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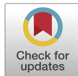

3D printed tissue models: From hydrogels to biomedical applications

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ABSTRACT

The development of new advanced constructs resembling structural and functional properties of human organs and tissues requires a deep knowledge of the morphological and biochemical properties of the extracellular matrices (ECM), and the capacity to reproduce them. Manufacturing technologies like 3D printing and bioprinting represent valuable tools for this purpose. This review will describe how morphological and biochemical properties of ECM change in different tissues, organs, healthy and pathological states, and how ECM mimics with the required properties can be generated by 3D printing and bioprinting. The review describes and classifies the polymeric materials of natural and synthetic origin exploited to generate the hydrogels acting as “inks” in the 3D printing process, with particular emphasis on their functionalization allowing crosslinking and conjugation with signaling molecules to develop bio-responsive and bio-instructive ECM mimics.

1. Introduction

With the advent of 3D printing, new opportunities for tissue engineering and 3D *in vitro* advanced tissue models are opened. The basic concept of 3D bioprinting takes inspiration from fundamental approaches employed in tissue engineering and regenerative medicine, based on the capacity to develop hydrogels able to host different cell populations, crosstalk with them and induce their fate by controlling both physical and biochemical signals.

These hydrogels exploited for 3D printing are known as “inks” or “bioinks”. An ink has been defined by Groll et al. as *‘a biomaterial is used for printing and cell-contact occurs post-fabrication’*, whereas the bioink has been defined as *‘a formulation of cells suitable for processing by an automated biofabrication technology that may also contain biologically active components and biomaterials’* [1].

One of the major gaps in the production of advanced 3D tissue models is related to the difficulty to generate inks suitable for the 3D bioprinting process and at the same time able to provide the signals inducing different cells to produce new matrix, ideally reproducing the specific in vivo cell environment of interest. Indeed, the traditional approach employed to develop inks and bioinks is mainly focused on the control of the physical properties of the hydrogels, neglecting the biochemical signals.

The first 3D bioprinted models were based on alginate in which the hydrogel formation occurs by physical crosslinking employing divalent cations [2]. More recently, a variety of natural and synthetic polymers have been exploited, functionalising and crosslinking them in order to generate hydrogels able to generate personalized artificial 3D tissue models with superior biochemical and physical properties and with the capacity to undergo dynamic modulation. This review will collect the latest concepts on hydrogels suitable for the development of static and dynamic 3D printable ECM mimics for tissues engineering. Noteworthy, the artificial tissues generated with the 3D printing approaches can incorporate drugs and allow their controlled release to avoid local infections during the regenerative processes, or even to kill residual cancer cells if implanted in tumor resections.

Abbreviations: ASGPR, asialoglycoprotein receptor; CRC, colorectal cancer; CSPGs, chondroitin sulphate proteoglycans; dECM, decellularized extracellular matrix; ECM, extracellular matrix; ELPs, elastin like peptides; EMT, mesenchymal transition; FGF, Fibroblast growth factors; GAGs, glycosaminoglycans; HA, hyaluronic acid; HGF, hepatocyte growth factor; HSCs, Hematopoietic stem cells; HSPGs, heparan sulphate proteoglycans; IC, intrahepatic cholangiocarcinoma; IOB, IntraOperative Bioprinting; MMPs, metalloproteinases; PCL, polycaprolactone; PEG, polyethylene glycol; PGA, polyglutamic acid; PGs, proteoglycans; PLA, polylactic acid; PTMs, post-translational modifications; TGF-β, transforming growth factor beta; TIMPs, tissue inhibitor metalloproteinases; TME, tumour microenvironment; VEGF, Vascular-Endothelial Growth Factor; WTA, walking track analysis.

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2. ECM: one of the major players of the microenvironment

The ECM components include structural proteins (i.e. collagens and elastin), glycoproteins (i.e. fibronectin, laminin), glycosaminoglycans (GAGs) (i.e hyaluronic acid) and proteoglycans (PGs) (i.e. Heparan sulphate proteoglycans (HSPGs), Chondroitin sulphate proteoglycans (CSPGs)) [3,4]. Around 30% of proteins in the human body are constituted by collagens, a family of ubiquitous proteins with 28 identified subtypes [5]. Collagens includes proteins with differential structural organization [3], post-translational modifications [6], parental receptors / proteins [6] and consequently biological functions [7].

Furthermore, the extracellular space is rich of several macromolecules like enzymes and growth factors that actively participate in ECM remodelling, with an impressive impact in cell signalling and microenvironmental regulation [8,9]. The most representative examples of ECM enzymes include metalloproteinases (MMPs) and tissue inhibitor metalloproteinases (TIMPs), involved respectively in ECM degradation and proteinases regulation [10].

All these components, and their balance contribute to the development of healthy and pathological states. Even if several ECM macromolecular components have been characterized and extensively reported in literature [11–19], our knowledge on ECM composition, remodelling and instructive role is still limited. The concept of "matrisome" is acquiring importance, it refers to ECM signatures related to specific diseases or aberrant phenomena like fibrosis or inflammation [12,15,20]. The ECM signature, however, is related and responsible not only of pathological events, but also, and equally important, to the evolution of the tissues in terms of morphogenesis [10].

The identity of ECM components, their concentrations and their structural organization varies in different tissues and organs and in different developmental stages of the same tissue.

In tumour microenvironment (TME) the mechanical properties and architecture of the ECM are strongly related to tumour malignancy and metastatic potential [21]. Even in fibrosis the ECM play a key role being one of the main driver of the aberrant deposition of ECM components with consequential organ functionality impairment [22]. The mechanical role of ECM is well studied and the impact of ECM stiffness in mechanotrasduction pathways is well recognized. Several works highlighted the importance of physical forces in driving cell adhesion, organization, metabolism and signalling [23–25] . The mechanotrasduction process is one of the most studied ECM-cell interaction mechanism regulated by cell surface integrins [26]. It is a dynamic mechanism regulating newly synthetized ECM components with a fine control of deposition rate and identity [23]. The instructive role of ECM in the maintenance of tissue homeostasis or in the induction of pathological conditions is exploited also through other mechanism of control, including cell receptor interactions [27]. It is well known that significant mutations of genes encoding for ECM components result in pathological phenomena with disruptive effect for organ and tissues functionalities [13]. Misfolded ECM proteins are associated to dysregulation of tissue functions and affect the physiological crosstalk with cell surface receptors [28,29]. Furthermore, both aberrant proteolytic phenomena or over expression of specific components induce aberrant cell fates or unfunctional cell development through physical and biochemical signalling [30]. The ECM proteins interact with cell receptors exploiting: a) functional structural organization, b) peptide sequences, and c) post-translational modifications (PTMs). Collagens are the most abundant components of ECM and the GFOGER hexapeptide interacts with different integrins including $\alpha2\beta1$, $\alpha1\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$ [31]. These interactions are fundamentals for the maintenance of cell adhesion, migration and spreading, and disruption of these interactions are observed in several pathologies, including cancer and inflammation or fibrosis [32]. A large plethora of ECM-cell interactions are mediated by other proteinaceous components of ECM, including the glycoproteins fibronectin and laminin. Both are characterized by peptide sequences, like RGD for fibronectin and IKVAV for laminin, recognized by cell surface receptors. RGD, discovered for the first time in fibronectin but present also in other proteins of the ECM, acts as adhesive sequence interacting with a subset of integrins [33]. IKVAV has multiple functions spanning from cell adhesion to neurite outgrowth in primary neuronal cell lines [34]. Hyaluronic acid (HA) is ECM polysaccharidic component interacting with CD44 and RHAMM cell receptors involved in both physiological or pathological processes [35]. CD44 acts as mediator between extracellular space and intracellular cytoskeleton, inducing multiple functions like pericellular matrix assembling, wound healing in physiological conditions, cell spreading and metastasis in pathological niches [36]. RHAMM is a multifunctional receptor involved in control of differential cell responses including cell motility and cell signalling pathways during injuries and pathological phenomena [37,38].

Proteoglycans, enzymes, growth factors and a wide range of other bio- and macromolecules induce signalling cascades with regulatory roles as reported in Table 1 [39].

Even if the main and intrinsic role of ECM is cell hosting and cell fate modulation, not surprisingly it is also involved in microbial infection processes. The first stages of infections occur in the extracellular space through biomolecular interactions and lytic activities [71]. Pathogens interact and with several extracellular glycoproteins, structural proteins and proteoglycans, including collagen, fibronectin, laminin and decorin, to colonize tissues and evade the host immune system [72–75] .

ECM remodelling is a dynamic mechanism which allows different cell populations to survive and exploit their functions in a more suitable microenvironment. Cell populations include stem and differentiates cells, cells of the immune system and even microbial populations. When the physiological interactions or remodelling of these components becomes aberrant, chronic or acute pathological phenomena occur [76]. It is more and more evident that the ECM properties are not just consequences of specific tissue stages but have a pivotal role in the progression of tissues morphogenesis and in the maintenance of functionalities. Table 2 reports examples of ECM characteristics related to different pathologies. In this view, the design of 3D bioprinted constructs to generate artificial tissues must find inspiration from the properties of ECM in different organs, in physiological and pathological states, and requires a panel of "inks" and chemical protocols for a tailor-made construction.

The capacity to generate mimetics of pathological tissues in a personalised way, once the properties of their ECM are known, allows to perform in vitro drug screening in 3D tissue models more adherent to the specific human pathology, with respect to the tests performed on mice

Table 1
Major biomolecules with regulatory roles.

Signalling molecules	Regulatory roles	Refs.
HSPG	pluripotent cells self-renewal maintenance; cell fate modulation	[40,41]
Agrin	HSCs survival and proliferation; cell interactions and synaptogenesis; epithelial-to-mesenchymal transition in heart development	[42–44]
Decorin	Pathways regulation in kidney, muscle, hematopoietic, and neural stem cells	[45–50]
Perlecan	Intestinal stem cells proliferation; regulation of neural stem cells.	[51,52]
Tenascins	Multiple functions in hemapotoietic, neural, and skin microenvironment	[53–55]
MMPs	ECM degradation and remodelling; multiple signals	[8,56]
ADAM and ADAMTs	ECM degradation and remodelling; tissue morphogenesis; multiple roles.	[57,58]
galectins	cell proliferation, differentiation, migration, and survival	[59–61]
VEGF	Angiogenesis, cell development; multiple functions	[62–64]
FGF	Cell development, cell fate induction, differentiation	[65,66]
TGF-β	Cell proliferation, cell differentiation and migration, ECM remodelling	[67,68]
HGF	Stem cell differentiation, cell migration	[69,70]

Table 2
Examples of ECM modulation in pathological conditions.

Tissue / Organ	Δ selected ECM components	Pathology	Source	Refs
Brain	Increase of hyaluronic acid, brevican, tenascin-C, fibronectin and thrombospondin. Decrease of versican.	Glioma	various	[77], [78]
Breast	Increase of type I and III collagen, fibronectin, tenascin C, periostin, osteopontin, SPARC, thrombospondin-1, syndecan I, MMP-2, −3, −9 and − 14 Decrease of type IV collagen, Laminin-11	Breast Cancer	various	[79]
Colon	Increase of Type I Collagen; MMP-2, and MMP-9. Decrease of ype IV collagen and TIMP-3	Colorectal cancer (CRC)	ECM obtained by human CRC samples (acellular technology)	[80]
Colon	Increase of heparan and chondroitin sulphate. Decrease of decorin.	Colorectal cancer (CRC)	Fixed tissues from human samples	[81]
Gastric	Increase of Type I and Type IV Collagen, Laminin, Fibronectin; Tenascin, Versican, Decorin, Byglican; MMP-2, MMP-9, MMP7; Galectin-1	Gastric cancer (GC)	Various	[82]
Gastrointestinal system	a) Increase of, fibronectin-3, Tenascin precursor, EMILIN-1 precursor, Vimentin Decrease of ADAMSTS1 b) increase of hyaluronic acid; total and type III and V collagen.	Inflammatory Bowel Diseases -Crohn's disease (CD)	a) intestinal fibroblast–derived ECM; b) various	a) [83], b) [84]
Ileum	Increase of neutrophil collagenases, MMP-9, MMP-10. Decrease of elastin and collagen	Inflammatory Bowel Diseases -Ulcerative Colitis (UC)	Colonic intestinal biopsies from UC patients – extraction of protein content for proteomic analysis	[84]
Liver	a) increased elastin and collagen. b) in liver metastasis: increased Type IV collagen, tenascin, laminin-511, hyaluronic acid	Cancer	a) surgically resected hepatocellular carcinoma (HCCs); b) various	a) [85], b) [86]
Liver	Increase of Type I and type III collagen, laminin fibronectin. In HCV infection fibrosis increase of type I and II collagen, elastin, MMP-2 and versican	Chronic liver diseases / liver fibrosis	various	[87] [88] [89]
Lung	Increased type IV collagen (NSCLCs), laminins, galectin-1; fibromodulin (SCLC); hyaluronic acid. Decrease of decorin.	Cancer	various	[90]
Pancreas	Increase of collagens I and III; collagen VI; Fibrillin-1 (FBN-1), fibronectin (FN1), fibrinogens (FGA, FGB, and FGG), and periostin (POSTN)	Pancreatic adenocarcinoma	Human PDAC tissues – extraction of protein content for proteomic analysis (ECM enriched)	[91]
Pancreas	a) Increase of extra-islet deposition of the glycosaminoglycan HA. Decrease of heparan sulfate (HS) b) Reduced laminin α5	Type 1 diabetes (T1D)	Various	a) [19]; b) [92]

models.

The characterization of ECM properties, in physiological and pathological states, is not an easy task and is still limited due to the many variables involved. Information is required on: a) the identity of ECM components for different organs and at different ages; b) the interactions occurring at the extracellular level; c) PTMs modifications affecting cell-ECM and ECM-ECM interaction. Even if in the last years proteomic [4,20,93] and biochemical [79,94,95] approaches revealed important information, the intricate puzzle of "matrisome" [20] is still unresolved being characterized by a plethora of actors with different biomolecular codes.

3. Cell-friendly polymers: the importance of biomaterials nature and crosslinking

The selection of polymers employable to develop 3D bioprinted constructs is guided by their compatibility with different cell populations. With the advancement of 3D printing processes and

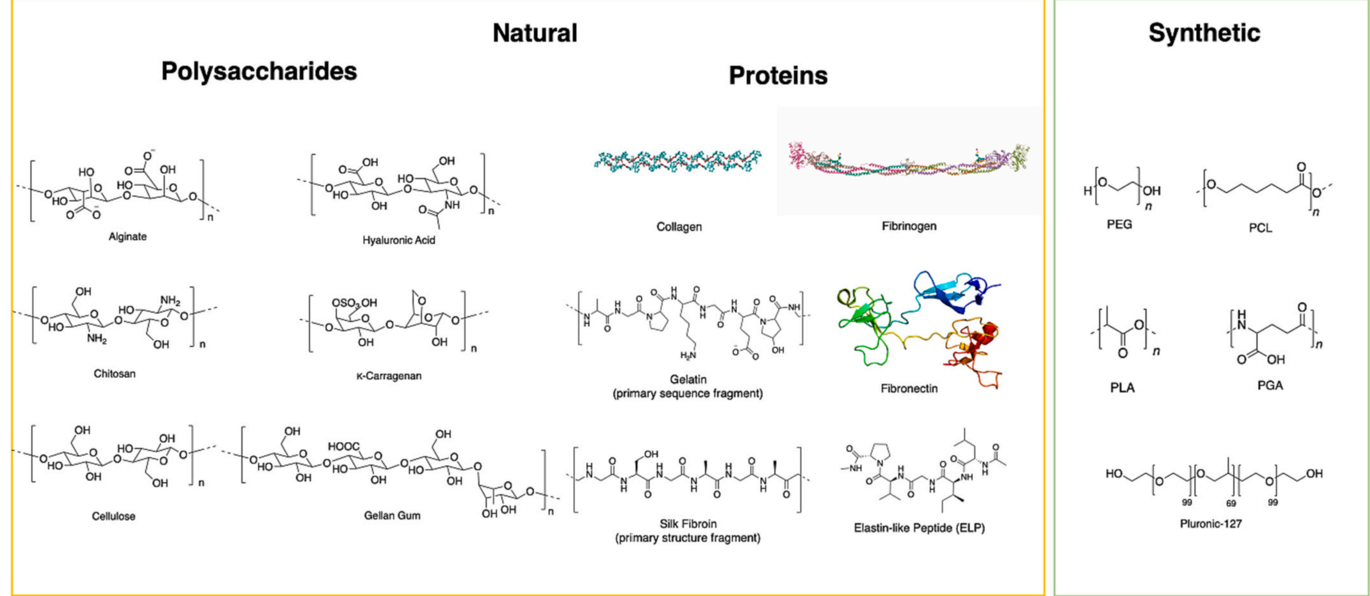

Fig. 1. Selected examples of natural (polysaccharides and proteins) and synthetic polymers employed for the formulation of inks and bioinks. Adapted from [118].

instruments, also a large number "inks" based on natural and synthetic polymers (Fig. 1) have been classified, defined and standardized [2–4]. The classification of polymers is based on their origin, including natural and synthetic polymers.

Natural polymers include polysaccharides [94,96], like hyaluronic acid [97,98], alginate [99], chitosan [100], cellulose [101], gellam gum [102], and proteins [103,104] like collagen [105], gelatin [106], elastin [107] and elastin like peptides (ELPs) [108], fibrinogen [109] and silk fibroin [110]. Biocompatible synthetic polymers with adequate hydrophilic properties, like polyethylene glycol (PEG) [111], Pluronic-127 [112], polycaprolactone (PCL) [113], polylactic acid (PLA) [114], polyglutamic acid (PGA) [115], are also largely employed for the generation of printable constructs [116,117].

Natural and synthetic polymers have different properties, advantages and limitations. The advantage of natural polymers, with respect to synthetic ones, consists of their intrinsic biocompatibility and appropriate cell crosstalk. Synthetic polymers lack biomolecular motifs needed for cell fate modulation, but offer improved control of mechanical and structural properties [118].

Combining natural and synthetic polymers different hybrid inks for 3D printing and bioprinting have been developed, suitable to generate 3D ECM mimetics with the required tailorable morphological and cell-specific properties [119–125].

In the bioprinting approach, cell populations or organoids are embedded in the hydrogel or hydrogel precursors (Fig. 2 A) before, during or after the printing process [1,126]. Cell viability must be guaranteed during the bioprinting process and, on the other and, the presence of leaving cells in the ink can compromise his correct printability and finally the structural fidelity of the construct. Accurate control is required and the crosslinking methodologies, properly selected and balanced, can increase the printing fidelity. In some cases, the cells can be added after the printing process once the scaffold has been generated (Fig. 2 B).

The rheological properties of the polymers are mostly important for the selection of the printability window and the reliability of 3D bioprinting process [127]; on the other hand, the biocompatibility of and capacity to crosstalk with cells are essential for the functionality of the final construct [128,129]. The morphology of the construct must be sufficiently stable over the time but at the same time must allow future modifications. The extracellular matrix is a dynamic environment, in which matrix metalloproteases (MMPs) are responsible for dynamic remodelling [5,96,97]. Dynamic bioresponsive ECM mimic can be generated by selection of adequate crosslinking strategies involving not only biodegradable interconnections but also week interactions and reversible covalent linkages [130].

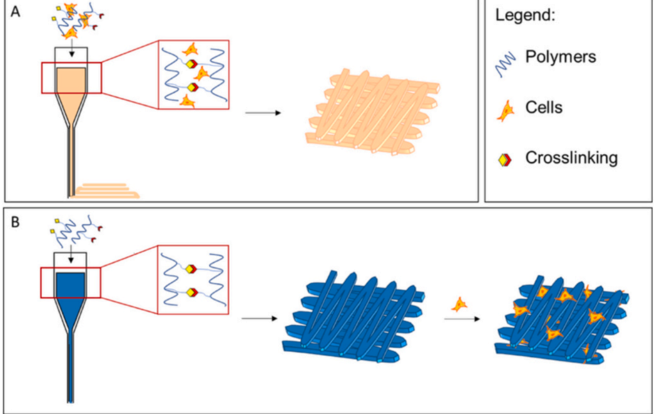

Fig. 2. A) Bioink and B) Ink. Adapted from reference [1].

3.1. Physical and chemical crosslinking of bioprintable polymers

The first of 3D bioprinted constructs were generated using bioinks able to generate physical crosslinking, defined "physical hydrogels". Physical hydrogels are obtained from polymers interconnected by different interactions, including ionic and hydrophobic interactions or thermal transitions [118]. Alginates have been extensively employed as physical hydrogel, also in combination with other polysaccharides and crosslinked by addition of divalent cations (Ca^{2+}, Ba^{2+} and Mg^{2+}) [99,131]. Self-assembling peptides have been recently explored as biocompatible bioinks allowing an easy control of gelling varying the pH or adding divalent ions [132]. Synthetic polymers like Pluronic F127 [133] were employed for their ability to form hydrogels by controllable thermal transition [134]. Pluronic F127 shows an inverse thermal jellification, becoming hydrogel with the increase of temperature, the hydrogel properties can be tailored varying the polymer concentration and the molecular weight. Furthermore, the mechanical properties of the obtained hydrogels can be improved by covalent crosslinking, as reported for example by its functionalization at the extremities as acrylate esters and UV induced polymerization [112]. The ability of Pluronic F127 to form hydrogels at 37° degrees is also optimal as sacrificial structures to generate 3D constructs with separate channels and compartments [133].

Even if physical hydrogels present clear advantages in the ease of preparation of the constructs, the limited number of polymers employable and the difficulty to control accurately mechanical and biochemical properties compromises the applications. Recent efforts are mostly focussed on the development of hybrid or multicomponent biomaterials in which physical and chemical crosslinking strategies are combined, also taking advantage of reversible covalent bonds, will know in organic chemistry, and supramolecular chemistry, to generate dynamic hydrogels [119,127,132,135,136]. The accurate selection of crosslinking methods is dictated by the final application, for example in vivo implantation for regenerative medicine could require scaffold biodegradable once implanted. Dynamic cross-linkages, reversible in specific cell culture conditions, provide dynamic ECM mimetics more similar to the in vivo ones, improving also cell motility and cell fate modulation and morphogenesis [136]. Self-repairing materials are based on supramolecular or reversible linkages, showing mechanical dynamic and customizable properties [120,121]. The combination of static and dynamic linkages was employed in implantable biomaterials to improve the scaffold integration in tissues and the adaptation in the implant site. Amaral et al. described a bioink based on the dynamic linkage between laminarin functionalized with boronic acid and alginate (Fig. 3A) [137]. The obtained hydrogel showed improved morphological and rheological properties and high biocompatibility with different cell lines, including MC3T3-E1 osteoblast precursors, L929 fibroblasts and MDA breast cancer cells. Interesting dynamics bioinks were developed employing nanocomposites based on amine functionalized silica nanoparticles combined with oxidized alginate and gellan gum [138]. In this case a covalent dynamic reversible crosslinking occurred by reaction of the amino groups of silica nanoparticles and the aldehyde of oxidized alginate, whereas gellan gum provided thermal gelation. The developed hybrid system showed improved rheological properties, advanced printing fidelity and *in vitro* and *in vivo* compatibility with chondrocytes.

Tallia et al. developed 3D printable scaffolds with porous channels of ~200 μm using inks based on silica, poly(tetrahydrofuran and poly (γ-caprolactone) with superior mechanical and self-healing properties employable for cartilage tissue engineering applications (Fig. 3B) [139].

New multifunctional biomaterials with superior dynamic properties have been generated exploiting dynamic reversible linkages [140].

The validation of cell-compatible crosslinking and conjugation methodologies represent an essential toolbox for the development of advanced customizable inks. Table 3 collect examples of physical and covalent crosslinking methodologies, including the dynamic and reversible ones.

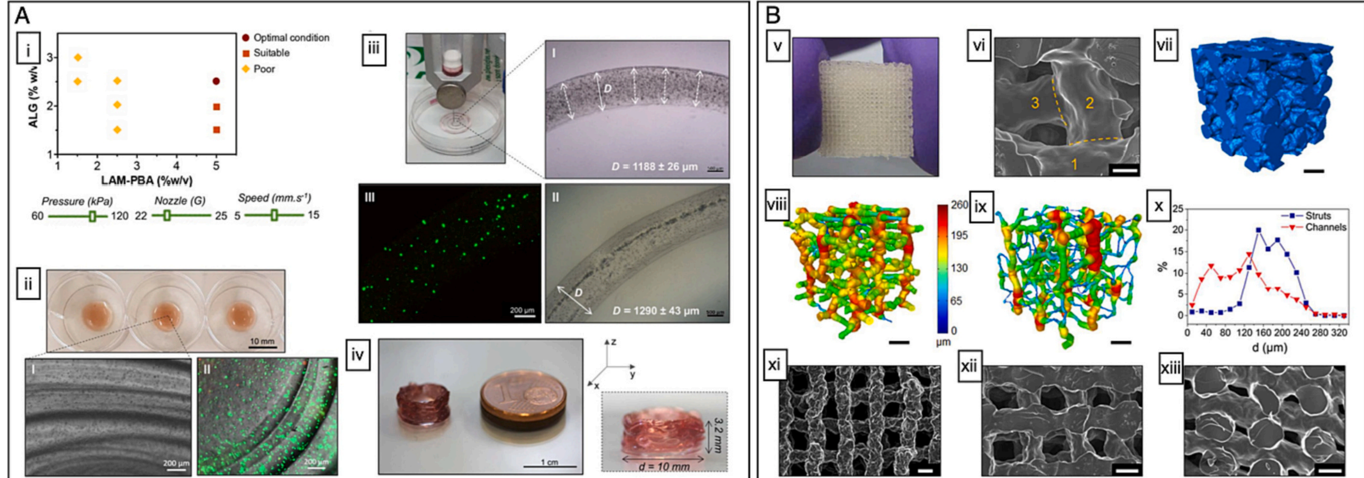

Fig. 3. A) Reproduced with permission from Amaral et al. [137], i) Printing conditions tested and phase diagram for optimal ink development in terms of printability, extrudability and homogeneity. ii) Digital photographs and bright-field micrograph of LAM-PBA$_5$/ALG$_{2.5}$ disk-shaped cell-laden constructs illustrating the strut concentric pattern still evident after three days in culture and fluorescence microscopy conjugated with bright-field imaging of MC3T3-E1 cells within LAM-PBA$_5$/ALG$_{2.5}$ hydrogel following live/dead staining. iii) 3D bioprinting of well-defined solid filaments with a spiral pattern. iv) Bioprinted tubular construct demonstrating the potential of the bioink to fabricate 3D structures. B) reproduce with permission from Tallia et al. [139] v) example of a scaffold with a grid-like 3D porous structure and vi) SEM image of a horizontal section showing the interaction among struts belonging to three different layers; vii) example μCT image of a 3D printed Si80-CL scaffold; viii-x) 3D rendering of the struts used for the μCT image analyses; xi-xiii) SEM images of top surface, horizontal (*x*–*y*) section and vertical (*z*–*y*) section, respectively, of a 3D printed Si80-CL scaffold. Scale bars: 100 μm in (vi); 250 μm in (vii-ix); 200 μm in (xi-xiii).

The many variables that can be adopted to generate 3D printed artificial tissues foresee the development of a combinatorial approach, in which the nature and ratio of polymeric components, the crosslinking nature and degree, differently combined, will generate a library of constructs ideally resembling the ECMs of different tissues in healthy and pathological states.

3.2. *Control of hydrogel properties*

The "instructive" role of ECM in cell fate is being exercised not only by the physical properties of the material but also by its chemical composition and by the presence of different biomolecules providing specific interactions and signal cascade. The development of functional ECM mimetics therefore must include such bioactive entities, the content, concentration and gradient of which will strongly modify the functionality of the construct [184]. In this perspective, a wide range of signalling biomolecules must be included in the construct, taking instructions from the natural ECMs of specific tissues, a further variable increasing the complexity of the scenario. In this context Nature exploits two main actors: fragments of polymeric molecules, in particular peptide sequences such as the well-known RGD, and effector proteins such as grow factors or enzymes. Their role is multifarious including the correct cell-matrix adhesion, essential for cell survival, the interaction with cell receptors (integrins, selectins, …) to start a signal cascade, or even an enzymatic process inducing functional changes. GAG hydrolases, for example, are responsible for the release in the ECM of growth factors sequestrated by GAG [185]. Examples of bioactive motifs, that can be conjugated to synthetic or natural polymers for the induction of a plethora of biological functions, includes peptides derived from ECM proteins or paracrine factors in the extracellular space. The most employed peptide sequences, inducting fundamental biological events like cell adhesion or differentiation, or specific morphological and functional features, are reported in Table 4.

Peptide sequences can be employed also to assist and promote the physiological ECM remodelling through the action of proteolytic enzymes. With this aim several inks containing MMP sensitive sequences like GCRDGPQGIWGQDRCG or CGPQGIWGQC have been developed [186,187].

Peptide sequences are largely present in commercially available proteinaceous biopolymers (collagen, elastin, ….) that can be used in different ratio to generate bioinks with their required content in the construct. Alternatively, such sequences can be conjugated to not proteinaceous polymers, exploiting different strategies depending on the functional groups available for the conjugation to the polymer backbone.

Among the other signalling molecules, enzymes should maintain their integrity to guarantee the functionality (even if synthetic enzymes have been developed), receptor proteins need the integrity of the receptor site. If the signalling biomacromolecule is commercially available, the easiest way consists in its absorption into the biomaterial. This approach is very easy but do not impede the progressive release with loss of its activity over the time. An alternative strategy consists in the conjugation of the entire biomacromolecules or eventually their active/functional fragments, a more complex and expensive approach that guarantee more reliable and structurally controlled constructs. Different conjugation methodologies can be applied taking inspiration by chemoselective reactions already employed for chemical biology and biomaterials related applications [205].

One of the major challenges still occurring for the development of in vitro tissue consists in the vascularization of the obtained constructs. Several authors reported the integration of vascular components, such as VEGF, in different stages of the 3D bioprinting process [206,207] .

To improve the biocompatibility of biomaterials or increase the adhesion of embedded cells, also other strategies have been adopted, including the conjugation of the selected polymers with adhesive molecules like dopamine [208,209].

The glycans content of ECM is acquiring importance, several evidence indicate that glycans are main actors in the induction of cell fate [206,207] and gained a leading role in nanomedicine [206]. N- and O-glycosylation of ECM and associated proteins is a dynamic process and its role in cell development, morphogenesis and pathological events is more and more evident [210–212]. The term glycosignature has been coined to define the great variety of glycan contents at cell surfaces, ECM and circulating proteins (including Ig) in different conditions. The glycosignature vary with organs, individuals, age, diet, healthy of pathological states [213,214] in a cause effect relation still to be fully

Table 3
Conjugation methods employed for the static and dynamic crosslinking of inks and hydrogels.

Static Covalent Crosslinking	Conditions	Refs
Chain photopolymerizations - Methacrylation	UV (365 nm), photoinitiators	[65], [98], [141], [142], [143], [144], [145] and many others
Thiol-ene (Acrylate; Norbornene)	UV (365 nm), photoinitiators	[146] [147] [148] [149] [150] [151] and many others
Thiol-yne	UV (365 nm), photoinitiators	[151], [152]
Thiol-Maleimide	pH 5.5–7.4	[151], [153], [154], [155]
Vinyl Sulfone - Thiol (pH 7.5-8.0)	pH 7.4–8.0	[151], [156], [157], [111]
Diels-Alder	pH 5.5–7.4, reversible at high temperatures, tailorable kinetics (diene selection)	[158], [159], [160], [161],
	pH 7.4	[162]

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Table 3 (*continued*)

Static Covalent Crosslinking	Conditions	Refs
Strain-promoted azide-alkyne cycloaddition (SPAAC) N_3; pH 7.4		
Enzymatic Crosslinking **HRP** OH; HRP H_2O_2	pH 7.4 5 U/mL HRP	[163] [164]
Transglutaminase NH_2 Lys; NH_2 O Gln; TG	37 °C	[165] [166]
Dynamic Covalent Crosslinking	**Conditions**	**Refs**
Oxime and Hydrazone ligation X: O - Alkoxyamine, NH - Hydrazine R: H - Aldheyde, CH_3 - Ketone X: O - Oxime, NH - Hydrazone	pH 4.5–7.0, reversible	[167]
Imine ligation NH_2; O; N	pH 4.5–6.5	[168]
Boronate ester HO B OH; HO OH	pH 6.5–7.5, reversible at acidic pH	[137] [169]
Disulphide SH; SH; S–S	pH 6.5–7.6 red-ox, thiol exchange	[170]
Dynamic Physical Crosslinking		
Ionic	Divalent cations, pH control	[171]
		[172] [173]

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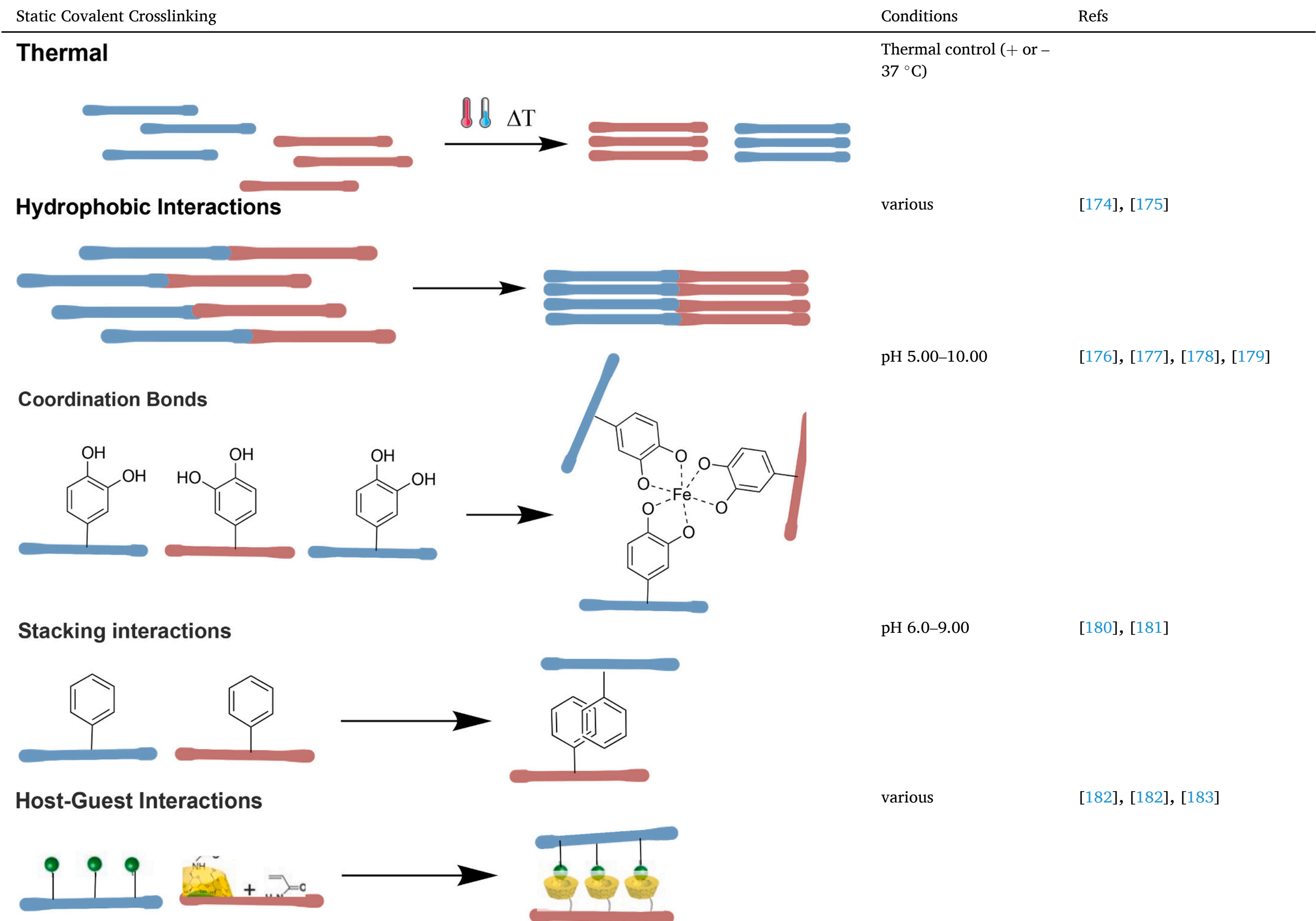
Table 3 (*continued*)



Static Covalent Crosslinking	Conditions	Refs
Thermal	Thermal control (+ or – 37 °C)	
Hydrophobic Interactions	various	[174], [175]
Coordination Bonds	pH 5.00–10.00	[176], [177], [178], [179]
Stacking interactions	pH 6.0–9.00	[180], [181]
Host-Guest Interactions	various	[182], [182], [183]

characterized. Clarifying the role of glycans in cell fate induction is therefore very complex and requires many data obtainable first observing the different cell behaviour in ECM mimics varying only for the glycan content. Glucose and galactose conjugated to 2D collagen films showed the ability to induce morphological and functional differentiation of F11 neuroblastoma cells [197]. 3D collagen hydrogels conjugated with glucose and galactose were employed with cells extracted from the ventral mesencephalon of Sprague–Dawley embryos. Galactose conjugated hydrogels resulted in an increase of astrocyte populations, with larger shape and greater width. In parallel, a significative decrease of astrocytes abundance was observed for glucose functionalised hydrogels [198]. Collagen functionalized with Neu5-Acα2–3-Galβ1–4Glc and Neu5Acα2–6-Galβ1–4Glc induced respectively the up-regulation of chondrogenic and osteogenic markers with hMSCs [199]. Whereases, tested *in vivo* with an osteoarthritic rat models, glucose and galactose functionalized collagens induced the recovery of functional motility as demonstrated by walking track analysis (WTA) [200]. Thanks to its ability to link the parental asialoglycoprotein receptor (ASGPR), β-galactose has been extensively employed for liver-based tissue engineering strategies using different biopolymers [215]. Arai et al. used alginate conjugated galactose to produce 3D culture platform for hepatocytes adhesion [203].

Others powerful glycans with signalling ability, includes sulphated oligosaccharides that in the ECM are involved in many fundamental roles, including the regulation of growth factors recruiting and release. To mimic the bioactive fragments of heparan and heparin sulphate (HS), Chopra et al. produced hydrogels modified with synthetic HS oligosaccharides to study their effect on induced pluripotent cell-derived neural stem cells (HIP-NSCs) modulation. The authors demonstrated the ability to control cell fate modulation tailoring oligosaccharides features [202]. Considering glycans as bioactive signalling molecules, it is important to take in mind that the glycosignature identity and abundance vary across different cells and tissues, physiological and pathological conditions, and different aging stages. 2D collagen functionalized with glucose has been evaluated for the ability to modulate the effect of glycosignature in lung tumour microenvironment. Glycosylated 2D collagens were employed to study the modulation of $CD133^{+}$ Cancer Stem Cell with A549 lung adenocarcinoma, H460 large cell lung carcinoma, LT73 primary lung adenocarcinoma cells. An increased modulation of $CD133^{+}$ CSC was observed with glucose functionalized collagen, demonstrating that the same glycan in a different microenvironment can induce different cell signals [201]. Recently Cadamuro et al. [204] showed that bioinks generated using clickable HA and glycosylated gelatin functionalized respectively with 3′-Sialylgalactose, 6′-Sialylgalactose and 2′-Fucosylgalactose with embedded HT29 cells induce diverse alteration of single cell proteome. Furthermore, patient derived tumoroids bioprinted with 6′-Sialylgalactose based ink induced higher expression of CD133 and LGR5 compared to the control, indicating that the specific glycosignature induces respectively a modulation of stemness phenotype and a correlation with cancer migration and metastases in colorectal cancer (CRC).

Even if the role of the ECM glycosignature is becoming more clear, particular attention on glycans identity is required representing a fundamental parameter to produce effective inks able to modulate cell fate. Furthermore, glycans conjugation strategy must be carefully designed in order to control their correct exposition for the interaction

Table 4
Bioactive and bioresponsive inks and bioinks.

Bioactive/bioresponsive factors	Polymers	Cells/applications	Refs
RGD GCRDGPQGIWGQDRCG	acrylated poly (ethylene glycol) (PEG)	hMSCs osteochondral regeneration	[186]
CGGGGRGDSP CGPQGIWGQC	pectin norbornene	fibroblasts	[187]
CRGDS or MMP sensitive gelatine-norborene (GelNB)	Methylcellulose	mMSCs	[188]
OGP	PCL	BMSCs, bone regeneration	[189]
GGGGRGDSP, VEGF and BMP-2	2:1 (*w*/w) alginate: methylcellulose, PCL	BMSCs, BALB/c OlaHsd-Foxn 1nu nude mice bone regeneration	[190]
VEGF	GelMA	NIH 3 T3, human umbilical vein endothelial cells; pig skin wound model, wound healing	[191]
KIPKASSVPTELSAISTLYL bone morphogenetic protein (BMP) peptide	GelMA	hDPSCs, dental tissue constructs	[192]
dopamine	GelMA	NSCs, Neural Regeneration	[193]
VEGF peptide mimic QK Cys-PEG6-KLTWQELYQLKYKGI	GelNB-GelSH	HUVECs, fibroblasts, iPSC-CM	[194]
Hydroxyapatite	Hydroxyapatite/ polycaprolactone nanoparticles NPs in gelatin, PVA	tibial bone defect model of New Zeland rabbit	[195]
Bioactive glass (45S5 Bioglass®)	Silk fibroin, gelatin	TVA-BMSCs, bone regeneration	[196]
α-glucose and β-galactose	2D and 3D type I collagens	F11, VM from (E14) Sprague–Dawley rats, neuronal regeneration	[197], [198]
Neu5Acα2–3-Galβ1–4Glc and Neu5Acα2–6-Galβ1–4Glc	2D type I collagens	hMSC, osteochondral regeneration	[199]
allyl-α-D-glucopyranoside allyl-β-D-galactopyranoside	2D type I collagens	Induced osteoarthritic Wistar rats, Cartilage regeneration	[200]
α-glucose	2D type I collagens	A549 lung adenocarcinoma, H460 large cell lung carcinoma, LT73 primary lung adenocarcinoma. Cancer stem cell modulation	[201]
HS oligosaccharides	PEG	HIP-NSCs, stem cell fate modulation	[202]
β-galactose	alginate	Murine primary hepatocytes, liver 3D cultures	[203]
3′-Sialylgalactose, 6′-Sialylgalactose and 2′-Fucosylgalactose	3D collagen and hyaluronic acid hydrogel	HT29 human colorectal adenocarcinoma, patient derived tumoroids. Colon cancer models	[204]

with the parental receptor, their functionalization density, and the spatio-temporal presentation of their signal.

4. Natural ECM in printable inks

One of the most innovative approaches for the generation of functional ECM mimics is based on the use of a natural ECM or on the incorporation of its powdered fragments in the printable biopolymer. The natural ECM contains many of the components useful to host the cells at the best and to modulate their fate. Organ decellularization protocols allow to obtain the entire architecture of the tissue of interest, however this approach has some relevant limitations. One is related to the difficulty to control the re-cellularization process, in which different cell types need to reach specific locations in the organ [216]. Furthermore, the efficacy of decellularization processes is tissue-dependent, and can result in partial loss of functional ECM biomacromolecules, structure variation, and tissue integrity with a consequent reduction in terms of functional biological properties [217].

Decellularized ECM (dECM) are becoming promising candidate as components of printable bioinks thanks to their content of ECM components, including the main structural protein, glycoproteins and growth factors. Nevertheless, the difficulty to control the 3D architecture and the morphology of the final construct limits the use of dECM alone for tissue engineering purposes [218]. Jakus et al. reported the production of “tissue papers” obtained combining freeze dried powdered dECM from different porcine and bovine sources and PLGA. The authors employed dECM obtained without enzymatic treatment to preserve the structural integrity and compared their properties with those of the original ECMs (Fig. 4). The results demonstrated the versatility of dECM based materials however, also in this case, limited re-cellularization efficacy was observed and the solvent casting method, which employs organic solvents, represented a further limitations [216].

3D Bioprinting offers a unique opportunity for homogeneous cell distribution during the manufacturing process, and therefore is the most promising methodological approach to employ dECM in combination with other polymers (Table 5). De Santis et al. reported the generation of a 3D bioprintable matrix based on alginate reinforced with dECM [219]; Pati et al. employed dECM obtained respectively from porcine cartilage and heart to produce bioinks for different 3D constructs. In detail, as showed in Fig. 5 the authors produced heart constructs, cartilage constructs and adipose constructs using respectively heart dECM (hdECM), cartilage dECM (cdECM) and adipose dECM (adECM) in combination with polycaprolactone (PCL). All the obtained constructs allowed the production of tissues analogues with increasing homogeneous cell distribution, and with adipogenic and chondrogenic potential [220]. To overcome the low printability of dECM and generate constructs with tailorable mechanical properties, alternative approaches using hydrogel based on different polymers and crosslinking methodologies of dECM are under study. Kim et al. proposed the reinforcement of liver porcine dECM with gelatin [221]. The developed dECM powder-based bio-ink (dECM pBio-ink) was compared with gelatin and dECM based inks (Fig. 5 ii) showing enhanced of mechanical properties and printability and maintaining a biocompatibility with endothelial cells and primary hepatocytes comparable to the conventional dECM. To better control the degradation rate and the mechanical properties of dECM reinforced inks, the dECM components were functionalized to obtain a photopolymerizable mixture. Lee et al. proposed a bioink based on alginate, methacrylate dECM (MA-dECM) and human adipose derived stem cells (hASCs) to generate bone constructs. With this approach the author demonstrated that the introduction of MA-dECM induces higher cell proliferation and osteogenic activity compared to alginate without dECM, even if the increase of MA-dECM reduced cell viability [222]. Also, enzymatic crosslinking methodologies are under study to improve the properties of dECM based inks. Sobreiro-Almeid employed dECM from porcine kidney using transglutaminase for the enzymatic crosslinking of ECM components taking advantage of an agarose

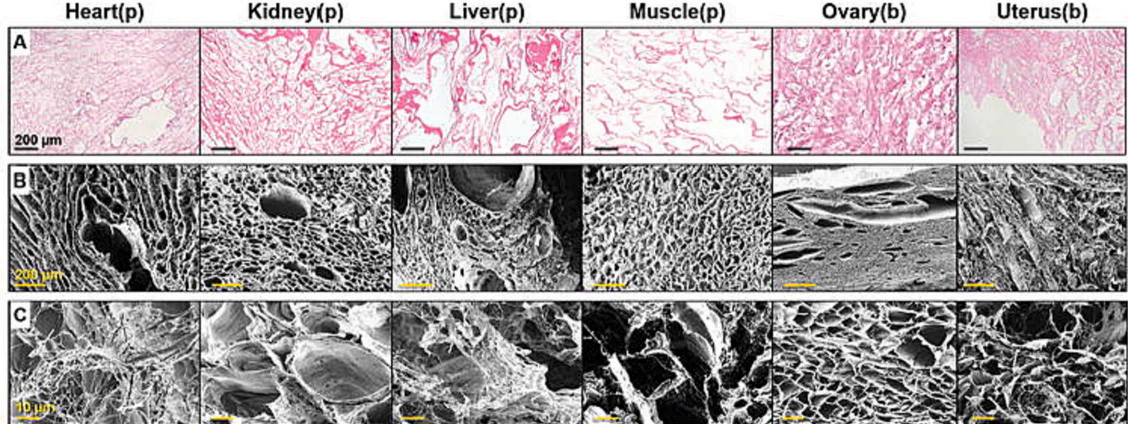


Fig. 4. A) Decellularized tissues from porcine (p) and bovine (b) sources stained with H&E. The decellularized extracellular matrix, which remain after the removal of cellular material, appears in pink. B) Scanning electron images of each decellularized tissue type prior to milling, highlighting the distinct, porous, and fibrous structures. C) Scanning electron images of dECM powder pieces that result from milling and which are used for the tissue paper fabrication**.** Reproduce with permission from [216]**.** (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
ECM mimics generated with dECM.

dECM	Polymers	Crosslinking	Cells/tissue model	Refs
mouse and human lung dECM	alginate	ionic - Ca^{2+}	MLE12 and A549, bEnd.3, HLSMCs, HBECs bioprinted airways	[219]
Porcine heart, cartilage and adipose dECM	PCL	thermal	hASCs and hTMSCs, L6, ATCC CRL-1458	[220]
porcine liver dECM	gelatin	thermal	HUVEC and primary mouse hepatocytes	[221]
Porcine bone methacrylated dECM	alginate	ionic – Ca^{2+} photopolymerization	hASCs bone	[222]
porcine kidney dECM	agarose supporting bath	enzymatic, transglutaminase	hRPCs, HRPCs	[223]
porcine cardiac dECM	collagen	thermal	Cardiac model -Neonatal rat cardiomyocytes (NRCM)	[225]
porcine liver dECM	sacrificial pluronic F-127	thermal	HUH7 spheroids	[226]
porcine cardiac dECM	Peg-diacrylate, Laponite-XLG nanoclay	photo-polymerization and ionic	HCFs, hiPSC derived cardiomyocytes, HS27A. Heart	[227]
Porcine auricular cartilage methacrylated dECM	–	photopolymerization	Auricular chondrocytes	[228]
Porcine pancreatic dECM	Alginate/dECM (pancreatic structure) Alginate/ Fibrinogen (vascular structure)	ionic – Ca^{2+}	Pancreatic islets, HMSC and MSCs. Vascularized pancreas construct	[229]
bovine Achilles' tendon dECM	–	thermal	NIH 3 T3	[230]
porcine heart, liver, and colon dECM	–	photocatalytic (ruthenium/sodium persulfate (dERS)) and thermal	hiPSCs, HepG2 cell line and human Caco-2	[231]
Porcine cornea and heart dECM	–	photocatalytic (ruthenium/sodium persulfate (dERS)) and thermal	hTMSCs and hiPSC-CMs	[232]

microparticle bath support. The developed methodology allowed to obtain a 3D bioprinted construct with defined morphology, high viability and differentiation of encapsulated renal progenitor cells (Figure 5iii) [223]. (See Fig. 6.)

Decellularization protocols and enzymatic treatments employed to obtain printable dECM partially disrupt the interactions between ECM components and remove biomolecules with important signaling role (i. e. sulphated glycans, proteoglycans), reducing the physical integrity and the functional activity of the final construct [224]. Batch-to-batch variation and limited availability of dECM from whole organs are further significant limitations. Noteworthy, dECM lacks specific PTMs (i. e. N- and O-glycosylation of proteins, sulphation patterns of PGs), the importance of which in cell fate modulation and pathological occurrence is now well established. Finally, the reproducibility of dECM, obtained by different sources and exploiting different protocols, remains an open challenge.

5. Applications of 3D printed and bioprinted models

The progress of 3D printing technologies and the increased knowledge of ECM features in both pathological and healthy states is leading to bioprinted constructs able to better mimic the morphological, biochemical and functional features of the tissues [124].

Initially, biomaterials-based approaches were limited to maintain the viability of cell cultures for regenerative treatments, in the last decades the objective were extended with the capacity to induce cell differentiation in 3D tissue in vitro models, in other words morphogenesis. New multifunctional biomaterials are employed today for 3D *in vitro* culture applications, as alternative to the traditional Matrigel®. 3D bioprinted models, tailorable upon request, present significantly higher performances if compared with traditional 2D cultures and tissue–like models, that are still in the market. The 3D bioprinted models are certainly much compliant with the *in vivo* situation, but their adherence to a specific healthy and pathological tissue, properly dosing all the

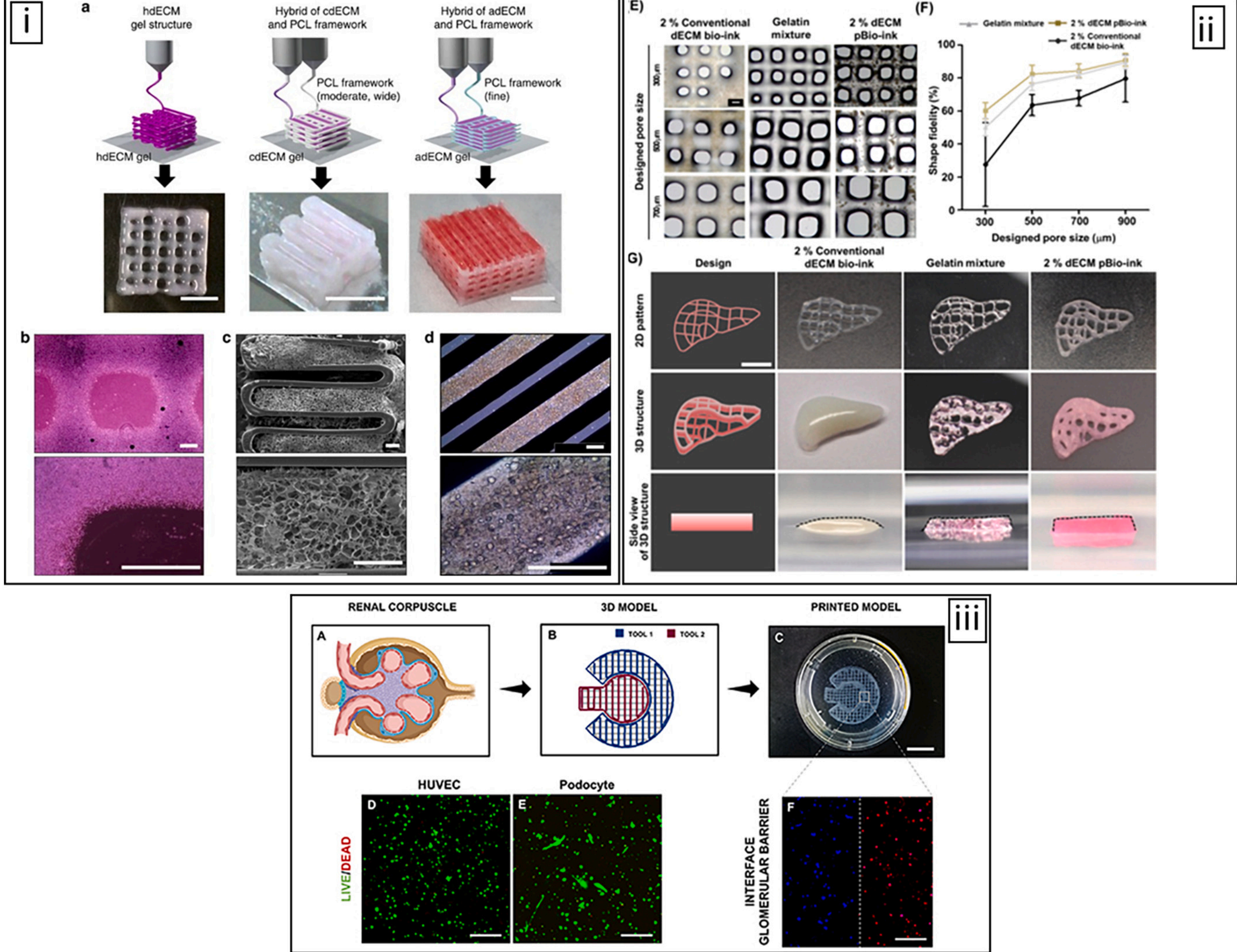


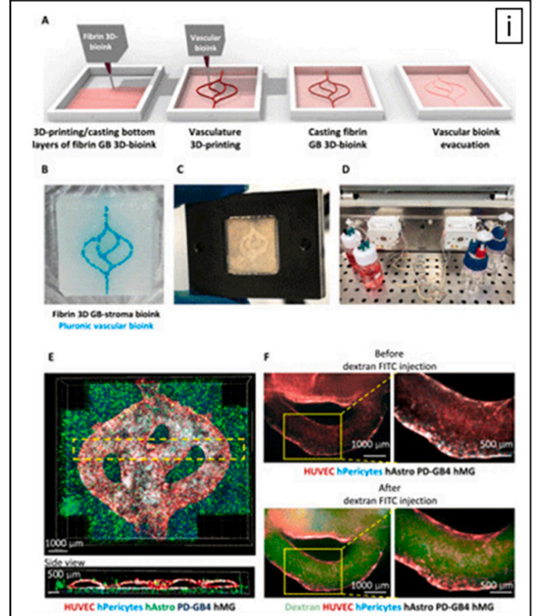
Fig. 5. i) Reproduce with permission from [220] Cell laden construct made of dECM and PCL employed as Bioinks ii) reproduce with permission from [221] Microscope image (E) (scale bar: 200 μm) and measured shape fidelity (F) of printed lattice patterns with varying pore sizes of 2% dECM pBio-ink. (G) Printing results of liver shaped structure with the three bio-inks. iii) Reproduce with permission from [223] Development of a pre-glomerular model. (a) Schematic representation of the renal corpuscle, where mesangial cells are represented in purple, endothelial cells are represented in red and podocytes are represented in blue. (b) representation of the model used as a printing code for the development of the model. (c) macroscopic image of the glomerular model after printing. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structural and biomolecular requirements, remains still challenging.

5.1. In vitro modeling applications

The capacity to reproduce 3D tissue models populated with human cells, controlling their physical and biomolecular properties, and determining the associated cell fate, will strongly contribute to our knowledge of key players in the pathogenesis of several diseases [118]. Furthermore, translational applications in regenerative medicine exploiting human cell cultures or even preformed tissues are evident. Finally, the availability of 3D in vitro engineered tissues models for both healthy and pathological conditions, can significantly contribute to the development of "animal free" protocols for drug discovery and testing. Noteworthy, the capacity to develop 3D tissue models reproducing the feature and containing the cells of a diseased organ of a specific patient, will move towards personalized therapeutic approaches.

In cancer research, the traditional 2D *in vitro* models fail to replicate the major tumor-stroma interactions and the heterogeneity of tumor components, whereas models obtained by 3D bioprinting include more sophisticate constructs better mimicking these interactions and the tumor microenvironment (TME) dynamics. 3D Bioprinted cancer models are revealing optimal solutions for personalized medicine applications using patient derived cells [233]. Mao et al. produced a 3D bioprinted intrahepatic cholangiocarcinoma (IC) model employing a bioink based on patient derived IC cells (ICCs) encapsulated in gelatin-alginate-Matrigel® hydrogel. The obtained 3D bioprinted IC model showed invasive and metastatic properties typical of IC, including stem-like properties of ICCs and cancer associated markers typical of epithelial to mesenchymal transition (EMT) [234]. Neufeld et al. proposed a 3D bioprinted brain tumor resembling different components of the TME and including perfusable blood vessels. With this aim, murine and patient derived glioblastoma cells, astrocyte and microglia embedded in fibrin-gelatin hydrogel were employed as bioink, whereas brain pericytes and endothelial cells were employed to build up blood vessels through the employment of Pluronic F127 as sacrificial ink. The developed model showed the ability to mimic cell invasion, gene profile and therapeutic response, resembling in this way many aspects typical of the in vivo models [235]. Many other examples of 3D bioprinted tumor models are under study, introducing several cellular components and analyzing the effect of different polymers used in the printing process



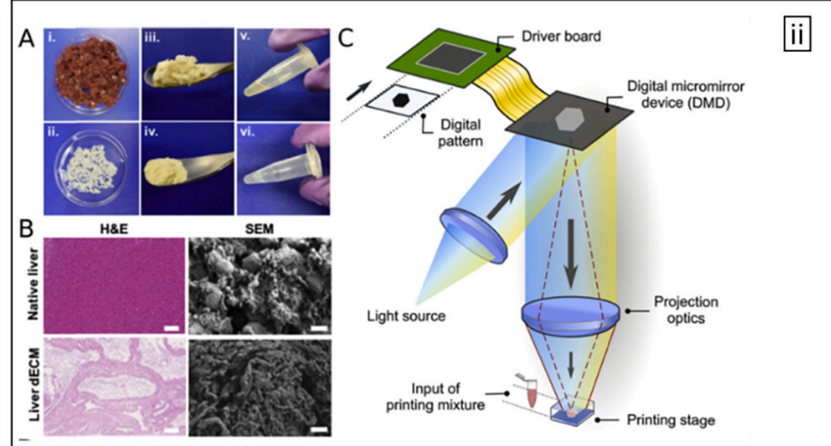


Fig. 6. i) Reproduce with permission from [235]; fibrin brain-mimicking 3D-bioink integrated with 3D engineered printed perfusable vascular network; ii) Reproduce with permission from [239] 3D bioprinting of photocrosslinkable liver dECM-based hydrogel: (A) Decellularization of porcine liver and processing into a printable solution; (B) Representative H&E stains and SEM images of the native liver and liver dECM showing full decellularization via removal of cells (scale bar = 100 μm) and preservation of intact collagen fibrils and ultrastructure (scale bar = 10 μm). (C) Schematic diagram showing the bioprinting of the dECM-based hexagonal scaffolds.

[236], [237], including the use of dECM [238].

The reproduction of healthy tissue models finds useful applications for cell cultures and regenerative medicine. Complex structural and functional tissues, like human intestinal tissues, are under study to produce functional 3D in vitro models. Human primary intestinal cells and myofibroblast thermo-responsive Novogel® Bioink, were employed to generated 3D intestinal tissues with physiological barrier function and the expression of functional transporters useful for toxicity studies and drug screening applications [173]. 3D bioprinted models have been exploited to study infections and tissue inflammations, for examples, 3D bioprinted models of mature biofilm constructs have been developed using alginate and bacteria based bioinks. The constructs were used for antimicrobial testing [240].

5.2. Regenerative medicine applications

The application of 3D bioprinting strategies for regenerative medicine represents an attractive and challenging topic [210,241,242]. 3D

Table 6
Examples of tissue and in vitro models obtain by bioprinting.

Polymers (MW)	Tissue model	Crosslinking	Cell Populations	Refs
gelatin-alginate-Matrigel™	intrahepatic cholangiocarcinoma tumor model	ionic	patient-derived ICC cells	[234]
Fibrinogen and gelatin	metastatic glioblastoma model	Enzymatic, transglutaminase. Vascularization in sacrificial Pluronic	U-87MG, T98G, U373 and HUVEC	[235]
decellularized cephalic parts of pig and collagen	glioblastoma model	Thermal	U-87MG and HUVEC	[238]
rat-tail collagen	human breast cancer	Thermo-responsive	MCF-12A, MCF-7 and MDA-MB-468	[244]
fibrinogen, alginate and gelatin	cervical tumor	physically crosslinked at 25 °C	Hela cells	[245]
Novogel®	Primary Human Intestinal Tissues Model	Thermo-responsive	Human intestinal epithelial cells (hIEC), Caco-2, Adult human intestinal myofibroblast (IMF)	[173]
Gelatin-metylfuran and chitosan methylfuran	glioblastoma model	Diels Alder	U87	[246]
Matrigel™	Lung air blood barrier model	thermal	A549 and EA.hy926	[247]
GelMa	Hepatic model	photopolymerization	HepaRG, LX-2, HUVECs	[248]
Collagen	heart model	thermal in gelatin microsphere bath (FRESH)	hESC-CMs, cardiac fibroblasts	[249]
GelMA & methacrylated hyaluronic acid (HAMA)	human heart valve	photopolymerization	VICs	[250]
alginate, gelatin and pectin	renal in vitro model	ionic	pmTEC, endothelial cells and fibroblasts from K8-YFP transgenic mice	[251]
alginate	Biofilm	Ionic (Ca^{2+})	(*Escherichia coli*, *Staphylococcus aureus* (MSSA), Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*)	[240]
Fibrinogen, collagen	Skin wound repair	Enzymatic (thrombin)	Fibroblasts and keratinocytes	[243]
Gelatin and alginate	gastric wound repair	Ionic (Ca^{2+}) and thermal	GES-1, HGSMCs	[252]

Bioprinting applications in regenerative medicine are mostly focused on osteochondral and cardiovascular diseases, even if also many other tissues are and can be generated (Table 6).

The ability to build up multicellular constructs with tailorable morphology can be advantageous for the generation of functional tissues and organ substitutes, even if tissue scalability and functionality still represent an open challenge. Furthermore, morphogenesis and tissue functionality are linked to different parameters, including structural features, type of cells embedded, and nature of the biomaterials employed in the 3D printing process. New trends in the field include the 3D bioprinting directly performed in vivo, the IntraOperative Bioprinting (IOB) - or *in situ* bioprinting - is showing interesting results for different tissues, including cartilage, skin and bone [211]. IOB provides 3D bioprinting directly in vivo, in the defective site, reducing both the manipulation of tissue graft and the implantation site. The development of IOB applications integrated with automated robotic and AI-assisted systems could effectively increase the translation opportunity of 3D bioprinting in therapies, taking advantage by the personalized medicine approach [212].

Albanna et al. applied 3D bioprinting system for autologous or allogenic dermal graft in murine and porcine models. Here 3D bioprinted dermal fibroblast and dermal keratinocytes embedded in bovine fibrin and type I collagen hydrogels, were bioprinted layer-by-layer in large injured sites leading to fasten regeneration of the skin [243]. Even if the authors observed differences between early formed and mature dermis layers, the proposed approach opens the way to new therapeutic opportunities with high translation potential (Fig. 7).

6. Drug release and drug screening applications

The exploitation of biomaterials for drug release has been widely described in recent reviews, to which we refer the readers [253–255]. Furthermore, the 3D printing technology has been recently exploited to develop drug delivery systems employing polymeric inks of different nature [256–260]. Here we want to highlight that the regenerative medicine applications can be implemented with the possibility to incorporate a drug during the 3D printing process, and the capacity of the obtained construct to release the drug even in a controlled manner. Key factors to control the drug release are the diffusive properties of the drug through the scaffold, which depends on the chemical and physical properties of both, and the biodegradability of the scaffold, eventually upon chemical, enzymatic and physical stimuli. Worthy of note is also the possibility to covalently link the drug to the polymeric components of the scaffold exploiting linkers cleavable upon specific stimuli [261–263].

Recently, there is a growing interest in 3D bioprinted tissue models employable for the development of patient-personalized drug screening and bioassay platforms [264]. In this context, even if 2D in vitro systems are still fundamental in drug screening and drug discovery processes, they lack important features of human tissues - including the ECM components and the 3D structural organization - that play fundamental role in drug permeation, delivery and efficacy [265]. Matsusaki et al. developed a 3D human micro-tissue chips platform using inkjet printing technology. The 3D tissue mimetics were layer-by-layer (LBL) printed using bionks based on fibronectin and gelatin with hepatocytes and endothelial cells, and allowed to obtain 3D liver constructs preliminary employed for drug screening applications [266]. 3D printed kidney

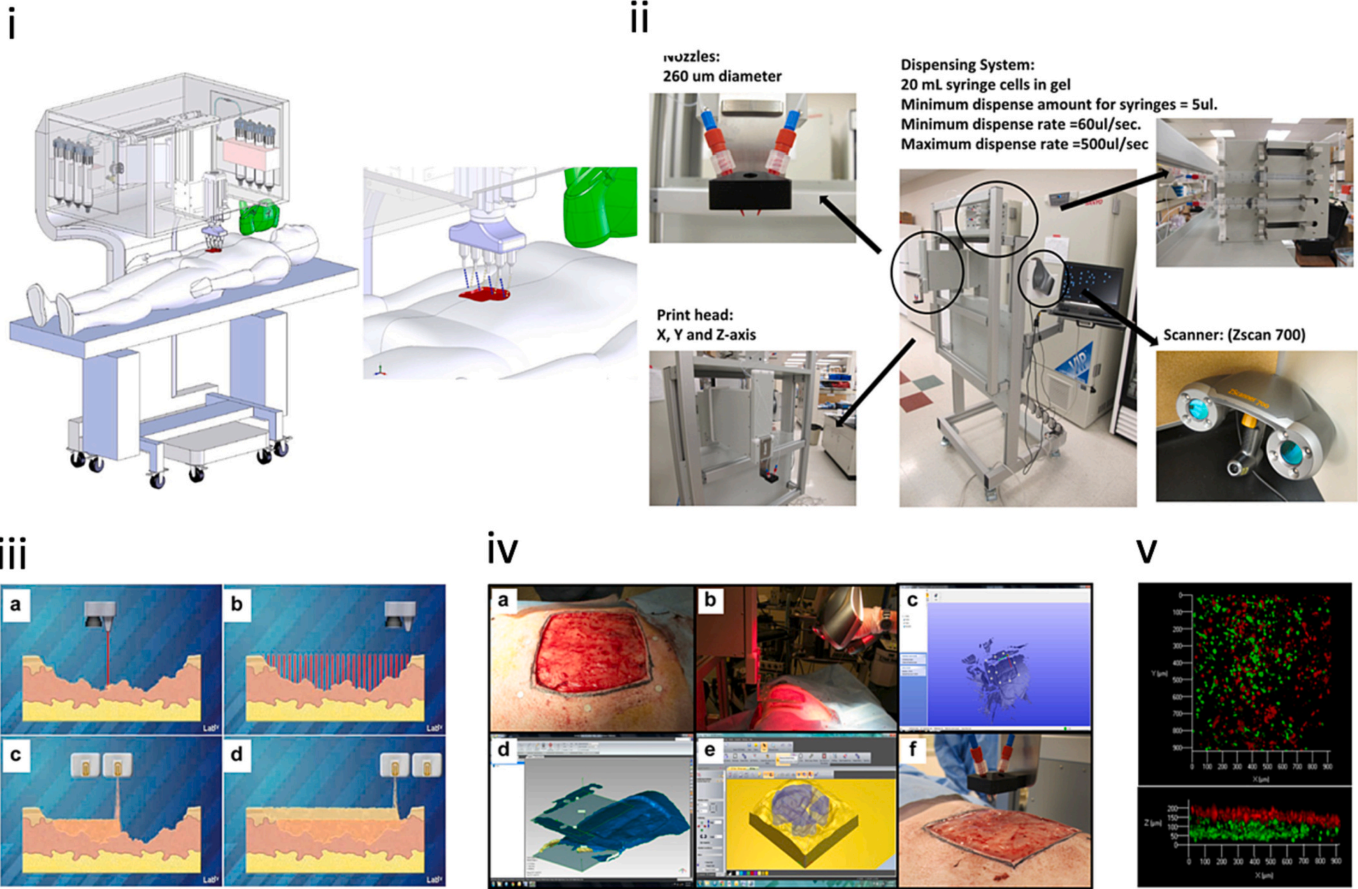


Fig. 7. Skin bioprinter prototype and in situ bioprinting concept: i) schematic demonstrating scale of the skin bioprinter; ii) Main components of the system; iii) skin bioprinting concept; iv and v) example of skin bioprinting process. Reproduce with permission from [243].

proximal tubule with epithelium lumen and surrounding stroma was generated by Homan et al. [267] using a silicone printed chamber with a bioink based on fibrinogen-gelatin with embedded fibroblasts and PF 127, as fugitive ink to form the tubular shape of the construct. The engineered 3D model was characterized and tested with the administration of cyclosporine A, in a dose-dependent manner demonstrating the epithelium disruption. In cancer related research, considering the importance of cell-ECM interaction in the tumorigenic microenvironment, 3D bioprinted models are currently under study. Zhao et al. developed human cervical cancer 3D bioprinted model using fibrinogen-gelatin-alginate bioinks loaded with cancer cells that showed a less sensitive response to paclitaxel, compared to the traditional 2D-in vitro cultures [245]. Dai et al. developed a 3D bioprinted model of glioma using a bioink solution based on fibrinogen, gelatin, and alginate with embedded glioma stem cells. Also in this case, the 3D bioprinted glioma model showed higher resistance to temozolomide compared to the 2D in vitro system [268]. Taiarol et al. employed 3D bioprinted glioblastoma models generated using gelatin-chitosan bioinks loaded with different glioblastoma cells to characterize the efficacy of liposomes loaded with Givinostat, a pan-HDAC inhibitor [269].

7. Open challenges and future perspectives

3D Printing and bioprinting processes are emerging as powerful technologies applicable to different biomedical fields. In the field of regenerative medicine, these technologies allowed the development of more accurate and functional 3D tissue models and even organ mimetics employable in advanced biological assays and in personalized drug testing. It is now clear that the progress in the field require a deeper knowledge on the physical and biomolecular features governing the ECM properties and functions, in different tissues/organs, healthy and pathological states, and even age, diet and environmental conditions. This knowledge must conduct to the capacity to generate by 3D bioprinting personalized human tissue models for the many applications reported in this chapter and future imaginative ones, including the reproduction of functional organs. The challenge consists in the capacity to rationalise the correlation between cell fate and the many variables of the ECM construct, and in the accurate reproduction of the multifarious structural and biomolecular features in 3D models. It is noteworthy that there are two actors in the 3D tissue construction process: the scientists with their capacity to generate appropriate biomaterials and 3D manufacturing process, and the cells that, "properly educated" by the manufactured construct, will proceed in the construction like an expert architect.

Data availability

No data was used for the research described in the article.

Acknowledgements

3D-Gastrointestinal cancer Models in microfluidics devices to disclose the tumour Glycosignature role in disease progression and therapy response - FAQC-22-UNIMIB. Italian Ministry of Health (Grant No. RF-2016- 02362946); POR-FESR 2014-2020, Innovazione e Competitivit`a, and Progetti Strategici di Ricerca, Sviluppo e Innovazione, IMMUN-HUB; EC, H2020-NMBP-15-2017-GA-760986, (iNanoBIT).

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