion	Temperature	Shape memory behavior	-	-	Vascular stents	[37]
GelMA/PEGDA [h]	Hybrid	3D printing: Beam-scanning stereolithography	Mechanical stimuli	Shape morphing behavior	hiPSC-derived CMs [l] hECs [m] hMSCs	Cell seeding post-fabrication	Cardiac TE	[34]
GelMA/Gel-COOH-MA	Natural-based	3D printing: Inkjet	Moisture / Humidity	Shape morphing behavior	HUVECs [n]	Cell seeding post-fabrication	Various TE applications (including intestinal, lung fat and linear tissues)	[60]

[a)] Polydopamine; [b)] Methacrylated Gelatin; [c)] Soybean oil epoxidized acrylate; [d)] Methacrylated hyaluronic acid; [e)] Methacrylated alginate; [f)] poly(*N*-isopropylacrylamide); [g)] Polylactic acid; [h)] Poly(ethylene glycol) diacrylate; [i)] Human primary knee articular chondrocytes; [j)] Human bone marrow mesenchymal stem cells; [k)] Mouse bone marrow stromal cells; [l)] Human induced pluripotent stem cell-derived cardiomyocytes; [m)] Human endothelial cells; [n)] Human umbilical vein endothelial cells.

3. Stimuli in 4D Bioprinting

In general, any material that comprises stimuli-responsive properties has the ability to bear certain changes when a given stimulus is applied.[13] These changes can occur in terms of alteration of biomaterials chemical, physical, mechanical, or electromagnetic properties, changes in constructs shape, size or even ability to perform dynamic movement, depending on the type of stimuli-responsive properties exhibited by the materials/constructs as a whole and the type of stimulus applied. Often the combination of several polymers, responsive to different stimuli, can also yield multi-stimuli-responsive constructs, which allows to produce extremely versatile biomaterials. Interestingly, from an applicability point of view, their ability to undergo transformation of their physicochemical properties (e.g., viscoelastic behavior, mechanical properties, etc) or shape (e.g., self-folding, self-assembly, morphing) in response to a particular stimulus (e.g., temperature, humidity, light, enzymes, etc) renders them suitable for simulating the dynamic and anisotropic nature of native tissues and organs in engineered 4D bioconstructs in the time dimension.[2],[29]

3.1. Stimuli classification

The time-dependent changes characteristic of 4D bioprinting can be induced through a variety of different stimuli, which in the context of biomedical applications require special attention, since they must not be harmful for the cells or tissues. There are two possible approaches to induce programmed responses in 4D bioconstructs: (i) the use of physical or chemical stimuli or (ii) cell responsive systems. The most common one is the bioprinting of materials that are intrinsically responsive to physical or chemical stimuli (e.g., temperature, pH, water, light, magnetic field, electric field)[13] (Figure 1).

Cell responsive systems, usually based on the concept of "cell origami", have also recently emerged, to induce shape change in cell-laden micro/macrostructures by taking advantage of the contractile forces that occur naturally, referred to as cell traction forces (CTF). Here, CTF is exploited as a biological stimulus to provoke folding of a cell-laden two-dimensional structure into a three-dimensional structure, according to specific pre-designed patterns, to achieve different geometries. Figure 3 exemplifies the application of this strategy in a work developed by Kuribayashi-Shigetomi *et al*..[85] Although in this case the authors explored CTF to promote bending of microplates with flexible joints, it is expected that the same principle can be employed in cell rich 3D bioprinted structures/constructs as the substrates. The presence of hinges in these bioconstructs can also be explored to facilitate the shape change into the intended geometries.[29] When applying this technique onto bioprinted constructs, it is important to bear in mind that a certain degree of freedom within the matrix is necessary to allow this cell-induced shape change process to occur. Additionally, the mechanical properties of the bioconstruct, particularly porosity,[86] viscoelasticity[87] and stiffness,[88] are other important aspects to be considered. For example, the material cannot be too soft that it cannot withstand the environmental conditions in which it will be applied, and at the same time cannot be too stiff, otherwise the cells might not be able to reshape it. Besides being able to promote shape change, the cellular process taking place after bioprinting also lead to the progressive maturation of the tissue construct, which may endow the biomaterial with certain functionalities over time.[63] Interestingly, shape changes such as self-folding, can also be obtained by using multiple components with different volume expansion properties (e.g., swelling ratio or thermal expansion) or by using single component-based hydrogels with a differential gradient in their intrinsic properties.[7]

Both physicochemical stimuli-induced changes and cellular-induced shape change or tissue maturation play a key role in achieving 4D biomaterials which mirror native tissues more accurately. However, within the scope of 4D bioprinting, the cellular-induced shape change

approach is much less frequently employed than the use of smart materials sensitive to physicochemical stimuli.[29]

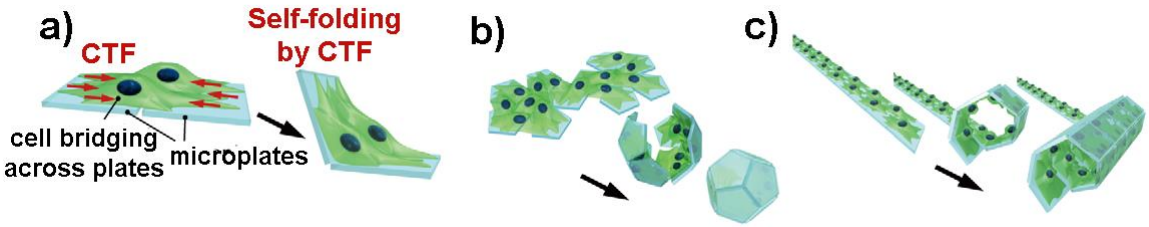

Figure 3. a) Folding process by cell traction forces (cell origami): a) after adhering and stretching across the microplates in which cells are seeded, cell traction forces generated towards the center of the cell causes the folding of these structures; b) schematic of self-folding into a dodecahedron structure; c) schematic of self-folding into a cylindrical tube structure. Adapted with permission.[85] Copyright 2012, Plos One.

3.1.1. Temperature

Among the different stimuli, temperature has been one of the most studied over the years, particularly for exploring shape memory.[32] In fact, there are several polymers known to possess temperature sensitive properties. However, considering that most of them are of synthetic origin, only a few of them hold the intrinsic properties required to produce bioactive biomaterial inks/ 4D bioinks. In this context, the use of temperature as the shape or functionality change inducing stimuli is only viable when this transition occurs at temperatures close to the physiological one, which limits the number of thermo-responsive polymers that could potentially be employed for 4D bioprinting applications, since those which require temperatures considered to be too extreme for cell survival are not suitable for most biomedical applications.[29] The combination of thermo-responsive macromolecules with biopolymer, such as polysaccharides, is a strategy to induce temperature sensitive properties in natural systems.[89] PNIPAAm is a common example of a synthetic thermo-responsive polymer widely used for tissue engineering and drug delivery applications.[28] Recently, Miao *et al*. reported on the use of a naturally-derived material, soybean oil epoxidized acrylate

(SOEA) , to print biocompatible constructs with temperature responsive shape memory effect, where after fixation of a temporary shape, the recovery of the permanent shape of the material, takes place at the physiological temperature (37 °C).[35] Mammalian derived gelatin is another example of a thermo-responsive naturally derived polymer, that can experience reversible sol-gel transitions upon temperature variations (at temperatures above 30 °C gelatin is in a soluble state, while at temperatures below 25 °C gelatin is in a gel state).[61] Importantly, these temperature ranges are highly dependent of gelatin origin, with fish skin gelatin presenting an entirely different temperature profile (for example: 4-8 °C gelling; 16-18 °C melting[90]), which is also dependent from the type of fish living areas (i.e., cold or warm waters).

3.1.2. pH

pH is another parameter that can be altered to trigger modifications (including, but not limited to shape memory effect) of some polymers or hydrogels. Biopolymers containing ionizable chemical groups in their structure are susceptible to pH changes in their environment.[91] For instance, the configuration of natural proteins like collagen , gelatin and keratin undergoes globule-to-coil transition upon pH alteration. Macroscopically, these changes can translate into behaviors like swelling, shrinking or bending.[32]

3.1.3. Moisture/humidity

Liquid-responsive materials which alter their shape or properties when in the presence for example, of water or cell culture medium, are very useful for applications in the sphere of actuator materials and soft robotics, due to their swelling/deswelling behavior, that can be used to impart motion through reversible bending.[9] In general, this feature is accomplished

by employing a single anisotropic material, with a differential gradient in their swelling ratio, or multiple materials with different volume expansion properties.[7] For example, chitosan undergoes a reversible glass transition with the presence of moisture,[92] that was used to produce scaffolds with shape memory induced by hydration.[93] We believe that this characteristic could be found in other biopolymers and further explored in the context of 4D bioprinting.

3.1.4. Light

The use of photo-responsive materials provides an opportunity to exploit light as an on-demand, user programmed stimuli to generate modifications on constructs shape or size, including contraction, bending or volume changes.[32],[94] Light can also be used as a heat source, producing thermal energy that may trigger a localized change on photothermal-responsive groups or molecules. This mechanism has been applied to trigger shape memory effect on some polymers and hydrogels and will be further discussed in the following sections.[7]

3.1.5. Magnetic and electrical fields

Several studies have also focused on the prospect of employing magnetic and electrical fields as a means to drive certain alterations within biomaterials. In the case of magnetic stimulus, the use of composites is generally required to impart the magnetic responsiveness onto the biomaterials.[95] Iron and iron oxide nanoparticles are common examples of additives used to prepare materials sensitive to magnetic fields.[59],[58],[96],[97] Besides being used to promote alterations after printing, the application of a magnetic field during the bioprinting process of magnetized bioinks can be harnessed to control the orientation of the magnetic responsive

particles, thus attaining bioconstructs with anisotropic properties, that can be directed to a given direction according to the envisioned final application.[58] In the case of electrical stimulus, the electric responsiveness may derive from the use of electrically conductive polymers or additives with electrical conductive properties (e.g., graphene, carbon nanotubes and other metal nanoparticles).[16] These types of biomaterials are currently being explored for the development of smart nerve guidance conduits.[22]

3.1.6. Biomolecules

Biological stimulus, namely the presence of certain biomolecules, represent an additional possibility to induce progressive changes to the bioprinted constructs. For instance, Devillard *et al*. developed a 4D printed hydrogel loading two different enzymes, alkaline phosphatase and thrombin, which promoted calcification and fiber formation over time, respectively, thus imparting calcification and vascularization functionalities to the bioconstruct post-printing.[98]

3.1.7. Mechanical forces

Finally, the use of mechanical stimuli (i.e., pressure, deformation, load) also poses an interesting alternative to promote changes within mechano-responsive materials. Hydrogel systems have been commonly studied for this purpose, and they have been known to be able to change some of their physicochemical properties such as strength,[99][102] viscosity,[100][102] color[101][102] and topography[103] upon mechanical stimulation.

Given that in general biological systems are constantly subjected to mechanical stimuli, and that mechanical cues from the surrounding environment are recognized by cells and trigger a specific cellular response (i.e. the so termed mechanotransduction), this stimuli has been broadly reported in the literature.[104],[105],[106] Exploiting the mechanical responsiveness of

artificial systems can be a great asset for the development of smart biomimetic materials, particularly for wound repair scaffolds, drug delivery systems, fabrication of artificial tissues or biosensors.[102]

When trying to achieve biomimetic artificial tissues, either the use of multiple stimuli-responsive materials or the combination of several materials which respond to different stimuli (for example gelatin, which exhibits both temperature and pH responsiveness), are most often a more interesting option to better recapitulate the complex interactions and transformations that occur in native ECM.

3.2. Shape Memory and Shape Morphing

Within the group of stimuli-responsive materials, two different types of behavior regarding shape modifications can be distinguished, specifically shape memory behavior and shape morphing behavior (Figure 1). In terms of 4D bioprinting applications, the use of stimuli-responsive hydrogels either with shape memory or shape morphing abilities has emerged as a viable option.

The shape memory ability involves the transition between a temporary and a permanent shape, in a pre-programmed manner, upon exposure to a specific stimulus.[107] Shape memory hydrogels typically comprise two types of crosslinking networks – a covalently crosslinked network, which is responsible for fixing structures permanent shape, as these are irreversible bonds, and a supramolecular or reversible crosslinked network which will, at a first instance, fix structures as a temporary shape. Then upon exposure to a determined stimulus (e.g., temperature, pH, light), the material will to return to its original permanent shape, as these are reversible bonds.[108] Some shape memory materials can sustain several cycles of temporary shape deformation/fixation and permanent shape recovery, showing reversibility and

possibility to repeat the stimuli-responsive behavior several times, while others can even be pre-programmed to enable more than one shape change – multiple shape memory effect.[109] On the other hand, the shape morphing ability comprises an irreversible change of the material's properties or morphology in response to a particular stimulus.[110] An example of such structures is provided in the study conducted by Luo *et al*..[57] The key difference between shape memory behavior and shape morphing behavior is that the first is reversible and the construct can therefore return to a previous form, while the latter is irreversible. Biomaterials capable of undergoing shape memory or shape morphing effects have gathered considerable attention for biomedical applications, mainly due to their ability to adapt to specific defect sites and potential for implantation through minimally invasive methods.[16] The shape morphing and shape memory processes in 4D bioprinted constructs are depicted in Figure 4 a) and b), respectively, where the reversible nature of the shape memory behavior and irreversible nature of shape morphing behavior are evidenced.

The anisotropic and reversible shape memory behavior of stimuli-responsive hydrogels is also the basis for most studies conducted on hydrogel-based soft actuator materials.[111] In this context and inspired by skeletal muscle movement, Bakarich *et al*. developed an alginate/poly(*N*-isoprolacrylamide) (PNIPAAm) hydrogel, where the reversible temperature-responsive volume transitions of the PNIPAAm network endowed this material with thermo-responsive actuation behavior. Through the 4D printing of supporting materials, a prototype for a smart valve was produced, for controlling water flow, triggered by changes in the water temperature.[68]

Hydrogel-based soft robot actuators with dynamic movement triggered by exposure to temperature variations,[112] electric fields[113] and magnetic fields,[114] have also been reported. Studies employing these unique concepts and fully natural origin biopolymers are still scarce but if materialized they may enable a unique set of opportunities and open new avenues for advanced biomedical applications that can take advantage of such dynamics.

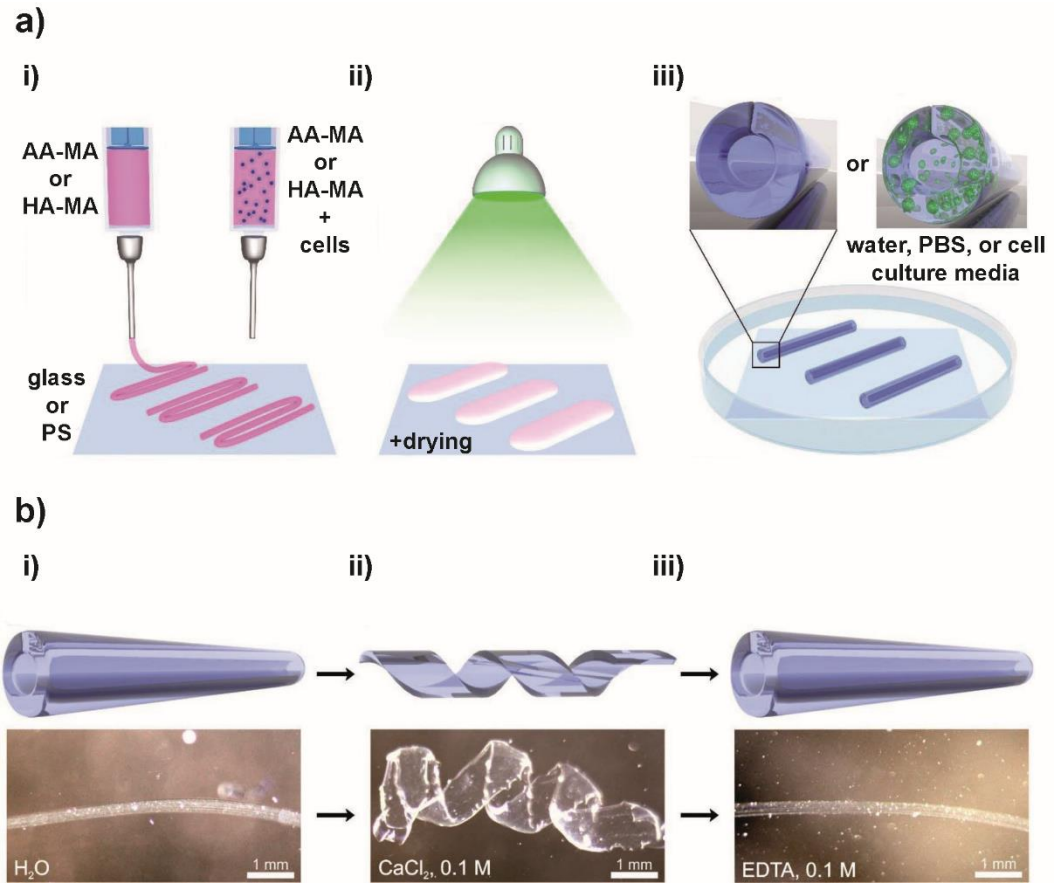

Figure 4. a) Illustration of the shape morphing process in a methacrylated alginate hydrogel: i) printing/bioprinting step; ii) photo-crosslinking with green light, where a differential crosslinking degree is created (higher at the top, since it absorbs more light); iii) folding into tubes induced by differential swelling degree when in contact with a solution, due to the differential crosslinking degree. b) Illustration of the shape memory process in a methacrylated alginate hydrogel: i) permanent shape, fixed by an irreversible covalent crosslinking network – photoinitiated covalent bonds due to the presence of methacrylic groups; ii) temporary shape, fixed by a secondary reversible crosslinking network – alginate crosslinking with calcium ions; ii) return to the permanent shape, after the removal of calcium ions by an EDTA solution. Adapted with permission.[33] Copyright 2017, Wiley-VCH.

4. Biomedical Applications

Although 4D bioprinting is a relatively recent technology its unique features contribute for its ever growing arrays of applications in many fields including as TERM, biomedical devices, and soft robotics/actuators, etc.[115] Some of the most relevant biomedical related applications

in which 4D printing/bioprinting is currently being employed are indicated in Figure 5 and will be outlined hereafter.

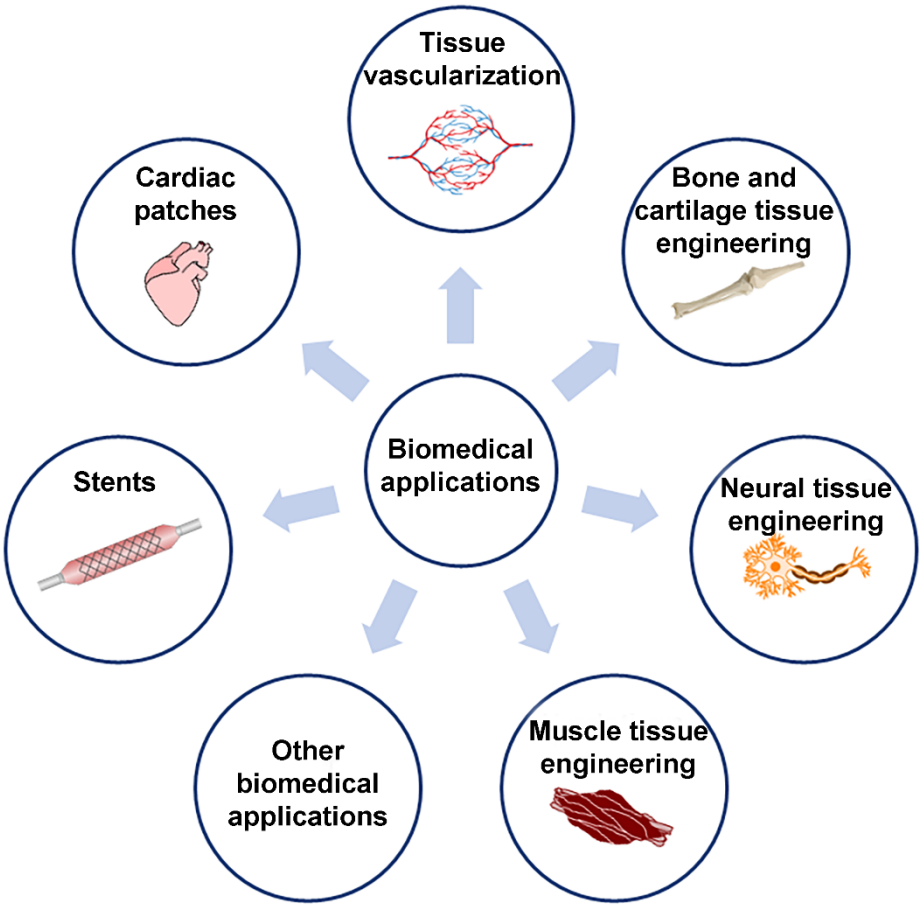

Figure 5. Scope of the possible biomedical applications for 4D bioprinting.

4.1. Tissue vascularization

The *in vivo* performance of engineered tissues depends significantly on the ability of seeded or encapsulated cells to survive, proliferate, integrate into host tissues, and eventually carry out their natural functions. To assure full functionality, an essential aspect that must be considered is the vascularization of artificial tissues, in order to allow gas (e.g., O_2 and CO_2), nutrients, proteins, and waste products exchange.[13] From this perspective, it is understandable that many research efforts are focusing on exploring bioprinting for

developing new ways to produce vascularized tissues or to induce the production of blood vessel-like tubular structures.[116]

Extrusion 3D bioprinting based methods have been investigated to fabricate hollow tubular constructs, however the high shear forces that are employed in these approaches to produce tubes of smaller diameter can be deleterious for cell viability. The use of 4D bioprinted materials, where the tubular structure is formed after printing (i.e., by self-folding) has been explored as a viable option to circumvent this problem. Several studies showed promising results, namely the hollow tubular cell-laden structures developed by Kirillova *et al.*.[33] In this report, the authors developed self-folding hollow tubular structures from bioprinted planar hydrogel sheets, based on the disparity of the crosslinking degree observed between the top and bottom layers of bioprinted materials. Two modified biopolymers, specifically methacrylated alginate (AA-MA) and methacrylated hyaluronic acid (HA-MA) were tested to produce bioprinted hydrogel films, in which the photo-crosslinking reactions were initiated using visible green light. Since the top layer of these hydrogels absorbs a larger amount of light than the bottom layer, the crosslinking degree was higher at the top, which caused these structures to bend into hollow tubes when immersed in water, phosphate-buffered saline (PBS) and cell culture media. The mouse bone marrow stromal cells encapsulated within these tubes showed homogeneous distribution and good viability after 7 days. Self-folding tubular constructs with inner diameters ranging from 20 to 150 µm, which are quite similar to the size of small blood vessels, were obtained (Figure 4).

Interestingly, the authors also found that when the folded AA-MA hydrogel was placed in a $CaCl_2$ solution, the crosslinking interactions between the alginate chains and Ca^{2+} ions led to its unfolding, as a result of the deswelling induced by this additional crosslinking mechanism. Refolding could then be restored by immersing the unfolded hydrogel in an EDTA solution, which captured the Ca^{2+} ions, and consequently disrupted the Ca^{2+}-alginate crosslinking

network. This approach shows that besides self-folding, the AA-MA hydrogel was capable of undergoing a reversible shape modification.

Using another strategy, Luo *et al*. produced a near-infrared (NIR) responsive shape-changing hydrogel, that transformed from planar into a tubular structure.[57] Alginate and polydopamine were the constituents of the bioinks used to produce these hydrogel constructs, and the self-deformation behavior into tubular structures was driven by NIR-induced dehydration, upon laser irradiation. The switch was induced by the photothermal effect of NIR, which led to a temperature increase with the printed hydrogel and consequent loss of water, resulting in the shrinkage and folding of the alginate/polydopamine scaffold in specific directions. Furthermore, researchers were able to control the bending angle of the structure by adjusting the laser power, irradiation time and designed patterns of the printed construct, resulting in different shape changes (i.e., tubular and saddle-like forms). The combination of stimuli-responsive alginate/polydopamine biomaterial inks and cell-laden alginate/GelMA bioinks allowed to produce biphasic scaffolds, capable of sustaining shape morphing while also supporting cell survival.

PEG bi-layered hydrogels, consisting of two different molecular weight PEG polymers, have also been described to be able to self-fold into hollow tubes, promoted by the differential swelling of the hydrogel bilayers when in contact with an aqueous solution. These cell-laden structures showed long term cell viability.[117]

In a slightly different approach, Lewis *et al*. took advantage of the temperature responsive character of gelatin and pH responsive character of chitosan to fabricate 4D dynamic tubular constructs through the printing of stimuli-responsive hydrogels.[61] Here, an initial solid tubular structure was obtained via 3D printing of gelatin and chitosan (at 5 pH < 5). Afterwards, this material was immersed in a sodium citrate solution, leading to the formation of a second electrostatic crosslinking network due to the interaction of chitosan and citrate ions at low pH. By controlling the diffusion time of citrate ions into the structure, the

researchers managed to limit this secondary crosslinking network to a certain thickness of the cylindrical object, thus generating a dual network shell surrounding the single network core. By cutting off the tips of the cylinder structure and immersing it in warm water, the core will act as a sacrificial material and will be removed, with only the hollow tubular structures remaining in the end.

Considering the current efforts that are being made into the researching this topic, it is feasible to assume that, in the future, 4D bioprinting of vascularized architectures will be a widespread process, allowing to obtain vascularized multi-material constructs with multiple cell types, thus getting one step closer to the ultimate goal of creating tissue mimicking bioactive constructs. Once the vascular network engineering is refined, it may also be possible to engineer nervous and lymphatic components onto these scaffolds.[2]

4.2. Bone and cartilage tissue engineering

The repair of bone defects through bone tissue engineered structures has been established as a better approach than using the previously used standard bone grafts, as it prevents disease transmission and is not dependent on donor availability.[118],[119] Therefore, this has been a widely researched area in terms of potential biomaterials and strategies used for bone defect repair. Taking into account that bone defects are usually irregular and can vary in size, 4D bioprinting presents several beneficial features for this application, such as the opportunity to tailor the biomaterial to the specificities of tissues injuries, to enable progressive tissue maturation, to impart functionality onto the engineered constructs, and to attain complex construct architectures, with further similarities to the native bone tissue. The incorporation of different inorganic composites within bioinks, including silicates, hydroxyapatite, tricalcium phosphate (TCP) and bioactive glass, among others is also an asset to promote stem cells osteogenic differentiation and to mimic natural bone building blocks. Moreover, as previously

mentioned, the 4D bioprinting technique is also being exploited to create artificial tissues with vascular and nervous networks, which will substantially enhance its regenerative action upon implantation in the bone tissue.[2],[29]

In the interest of developing a biomaterial resembling the structure and functionality of vascularized alveolar bone, Devillard *et al*. fabricated a 4D printed PEGDA hydrogel.[98] Alkaline phosphatase and thrombin were mixed with the PEGDA polymer precursor, to be entrapped within the printed structure – Figure 6, (a) and (b), respectively. The authors found that over time, these enzymes could promote both calcification and fiber formation (comparable to blood vessels) on the printed construct – Figure 6, (c).

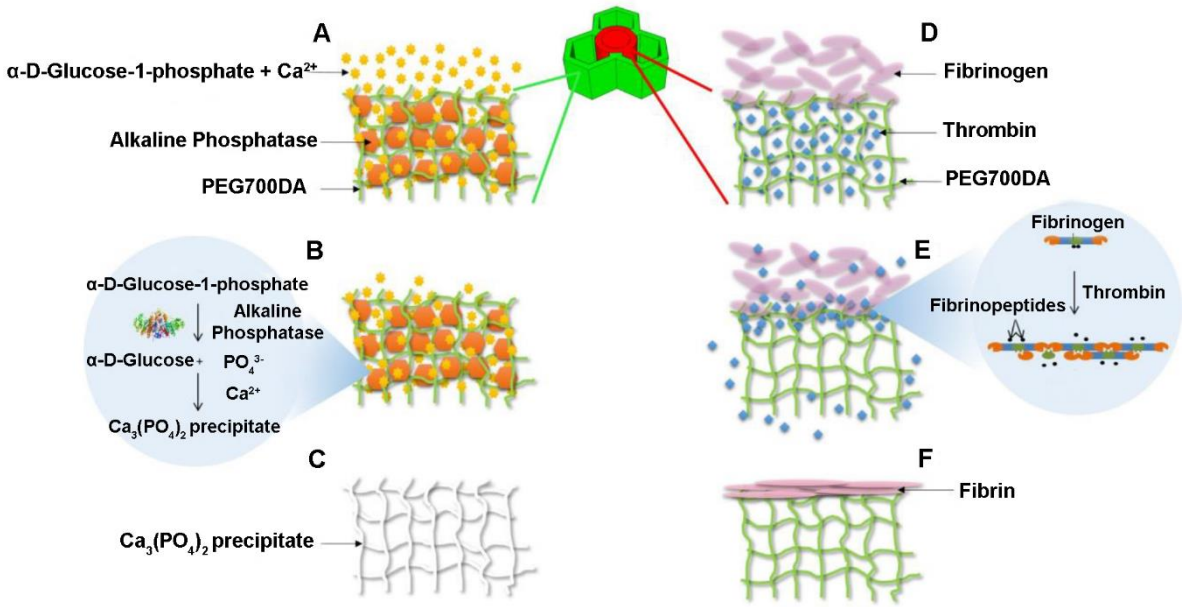

Figure 6. Schematic of 4D activity of a) calcification promoted by alkaline phosphatase and b) fibrin formation promoted by thrombin; c) 4D printed bioconstruct with calcification and fibrin formation activity, promoted by alkaline phosphatase and thrombin enzymes, respectively. Adapted with permission.[98] Copyright 2018, Wiley-VCH.

Besides bone, 4D bioprinting also finds application for cartilage tissue engineering. For instance, Betsch *et al*. took advantage of the magnetic responsiveness of a bioink composed of agarose, type I collagen, iron nanoparticles and human primary knee articular chondrocytes, to force the alignment of collagen fibers (due to unidirectional motion provoked in the iron

nanoparticles by the application of a magnetic field) at the moment of bioprinting. Since the native cartilage tissue is composed of several layers of collagen fibers aligned in distinct orientations, a heterogeneous construct composed of two distinct layers of horizontally aligned and randomly distributed collagen was produced. The study revealed that bi-layered constructs exhibited higher potential for cartilage tissue repair than constructs built with only one hydrogel layer. In this study, the time dimension is not employed exactly in the same way as it is viewed in other studies concerning 4D bioprinting – i.e., the hydrogel morphology and functionality change occurs during bioprinting, in response to its exposure to a magnetic field.[58] In the future, developments in this field are envisioned to include more complex structures for cartilage regeneration, including constructs for osteochondral repair.

4.3. Neural tissue engineering

Tissue engineered nerve grafts have become an increasingly viable option to treat peripheral nerve injury, by integrating the use of biomaterials with biological, physical and chemical cues, to promote nerve regeneration.[120] In this domain, Miao *et al*. reported the 4D bioprinting of a smart nerve guidance conduit from a bioink composed of soybean oil epoxide acrylate (SOEA) , graphene and human mesenchymal stem cells (hMSCs). The 4D functionality was achieved as a result of the light-induced graded internal stress, during the crosslinking step at the bioprinting stage, which later on caused the bioprinted structure to bend, when in contact with a solution. Graphene was used both as a promoting agent for hMSCs differentiation into neural cell types, and as an approach to enhance biomaterial conductivity. Since SOEA possesses shape memory property, with permanent shape recovery triggered by physiological temperature, this feature was further explored to perform dynamic self-entubulation and seamless integration of the tissue engineered nerve graft with the damaged nerve. Succinctly, the bioprinted planar sheet is sequentially exposed to ethanol and

water to induce the shape change into a tubular structure, which corresponds to the permanent shape of the biomaterial. Then, this structure is opened and flattened, fixing this as the temporary shape, to facilitate its implementation *in vivo*, recovering its permanent shape at body temperature on the defect site, thus wrapping itself around both stumps of the damaged nerve.[22] The *in vivo* implant processes aided by the shape memory process and its result are illustrated in Figure 7.

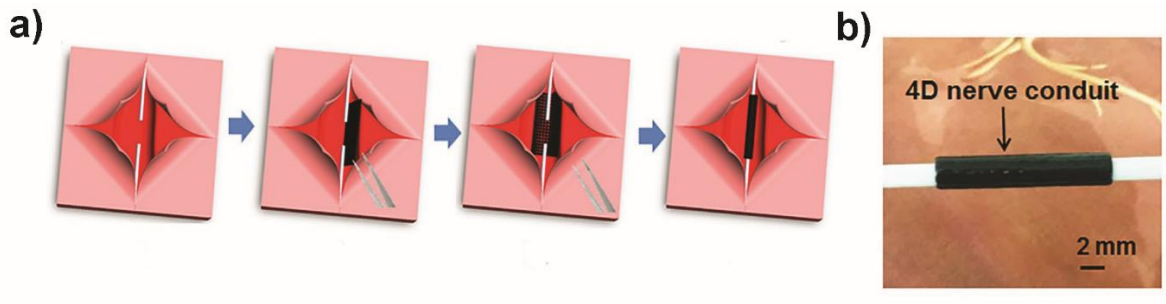

Figure 7. SOEA/graphene 4D construct for nerve regeneration: a) Implantation process of the SOEA/graphene nanohybrid through shape memory process – a temporary planar shape recovers its permanent tubular shape at physiological temperature, allowing the 4D construct to wrap itself around the two stumps of a severed nerve; b) 4D smart nerve conduit integrated in a severed nerve. Adapted with permission. [22] Copyright 2018, Wiley-VCH.

Recently, the 4D functionality was also employed in scaffolds produced by the electrospinning technique for the biofabrication of artificial nerve grafts. In this case, Apsite *et al*. produced scaffolds built from a top layer of uniaxially aligned polycaprolactone-poly(glycerol sebacate) (PCL-PGS) and a bottom layer of randomly aligned methacrylated hyaluronic acid (HA-MA) . This material proved to be capable of shape transformation from a flat to a tubular configuration upon immersion in an aqueous solution, and was suitable to sustain neural cells cultured on its top layer with high adhesion, viability and proliferation being obtained.[121]

4.4. Muscle tissue engineering

Skeletal muscles are a substantial component of the human body, and therefore, the accurate and viable reproduction of muscle tissue is a subject of much interest among the tissue engineering field.[36] Within this scope, Tognato *et al.* [59] reported the biofabrication of a multiple-stimuli responsive nanocomposite hydrogel, comprised of GelMA and iron oxide nanoparticles (IONPs) in the context of the magnetic force-based tissue engineering concept. In this work, the magnetic responsiveness of the bioink due to the presence of IONPs was harnessed to promote the aligned organization of the IOPs into filaments in the constructs. This anisotropic arrangement of the IONPs was stabilized by decreasing the temperature prior to hydrogel photo-crosslinking. It was observed that the C2C12 skeletal myoblasts within these scaffolds aligned to the same axes of the IONPs filaments, and differentiated into myotubes, proving that this kind of structure possesses the necessary cues to guide cell behavior in the direction of muscle tissue formation.

More recently, Yang *et al*. also sought to replicate skeletal muscle tissue, by using a combination of 3D and 4D bioprinting techniques. In this study, the researchers produced cell-laden GelMA fibers through a modified 3D printing process, which included an applied electrical field to stimulate cell alignment and myogenic differentiation. Then, a 4D printed gelatin film with shape morphing behavior was used to hold these fibers together in bundles – Figure 8 (a). Here, the self-folding behavior of gelatin resulted from the grooved pattern applied during the 3D printing process of the gelatin film, which caused it to experience different swelling degrees throughout its structure when placed in a liquid environment. By placing the cell-laden GelMA fibers on top of the 4D printed gelatin film and then exposing it to culture medium, the gelatin film folded, wrapping around the cell-laden GelMA fibers, thus creating a biomimetic skeletal muscle-like structure. The DAPI/MHC images obtained for these fibers after 21 days of culture evidence the alignment and myotube differentiation of the C2C12 cells within the fibers – Figure 8 (b).[46]

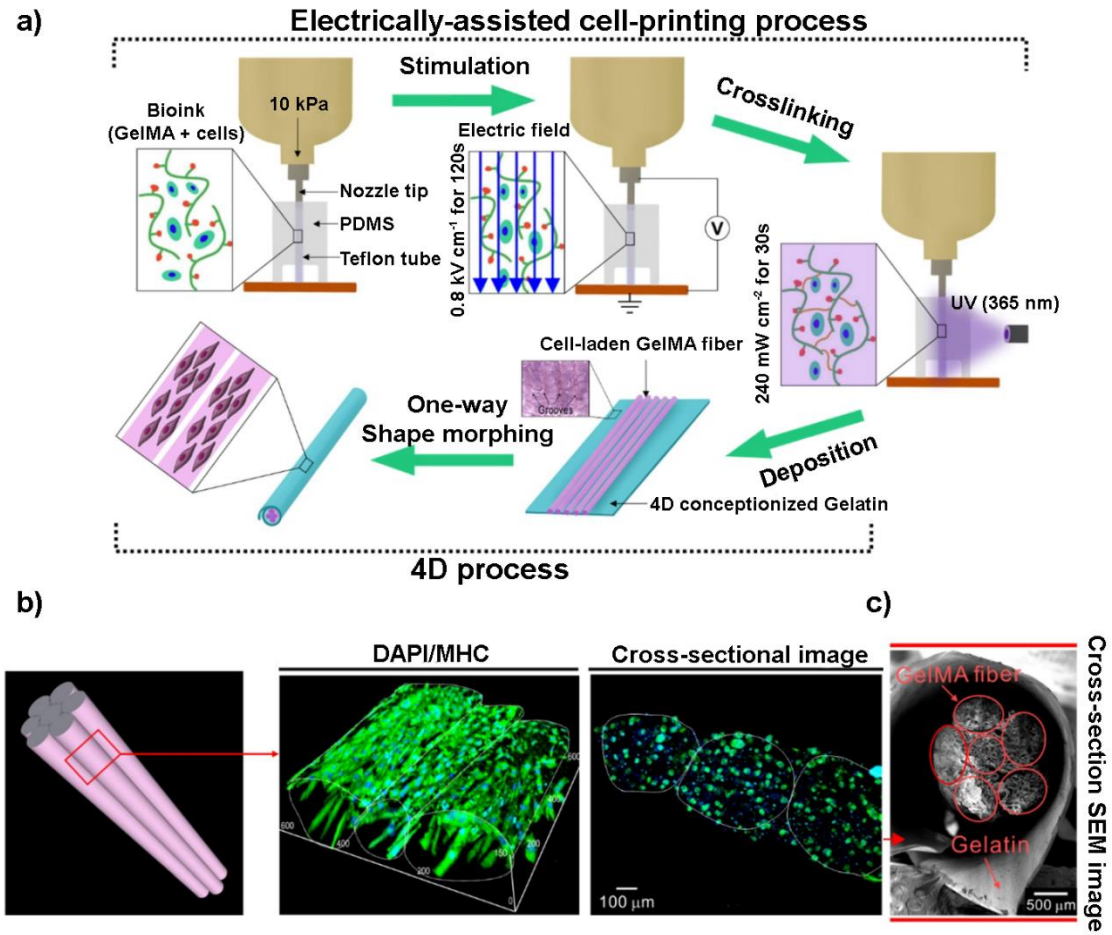

Figure 8. a) Schematic representation of the electrically assisted bioprinting and 4D process employed; b) DAPI/MHC images of cell-laden GelMA fibers after 21 days of culture; c) cross-sectional SEM image of the cell-laden GelMA fibers enwrapped in the gelatin outer structure. Adapted with permission.[46] Copyright 2021, Ivyspring International Publisher.

4.5. Cardiac patches

Seeing that cardiac problems are to this day one of the major health concerns worldwide, there has been a growing necessity to develop increasingly functional and improved means to treat damages in cardiac tissues.[122]

In a recent study, Cui *et al*. focused on the development of cardiac patches produced from cell-laden smart hydrogels via 4D bioprinting, in order to obtain a biomaterial capable of fully adapting to the native physiology and function of the heart. Cui's team aimed at obtaining a cardiac patch with a curvature similar to the heart curvature, with the possibility to reversibly

stretch and shrink, according to the movement of the heart in its cardiac cycle. The shape morphing ability of the 4D bioprinted GelMA and PEGDA construct was achieved due to the uneven crosslinking of the bioprinted structure during the bioprinting process – the higher crosslinking density on the bottom layer allows the structure to bend into the desired curved conformation. Furthermore, the four-dimensional ability of this anisotropic patch to transition between flat and curved architectures promoted an excellent integration with the dynamic process of the beating heart.[34]

Following up on this work, Wang *et al*. developed a 4D cardiac patch with NIR light induced shape memory behavior.[123] To this end, researchers prepared a bioink composed of temperature responsive shape memory polymers, namely bisphenol A diglycidyl ether (monomers), poly(propylene glycol) bis(2-aminopropyl) ether (crosslinker) and decylamine (crosslinking modulator), along with graphene as an additive nanomaterial. Due to the photothermal effect triggered upon NIR light exposure, this construct was able to change its shape from a flat to a curved configuration (Figure 9, b), in order to adjust to the natural cardiac tissue morphology. The addition of graphene onto this construct promotes heat absorbance, thus facilitating the photothermal induced shape change process. The 4D shape changing feature allows the cardiac patch to adjust its curvature according to the region of the heart where it will be applied, thus ensuring seamless and organ personalized integration and increased performance for myocardial tissue regeneration. The shape memory effect also enabled to perform uniform cell seeding onto the flat temporary shape of the bioconstruct, and the microgroove pattern printed onto this 4D biomaterial promoted cell alignment and mechanical support (Figure 9, a). When transitioning into the permanent curved shape, this cardiac patch is already laden with a uniform layer of aligned myofibers throughout its entire surface, thus preventing cell aggregation, which so far has been one of the biggest challenges to overcome when producing curved bioconstructs for cardiac tissue repair.

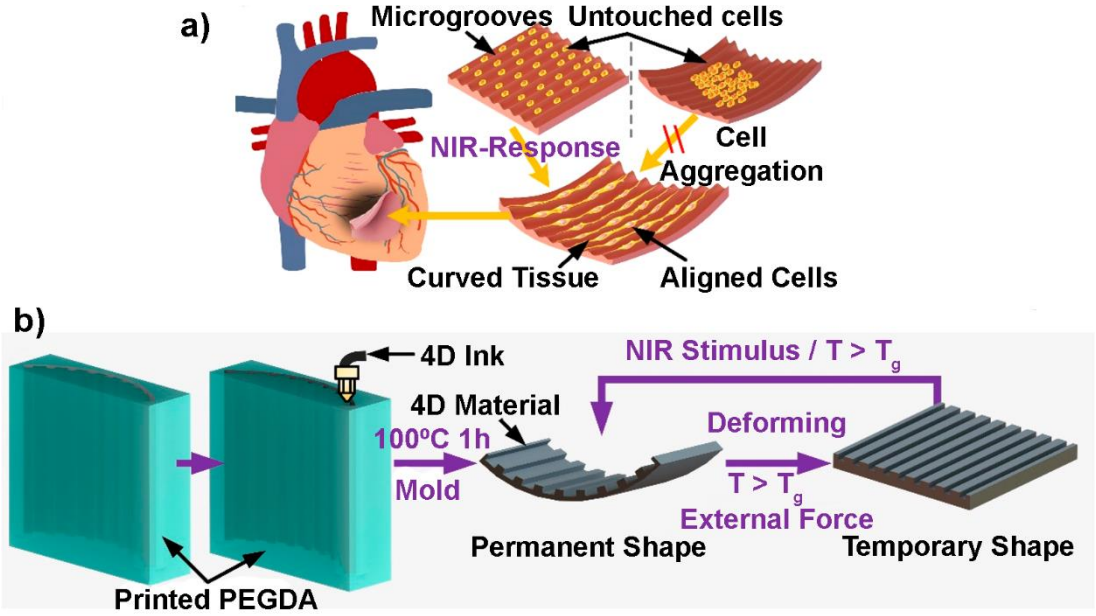

Figure 9. 4D Bioprinting of dynamic constructs for cardiac tissue engineering. a) Representation of the developed 4D cardiac construct to promote myocardial repair. The use of a construct capable of shape changing into a curved form as an alternative to a pre-curved construct prevents cell aggregation and stimulates uniform cell distribution and alignment, as well as organ-specific shape fitting; b) Schematic representation of the 4D cardiac construct production process and shape memory behavior. Adapted with permission.[123] Copyright 2021, ACS Publications.

4.6. Additional biomedical applications

Due to its versatility and advantageous features, the 4D bioprinting technique has been branching out in many biomedical applications, and several studies have reported the development of 4D bioconstructs that hold the potential to be applied in different tissues. For example, Luo *et al* reported the production of a multi-component shape morphing scaffold, by alternatively employing an AA/GelMA/human embryonic kidney cells bioink and an AA/PDOPA ink during the bioprinting process, thus obtaining a heterogenous scaffold.[57] The photothermal responsiveness of PDOPA allowed to induce shape morphing behavior (bending) onto the AA/PDOPA segment of the scaffold, through localized NIR light-induced heating, generating a curved structure, which could potentially be employed for cartilage and skin tissue repair, as well as cardiac patches for myocardial repair.

In a more recent study, Cui *et al*. developed a new approach to produce shape morphing 4D printed hydrogels from gelatin based bioinks (GelMA and Gel-COOH-MA).[60] In this approach, the bioprinting of the shape morphing hydrogel is performed on a glass slide coated with a sacrificial layer of alginate/gelatin, to allow detachment and folding of this structure in due time, after umbilical vein endothelial cells are properly seeded and cultured on its surface. After detaching, the different swelling rate of GelMA and Gel-COOH-MA in aqueous solutions causes the hydrogels to self-fold. By modifying a few parameters during the bioprinting process (which bioink is used for the top and bottom layers, and if there is rotation of bioinks, for example) it was possible to create a variety of conformations with these hydrogels, that could be employed in various applications. Constructs with grooves and ridges resembling the topographical traits of intestinal villi were obtained, which could possibly be used to engineer intestinal tissues. Hollow spherical structures were also obtained by promoting self-folding into polyhedron structures, which could be useful to mimic tissues such as lung alveoli or as lipid compartments characteristic of fat tissues. Tubular structures with potential to be used in linear tissues were also achieved.

With the increasing amount of research on this subject, certainly many more applications for 4D bioprinting will arise in a near future. Besides bioprinting, the four-dimension functionality is also finding applications in other biofabrication techniques, namely electrospinning,[121],[124] which indicates that this is becoming a transversal functionality to other methods.

5. Limitations and future perspectives

4D bioprinting technology is driven by the ever growing need to develop artificial biomaterials with closer resemblance to native tissues and organs dynamics, in an effort to enhance its potential as a solution for translational TERM applications. However, due to the

incredible complexity and dynamics inherent to the natural extracellular environment, the construction of biomaterials capable of accurately mimicking living tissues in their whole intricacy is a challenging endeavor, even with advanced fabrication techniques available at the moment.[17] Furthermore, considering that the biological environment may be different for each individual one must consider this added layer of complexity prior to translation into a clinical setting.[16]

The 4D bioprinting technology presents many beneficial aspects including the fact that cells can be distributed in a spatially controlled manner throughout the bioconstructs, the possibility to attain complex tissue alike structures (such as vascularized tissues, nerve graft conduits, and tracheal stents) and the ability to mimic the dynamic changes that occur in the natural tissues over time.[9] However, considering that the 4D functionality is still a rather recent technology, there are still many challenges and limitations to be overcome in this domain. One limitation of the bioprinting techniques in general, whether it is 3D or 4D, is that the physical entrapment of cells within the bioprinted constructs may hinder some cellular process, namely spreading, migration and organization, which may compromise the overall therapeutic performance of the constructs.[60] Controlling cell distribution within the bioprinted construct in the long term is also a very challenging aspect of 3D/4D bioprinting, since the cellular processes taking place post-printing may alter the cell distribution within the material, which may result in inhomogeneous cell distribution and even the formation of cell clusters within the bioprinted material.[125] Many of the studies carried out on 4D bioprinted constructs focus a lot more on the shape or functionality change process of the final material and end up lacking more comprehensive studies on how these changes affect complex cellular processes, oftentimes only performing biological assays based on staining techniques (e.g., live/dead viability assay). To safely move towards clinical applications, more advanced biological tests will most likely be required.[16] Besides cell viability and proliferation, *in vitro* studies should also comprise the evaluation of cell morphology, adhesion, differentiation

and activity, and then a transition into *in vivo* implementation will be necessary to better understand how the fabricated biomaterial responds when included in a living host system – analysis of inflammatory responses, assessment of biofunctionality, biodegradation and overall effectiveness are examples of crucial data that needs to be acquired by *in vivo* studies before moving towards human clinical trials.

There are also a number of limitations concerning the materials available to employ for 4D bioprinting. For one, it is essential that they comprise all the necessary characteristics to be used in biomedical applications, which have been discussed previously. Among these, the mechanical properties assume a significant role, and should be in good agreement with the final applications for which the 4D bioconstructs are intended, in order to guarantee an adequate performance. However, taking into account that the 4D functionality presupposes changes in the constructs over time, it is reasonable to assume that the mechanical properties may also be altered. Therefore, it is crucial for researchers to critically evaluate the mechanical properties of 4D bioprinted materials, considering the requirements for the different stages of the biomaterial's practical applications. For instance, in the case of shape memory hydrogels, the mechanical properties may vary significantly from the permanent to the temporary shape and may not recover completely after the permanent shape recovery. Typically, the mechanical performance, namely the elastic moduli, is lower in the permanent state as compared with the temporary state, which can limit some load-bearing applications of the construct. Moreover, in the cases of multiple shape memory effect, in which the materials can undergo shape deformation and recovery several times, their mechanical performance tends to decrease with the number of cycles carried out.[126] For the case of natural-based polymers macromolecular design strategies may be used to produce hydrogels with improved mechanical properties, that could be integrated in 4D bioprinted principles.[127] For that more work will be needed to build larger libraries of chemically modified natural polymers, including polysaccharides,[128] proteins[129] and human plasma derivatives.[130]

Furthermore, the 4D functionality requires the precursor material or a mixture to contain stimuli-responsive components, which further limits the number of candidates suitable to be explored for this new technology. Besides this, the stimulus needed to induce a specific change upon 4D bioprinted constructs must also be cell friendly, which means that materials responsive to any stimuli considered too extreme for cells (for instance, temperature and pH outside the physiological range) will not be adequate for the vast majority of applications related to TERM.[14],[41]

Although natural polymeric materials present several intrinsic advantageous properties for use in biomaterials fabrication, their use also entails some difficulties. For instance, their mechanical properties are often not suitable for highly demanding biomedical applications, and in some cases their fast biodegradation rate may pose as a disadvantage, as it reduces biostability of the construct. Common strategies to overcome such drawbacks include the combination of natural and synthetic materials, as well as chemical modifications using precision chemistry tools (i.e. covalent, dynamic covalent, guest-host moieties) to tune their physicochemical properties.[131]

Additionally, the currently developed bioprinters do not possess the necessary resolution to fabricate certain structures with high precision[16] (for instance, the diameter of the smallest blood capillaries ranges from around 5 to 10 μm, which in terms of 3D bioprinting resolution are values remarkably difficult to attain[132]). On the other hand, the bioprinting of very large constructs (such as bone tissue) in a high throughput manner is also an issue this technology is not yet prepared to solve.[2] Technological solutions, involving the combination of different materials (including the inclusion of distinct stimuli-responsive components in different regions of the construct) will continue to be developed in the coming years, as well as the integration of different techniques to process multi-scale and complex structures.[133]

Besides the technical challenges and safety assurance regarding 4D bioprinted constructs, there are other critical issues that need to be addressed to be able to fully implement 4D

bioprinting as a standard clinical practice. For instance, the evaluation of the cost effectiveness of this technique, the need for personnel training and the ability to comply with current legal and ethical requirements, are all factors that need to be considered of and settled before moving forward to a well-established clinical implementation.[134]

Despite the above-mentioned challenges, 4D bioprinting is still a very promising technology to achieve numerous breakthroughs within the biomedical field, being highly expected that the scientific community will continue to pursue new endeavors in this topic. Currently, the most pressing matters requiring progress in this field are: i) the development of new materials (or upgrade the functionalization of existing ones); ii) the development of more precise bioprinting methods; iii) the improvement of the biological assay component in these research studies, to eventually translate to clinical trials and ultimately, clinical applications.

6. Conclusion

The development of 4D bioprinting has led to incredible progress in several areas of tissue engineering, allowing to attain more complex and dynamic structures, with a better resemblance of the native tissues. Biocompatible stimuli-responsive shape memory and shape morphing hydrogels have been established as promising systems to apply with this technology, as they often provide a suitable support for cellular processes to occur, in addition to being able to be modified and combined with other materials to achieve the most favorable properties for different applications, making them remarkably versatile. To build such biomaterials, polymers of natural origin are being substantially explored for bioinks formulation, due to their inherent biocompatibility and biodegradability, intrinsic resemblance with natural tissues, possibility to tune their properties by chemical modifications and responsiveness to stimuli compatible with biological implementation. So far, the 4D bioprinting technique has allowed to introduce a series of beneficial new features into engineered tissues, such as vascularization, the ability to perform some biological functions and the integration of biophysical and biochemical cues to guide cell fate and behavior over time, ultimately promoting a better integration with the host tissues and regeneration of their functions. Given that this is still a rather recent technique, further studies on bioprinting techniques, precursor materials and eventually clinical trials hold the key to a widespread implementation of 4D bioprinting as a viable technique to meet some of the current limitations in TERM.

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References

[1] J. Groll, T. Boland, T. Blunk, J. A. Burdick, D. W. Cho, P. D. Dalton, B. Derby, G. Forgacs, Q. Li, V. A. Mironov, L. Moroni, M. Nakamura, W. Shu, S. Takeuchi, G. Vozzi, T. B. F. Woodfield, T. Xu, J. J. Yoo, J. Malda, *Biofabrication* **2016**, *8*, 013001.

[2] N. Ashammakhi, S. Ahadian, C. Xu, H. Montazerian, H. Ko, R. Nasiri, N. Barros, A. Khademhosseini, *Mater. Today Bio* **2019**, *1*, 100008.

[3] V. M. Gaspar, P. Lavrador, J. Borges, M. B. Oliveira, J. F. Mano, *Adv. Mater.* **2020**, *32*, 1.

[4] A. Subash, B. Kandasubramanian, *Eur. Polym. J.* **2020**, *134*, 109771.

[5] F. Pati, J. Gantelius, H. A. Svahn, *Angew. Chemie - Int. Ed.* **2016**, *55*, 4650.

[6] C. W. Hull, *(UVP, Inc., San Gabriel, Calif.), U.S. Patent 4,575,330,* **1986**.

[7] M. L. Bedell, A. M. Navara, Y. Du, Y. Du, S. Zhang, S. Zhang, A. G. Mikos, *Chem. Rev.* **2020**, *120*, 10744.

[8] C. K. Chua, *Int. J. Bioprinting* **2020**, *6*, 257.

[9] Q. Yang, B. Gao, F. Xu, *Biotechnol. J.* **2020**, *15*, 1900086.

[10] I. T. Ozbolat, M. Hospodiuk, *Biomaterials* **2016**, *76*, 321.

[11] J. Groll, J. A. Burdick, D. W. Cho, B. Derby, M. Gelinsky, S. C. Heilshorn, T. Jüngst, J. Malda, V. A. Mironov, K. Nakayama, A. Ovsianikov, W. Sun, S. Takeuchi, J. J.

Yoo, T. B. F. Woodfield, *Biofabrication* **2019**, *11*, 013001.

[12] D. G. Tamay, T. D. Usal, A. S. Alagoz, D. Yucel, N. Hasirci, V. Hasirci, *Front. Bioeng. Biotechnol.* **2019**, *7*, 164.

[13] J. M. Unagolla, A. C. Jayasuriya, *Appl. Mater. Today* **2020**, *18*, 100479.

[14] A. Lapomarda, G. Vozzi, *Biosci. Biotechnol. Res. Asia* **2019**, *16*, 15.

[15] S. G. Patrício, L. R. Sousa, T. R. Correia, V. M. Gaspar, L. S. Pires, J. L. Luís, J. M. Oliveira, J. F. Mano, *Biofabrication* **2020**, *12*, 035017.

[16] W. Zhou, Z. Qiao, E. Nazarzadeh Zare, J. Huang, X. Zheng, X. Sun, M. Shao, H. Wang, X. Wang, D. Chen, J. Zheng, S. Fang, Y. M. Li, X. Zhang, L. Yang, P. Makvandi, A. Wu, *J. Med. Chem.* **2020**, *63*, 8003.

[17] E. S. Bishop, S. Mostafa, M. Pakvasa, H. H. Luu, M. J. Lee, J. M. Wolf, G. A. Ameer, T. C. He, R. R. Reid, *Genes Dis.* **2017**, *4*, 185.

[18] C. Aronsson, M. Jury, S. Naeimipour, F. R. Boroojeni, J. Christoffersson, P. Lifwergren, C. F. Mandenius, R. Selegård, D. Aili, *Biofabrication* **2020**, *12*, 035031.

[19] J. Zhang, E. Wehrle, P. Adamek, G. R. Paul, X. H. Qin, M. Rubert, R. Müller, *Acta Biomater.* **2020**, *114*, 307.

[20] S. Wüst, R. Müller, S. Hofmann, *J. Funct. Biomater.* **2011**, *2*, 119.

[21] C. J. Ferris, K. G. Gilmore, G. G. Wallace, M. In Het Panhuis, *Appl. Microbiol. Biotechnol.* **2013**, *97*, 4243.

[22] S. Miao, H. Cui, M. Nowicki, L. Xia, X. Zhou, S. J. Lee, W. Zhu, K. Sarkar, Z. Zhang, L. G. Zhang, *Adv. Biosyst.* **2018**, *2*, 1800101.

[23] A. Sydney Gladman, E. A. Matsumoto, R. G. Nuzzo, L. Mahadevan, J. A. Lewis, *Nat. Mater.* **2016**, *15*, 413.

[24] C. de Marco, C. C. J. Alcântara, S. Kim, F. Briatico, A. Kadioglu, G. de Bernardis, X. Chen, C. Marano, B. J. Nelson, S. Pané, *Adv. Mater. Technol.* **2019**, *4*, 1.

[25] A. Melocchi, M. Uboldi, N. Inverardi, F. Briatico-Vangosa, F. Baldi, S. Pandini, G.

Scalet, F. Auricchio, M. Cerea, A. Foppoli, A. Maroni, L. Zema, A. Gazzaniga, *Int. J. Pharm.* **2019**, *571*, 118700.

[26] A. Melocchi, N. Inverardi, M. Uboldi, F. Baldi, A. Maroni, S. Pandini, F. Briatico-Vangosa, L. Zema, A. Gazzaniga, *Int. J. Pharm.* **2019**, *559*, 299.

[27] D. Han, R. S. Morde, S. Mariani, A. A. La Mattina, E. Vignali, C. Yang, G. Barillaro, H. Lee, *Adv. Funct. Mater.* **2020**, *30*, 1909197.

[28] B. Gao, Q. Yang, X. Zhao, G. Jin, Y. Ma, F. Xu, *Trends Biotechnol.* **2016**, *34*, 746.

[29] Z. Wan, P. Zhang, Y. Liu, L. Lv, Y. Zhou, *Acta Biomater.* **2020**, *101*, 26.

[30] M. A. Mohamed, A. Fallahi, A. M. A. El-Sokkary, S. Salehi, M. A. Akl, A. Jafari, A. Tamayol, H. Fenniri, A. Khademhosseini, S. T. Andreadis, C. Cheng, *Prog. Polym. Sci.* **2019**, *98*, 101147.

[31] A. Higuchi, Q. D. Ling, Y. Chang, S. T. Hsu, A. Umezawa, *Chem. Rev.* **2013**, *113*, 3297.

[32] Y. S. Lui, W. T. Sow, L. P. Tan, Y. Wu, Y. Lai, H. Li, *Acta Biomater.* **2019**, *92*, 19.

[33] A. Kirillova, R. Maxson, G. Stoychev, C. T. Gomillion, L. Ionov, *Adv. Mater.* **2017**, *29*, 1703443.

[34] H. Cui, C. Liu, T. Esworthy, Y. Huang, Z. X. Yu, X. Zhou, H. San, S. J. Lee, S. Y. Hann, M. Boehm, M. Mohiuddin, J. P. Fisher, L. G. Zhang, *Sci. Adv.* **2020**, *6*, eabb5067.

[35] S. Miao, W. Zhu, N. J. Castro, M. Nowicki, X. Zhou, H. Cui, J. P. Fisher, L. G. Zhang, *Sci. Rep.* **2016**, *6*, 27226.

[36] I. Apsite, J. M. Uribe, A. F. Posada, S. Rosenfeldt, S. Salehi, L. Ionov, *Biofabrication* **2020**, *12*, 015016.

[37] C. Lin, L. J. Zhang, Y. J. Liu, L. W. Liu, J. S. Leng, *Sci. China Technol. Sci.* **2020**, *63*, 578.

[38] M. Champeau, D. A. Heinze, T. N. Viana, E. R. de Souza, A. C. Chinellato, S. Titotto,

Adv. Funct. Mater. **2020**, *30*, 1.

[39] J. F. Mano, *Adv. Eng. Mater.* **2008**, *10*, 515.

[40] J. F. Mano, G. A. Silva, H. S. Azevedo, P. B. Malafaya, R. A. Sousa, S. S. Silva, L. F. Boesel, J. M. Oliveira, T. C. Santos, A. P. Marques, N. M. Neves, R. L. Reis, *J. R. Soc. Interface* **2007**, *4*, 999.

[41] Y. J. Li, F. H. Zhang, Y. J. Liu, J. S. Leng, *Sci. China Technol. Sci.* **2020**, *63*, 545.

[42] N. J. Castro, C. Meinert, P. Levett, D. W. Hutmacher, *Curr. Opin. Biomed. Eng.* **2017**, *2*, 67.

[43] G. Ying, N. Jiang, C. Parra-Cantu, G. Tang, J. Zhang, H. Wang, S. Chen, N. P. Huang, J. Xie, Y. S. Zhang, *Adv. Funct. Mater.* **2020**, *30*, 2003740.

[44] Z. Ren, Y. Zhang, Y. Li, B. Xu, W. Liu, *J. Mater. Chem. B* **2015**, *3*, 6347.

[45] L. Ren, B. Li, Y. He, Z. Song, X. Zhou, Q. Liu, L. Ren, *ACS Appl. Mater. Interfaces* **2020**, *12*, 15562.

[46] G. H. Yang, W. Kim, J. Kim, G. H. Kim, *Theranostics* **2020**, *11*, 48.

[47] A. B. Mathur, T. O. Collier, W. J. Kao, M. Wiggins, M. A. Schubert, A. Hiltner, J. M. Anderson, *J. Biomed. Mater. Res.* **1997**, *36*, 246.

[48] Monika, S. K. Mahto, S. Das, A. Ranjan, S. K. Singh, P. Roy, N. Misra, *RSC Adv.* **2015**, *5*, 45231.

[49] Q. Li, L. Sun, L. Zhang, Z. Xu, Y. Kang, P. Xue, *J. Biomed. Mater. Res. - Part A* **2018**, *106*, 408.

[50] S. C. N. D. S. Santos, Ó. E. Sigurjonsson, C. D. A. Custódio, J. F. C. da L. Mano, *Tissue Eng. - Part B Rev.* **2018**, *24*, 454.

[51] X. Cui, J. Li, Y. Hartanto, M. Durham, J. Tang, H. Zhang, G. Hooper, K. Lim, T. Woodfield, *Adv. Healthc. Mater.* **2020**, *9*, 1.

[52] W. J. Hendrikson, J. Rouwkema, F. Clementi, C. A. Van Blitterswijk, S. Farè, L. Moroni, *Biofabrication* **2017**, *9*, 031001.

[53] S. Miao, H. Cui, M. Nowicki, S. Lee, X. Zhou, X. Yao, F. Masood, M. W. Plesniak, M. Mohiuddin, L. G. Zhang, *Biofabrication* **2018**, *10*, 035007.

[54] K. Kim, J. H. Ryu, D. Y. Lee, H. Lee, *Biomater. Sci.* **2013**, *1*, 783.

[55] K. B. Fonseca, D. B. Gomes, K. Lee, S. G. Santos, A. Sousa, E. A. Silva, D. J. Mooney, P. L. Granja, C. C. Barrias, *Biomacromolecules* **2014**, *15*, 380.

[56] M. I. Neves, L. Moroni, C. C. Barrias, *Front. Bioeng. Biotechnol.* **2020**, *8*, 665.

[57] Y. Luo, X. Lin, B. Chen, X. Wei, *Biofabrication* **2019**, *11*, 045019.

[58] M. Betsch, C. Cristian, Y. Y. Lin, A. Blaeser, J. Schöneberg, M. Vogt, E. M. Buhl, H. Fischer, D. F. Duarte Campos, *Adv. Healthc. Mater.* **2018**, *7*, 1800894.

[59] R. Tognato, A. R. Armiento, V. Bonfrate, R. Levato, J. Malda, M. Alini, D. Eglin, G. Giancane, T. Serra, *Adv. Funct. Mater.* **2019**, *29*, 1804647.

[60] C. Cui, D.-O. Kim, M. Y. Pack, B. Han, L. Han, Y. Sun, L.-H. Han, *Biofabrication* **2020**, *12*, 045018.

[61] H. Wen, J. Li, G. F. Payne, Q. Feng, M. Liang, J. Chen, H. Dong, X. Cao, *Biofabrication* **2020**, *12*, 035007.

[62] C. C. Piras, D. K. Smith, *J. Mater. Chem. B* **2020**, *8*, 8171.

[63] A. K. Miri, A. Khalilpour, B. Cecen, S. Maharjan, S. R. Shin, A. Khademhosseini, *Biomaterials* **2019**, *198*, 204.

[64] K. Y. Lee, J. A. Rowley, P. Eiselt, E. M. Moy, K. H. Bouhadir, D. J. Mooney, *Macromolecules* **2000**, *33*, 4291.

[65] C. García-Astrain, L. Avérous, *Carbohydr. Polym.* **2018**, *190*, 271.

[66] B. Sarker, J. Rompf, R. Silva, N. Lang, R. Detsch, J. Kaschta, B. Fabry, A. R. Boccaccini, *Int. J. Biol. Macromol.* **2015**, *78*, 72.

[67] N. Ashammakhi, S. Ahadian, F. Zengjie, K. Suthiwanich, F. Lorestani, G. Orive, S. Ostrovidov, A. Khademhosseini, *Biotechnol. J.* **2018**, *13*, 1.

[68] S. E. Bakarich, R. Gorkin, M. In Het Panhuis, G. M. Spinks, *Macromol. Rapid*

Commun. **2015**, *36*, 1211.

[69] N. Huebsch, C. J. Kearney, X. Zhao, J. Kim, C. A. Cezar, Z. Suo, D. J. Mooney, *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 9762.

[70] A. Leucht, A. C. Volz, J. Rogal, K. Borchers, P. J. Kluger, *Sci. Rep.* **2020**, *10*, 5330.

[71] L. P. Ferreira, V. M. Gaspar, J. F. Mano, *Biomaterials* **2018**, *185*, 155.

[72] S. Li, X. Tian, J. Fan, H. Tong, Q. Ao, X. Wang, *Micromachines* **2019**, *10*, 765.

[73] N. M. Alves, J. F. Mano, *Int. J. Biol. Macromol.* **2008**, *43*, 401.

[74] F. Lin, H. R. Jia, F. G. Wu, *Molecules* **2019**, *24*, 4371.

[75] F. E. Montero, R. A. Rezende, J. V. L. da Silva, M. A. Sabino, *Front. Mech. Eng.* **2019**, *5*, 56.

[76] M. Kesti, M. Müller, J. Becher, M. Schnabelrauch, M. D'Este, D. Eglin, M. Zenobi-Wong, *Acta Biomater.* **2015**, *11*, 162.

[77] R. P. Babu, K. O'Connor, R. Seeram, *Prog. Biomater.* **2013**, *2*, 8.

[78] W. Zhao, F. Zhang, J. Leng, Y. Liu, *Compos. Sci. Technol.* **2019**, *184*, 107866.

[79] R. J. Morrison, S. J. Hollister, M. F. Niedner, M. G. Mahani, A. H. Park, D. K. Mehta, R. G. Ohye, G. E. Green, *Sci. Transl. Med.* **2015**, *7*, 285ra64.

[80] M. Zarek, N. Mansour, S. Shapira, D. Cohn, *Macromol. Rapid Commun.* **2017**, *38*, 1600628.

[81] A. Kirillova, L. Ionov, *J. Mater. Chem. B* **2019**, *7*, 1597.

[82] B. Narupai, P. T. Smith, A. Nelson, *Adv. Funct. Mater.* **2021**, 2011012.

[83] N. Ashammakhi, A. Hasan, O. Kaarela, B. Byambaa, A. Sheikhi, A. K. Gaharwar, A. Khademhosseini, *Adv. Healthc. Mater.* **2019**, *8*, 1801048.

[84] P. Lavrador, M. R. Esteves, V. M. Gaspar, J. F. Mano, *Adv. Funct. Mater.* **2020**, *2005941*, 1.

[85] K. Kuribayashi-Shigetomi, H. Onoe, S. Takeuchi, *PLoS One* **2012**, *7*, e51085.

[86] G. Bao, T. Jiang, H. Ravanbakhsh, A. Reyes, Z. Ma, M. Strong, H. Wang, J. M.

Kinsella, J. Li, L. Mongeau, *Mater. Horizons* **2020**, *7*, 2336.

[87] Y. Tan, H. Huang, D. C. Ayers, J. Song, *ACS Cent. Sci.* **2018**, *4*, 971.

[88] O. Chaudhuri, *Biomater. Sci.* **2017**, *5*, 1480.

[89] M. Prabaharan, J. F. Mano, *Macromol. Biosci.* **2006**, *6*, 991.

[90] S. R. Derkach, N. G. Voron'ko, Y. A. Kuchina, D. S. Kolotova, *Polymers (Basel).* **2020**, *12*, 1.

[91] G. Kocak, C. Tuncer, V. Bütün, *Polym. Chem.* **2017**, *8*, 144.

[92] J. F. Mano, *Macromol. Biosci.* **2008**, *8*, 69.

[93] C. O. Correia, J. F. Mano, *J. Mater. Chem. B* **2014**, *2*, 3315.

[94] A. Lendlein, H. Jiang, O. Jünger, R. Langer, *Nature* **2005**, *434*, 879.

[95] S. Gil, J. F. Mano, *Biomater. Sci.* **2014**, *2*, 812.

[96] J. C. Breger, C. Yoon, R. Xiao, H. R. Kwag, M. O. Wang, J. P. Fisher, T. D. Nguyen, D. H. Gracias, *ACS Appl. Mater. Interfaces* **2015**, *7*, 3398.

[97] F. Zhang, L. Wang, Z. Zheng, Y. Liu, J. Leng, *Compos. Part A Appl. Sci. Manuf.* **2019**, *125*, 105571.

[98] C. D. Devillard, C. A. Mandon, S. A. Lambert, L. J. Blum, C. A. Marquette, *Biotechnol. J.* **2018**, *13*, 1800098.

[99] S. R. Deshpande, R. Hammink, F. H. T. Nelissen, A. E. Rowan, H. A. Heus, *Biomacromolecules* **2017**, *18*, 3310.

[100] M. Shin, H. Lee, *Chem. Mater.* **2017**, *29*, 8211.

[101] H. Chen, F. Yang, Q. Chen, J. Zheng, *Adv. Mater.* **2017**, *29*, 1606900.

[102] J. Chen, Q. Peng, X. Peng, L. Han, X. Wang, J. Wang, H. Zeng, *ACS Appl. Polym. Mater.* **2020**, *2*, 1092.

[103] P. Lavrador, V. M. Gaspar, J. F. Mano, *Adv. Healthc. Mater.* **2020**, *9*, 1.

[104] B. D. Hoffman, C. Grashoff, M. A. Schwartz, *Nature* **2011**, *475*, 316.

[105] N. Wang, *J. Phys. D. Appl. Phys.* **2017**, *50*, 233002.

[106] W. Li, Z. Yan, J. Ren, X. Qu, *Chem. Soc. Rev.* **2018**, *47*, 8639.

[107] R. Liang, L. Wang, H. Yu, A. Khan, B. Ul Amin, R. U. Khan, *Eur. Polym. J.* **2019**, *114*, 380.

[108] J. M. Korde, B. Kandasubramanian, *Chem. Eng. J.* **2020**, *379*, 122430.

[109] J. Shang, X. Le, J. Zhang, T. Chen, P. Theato, *Polym. Chem.* **2019**, *10*, 1036.

[110] A. P. Piedade, *J. Funct. Biomater.* **2019**, *10*, 9.

[111] H. Ko, M. C. Ratri, K. Kim, Y. Jung, G. Tae, K. Shin, *Sci. Rep.* **2020**, *10*, 7527.

[112] J. Guo, R. Zhang, L. Zhang, X. Cao, *ACS Macro Lett.* **2018**, *7*, 442.

[113] G. H. Kwon, J. Y. Park, J. Y. Kim, M. L. Frisk, D. J. Beebe, S. H. Lee, *Small* **2008**, *4*, 2148.

[114] P. Zhu, W. Yang, R. Wang, S. Gao, B. Li, Q. Li, *ACS Appl. Mater. Interfaces* **2018**, *10*, 36435.

[115] Y. C. Li, Y. S. Zhang, A. Akpek, S. R. Shin, A. Khademhosseini, *Biofabrication* **2017**, *9*, 012001.

[116] Y. S. Zhang, A. Khademhosseini, in *Tissue-Engineered Vasc. Grafts* (Eds.: B.H. Walpoth, H. Bergmeister, G.L. Bowlin, D. Kong, J.I. Rotmans, P. Zilla), Springer International Publishing, Cham, **2020**, pp. 321–338.

[117] M. Jamal, S. S. Kadam, R. Xiao, F. Jivan, T. M. Onn, R. Fernandes, T. D. Nguyen, D. H. Gracias, *Adv. Healthc. Mater.* **2013**, *2*, 1142.

[118] A. R. Amini, C. T. Laurencin, S. P. Nukavarapu, *Crit. Rev. Biomed. Eng.* **2012**, *40*, 363.

[119] E. J. Sheehy, D. J. Kelly, F. J. O'Brien, *Mater. Today Bio* **2019**, *3*, 100009.

[120] X. Gu, F. Ding, D. F. Williams, *Biomaterials* **2014**, *35*, 6143.

[121] I. Apsite, G. Constante, M. Dulle, L. Vogt, A. Caspari, A. R. Boccaccini, A. Synytska, S. Salehi, L. Ionov, *Biofabrication* **2020**, *12*, 035027.

[122] J. Jang, H. J. Park, S. W. Kim, H. Kim, J. Y. Park, S. J. Na, H. J. Kim, M. N. Park, S.

H. Choi, S. H. Park, S. W. Kim, S. M. Kwon, P. J. Kim, D. W. Cho, *Biomaterials* **2017**, *112*, 264.

[123] Y. Wang, H. Cui, Y. Wang, C. Xu, T. J. Esworthy, S. Y. Hann, M. Boehm, Y.-L. Shen, D. Mei, L. G. Zhang, *ACS Appl. Mater. Interfaces* **2021**, in press.

[124] Q. Zhao, J. Wang, H. Cui, H. Chen, Y. Wang, X. Du, *Adv. Funct. Mater.* **2018**, *28*, 1801027.

[125] K. Hölzl, S. Lin, L. Tytgat, S. Van Vlierberghe, L. Gu, A. Ovsianikov, *Biofabrication* **2016**, *8*, 032002.

[126] H. Jing, L. He, J. Feng, H. Fu, S. Guan, P. Guo, *Soft Matter* **2019**, *15*, 5264.

[127] A. M. S. Costa, J. F. Mano, *Eur. Polym. J.* **2015**, *72*, 344.

[128] A. M. S. Costa, J. M. M. Rodrigues, M. M. Pérez-Madrigal, A. P. Dove, J. F. Mano, *J. Am. Chem. Soc.* **2020**, *142*, 19689.

[129] M. C. Gomes, J. F. Mano, *Biomater. Biosyst.* **2021**, *1*, 100010.

[130] S. C. Santos, C. A. Custódio, J. F. Mano, *Adv. Healthc. Mater.* **2018**, *7*, 1.

[131] S. Ullah, X. Chen, *Appl. Mater. Today* **2020**, *20*, 100656.

[132] D. C. Corbett, E. Olszewski, K. Stevens, *Trends Biotechnol.* **2019**, *37*, 1153.

[133] S. M. Oliveira, R. L. Reis, J. F. Mano, *Biotechnol. Adv.* **2015**, *33*, 842.

[134] J. Mason, S. Visintini, T. Quay, in *CADTH Issues Emerg. Heal. Technol.*, **2019**.

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4D bioprinting is becoming an increasingly valuable technique to produce biomaterials resembling living tissues. This approach, which relies on the post-printing modifications of the bioconstructs in response to certain stimuli, enables a closer replication of the dynamic nature of native tissues. Polymers of natural origin are being widely explored for this purpose, due to their inherently advantageous properties.

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Natural Origin Biomaterials for 4D Bioprinting Tissue-like Constructs