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TOPICAL REVIEW

Co-axial wet-spinning in 3D bioprinting: state of the art and future perspective of microfluidic integration

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Abstract

Nowadays, 3D bioprinting technologies are rapidly emerging in the field of tissue engineering and regenerative medicine as effective tools enabling the fabrication of advanced tissue constructs that can recapitulate *in vitro* organ/tissue functions. Selecting the best strategy for bioink deposition is often challenging and time consuming process, as bioink properties—in the first instance, rheological and gelation—strongly influence the suitable paradigms for its deposition. In this short review, we critically discuss one of the available approaches used for bioprinting—namely co-axial wet-spinning extrusion. Such a deposition system, in fact, demonstrated to be promising in terms of printing resolution, shape fidelity and versatility when compared to other methods. An overview of the performances of co-axial technology in the deposition of cellularized hydrogel fibres is discussed, highlighting its main features. Furthermore, we show how this approach allows (i) to decouple the printing accuracy from bioink rheological behaviour—thus notably simplifying the development of new bioinks—and (ii) to build heterogeneous multi-materials and/or multicellular constructs that can better mimic the native tissues when combined with microfluidic systems. Finally, the ongoing challenges and the future perspectives for the ultimate fabrication of functional constructs for advanced research studies are highlighted.

1. Introduction

Additive manufacturing (AM) technologies, commonly addressed as 3D printing, are revolutionizing all manufacturing fields, including biofabrication, where it can be used to fabricate 3D artificial tissues useful for regenerative medicine applications or *in vitro* tissue modelling [1–3]. This revolution is driven by the numerous advantages offered by these technologies. Among them, the most remarkable ones are the one-step fabrication of highly complex geometries that are not achievable with conventional (i.e. subtractive) computer-numerical control machines and the fast and cost-effective production of customised parts in small-scale [4, 5]. AM technologies enable to fabricate 3D objects by depositing a selected material in a *layer-by-layer* fashion [6]. In order to achieve high resolution and high shape fidelity within a 3D printed object, the building material needs to undergo a fast transition—generally from liquid to solid phase—as soon as it is deposited. The building unit can either be a droplet or a filament; the smallest the characteristic dimensions of this unit, the higher the resolution of the process. Differently work 3D-stereolitographic processes, that locally induce the solidification of photosensitive resins using laser pens, digital light projection or two-photon focal points of a light source [7].

In biofabrication, 3D bioprinting generally rely on the deposition of a building material, called *bioink*. A bioink is a physiological buffer (e.g. PBS, HEPES, etc.) containing living cells and one or more biopolymers that act as 3D extracellular matrix equivalent. During the bioprinting step, the supporting macromolecules

must either undergo a rapid sol-gel transition or a sharp transition from the fluid to the solid-like gel state *in situ* to allow an efficient 3D deposition, while maintaining mild and cell-friendly conditions during the whole process [8]. Compared to classic 3D cell culture strategies, based on scaffold seeding, bioprinting strategies offer the advantage to fabricate in one-step a cellularized 3D hydrogel construct of arbitrary geometry and controlled spatial organisation of cells, ready to be cultured *in vitro* or grafted *in vivo* [9].

Similarly to conventional 3D printing, the bioink can be deposited in the form of droplets or filaments. Droplets can be deposited using either inkjet [10, 11] or laser-induced forward-transfer (LIFT) technologies [12] while extrusion-based techniques deposit the bioink in the form of filaments, in a process called 3D fibre deposition [12].

Inkjet printing approaches have been one of the first strategies to be employed for building 3D cellularized construct [13, 14]. Such approaches—derived directly from inkjet desktop printers—use either piezoelectric microactuators or thermal microheaters to eject out micrometric-sized orifices droplets of material with diameters ranging between $5 \div 50\ \mu$m. Despite its simplicity, this technique must deal with the viscosity of the bioink, that should not exceed ~30 mPa s to allow the production of monodisperse droplets and avoid clogging issues [15]. This significantly restricts the range of processable materials and achievable cell densities, and represents an intrinsic limit of the technique. LIFT technology avoids the presence—and thus the possible clogging—of orifices by using planar slides loaded with the bioink [16, 17]; laser impulses induce the local propulsion of micrometric droplets out of the donor slide onto a receiving substrate. A time consuming process for slide fabrication and a more complex and expensive set-up of the machine, counterbalance the gained freedom in the composition of the bioink.

A good compromise is represented by extrusion-based techniques, which extrude the bioink out of needle-shaped printing heads using pneumatic or piston-driven actuators. So far, this is the most popular bioprinting approach, as it enjoys a simple set-up comparable with classic thermoplastic 3D printers and the constraints relative to the rheological properties of the bioink are less stringent than those related with inkjet techniques. Many different approaches have been developed for bioink 3D extrusion, based on the shear-thinning behaviour of the deposited material/receiving medium or on the direct writing of liquid bioinks into coagulation baths [18–23].

In this review, we will focus on a specific extrusion-based bioprinting technique based on the wet-spinning of bioinks through co-axial extruders. First, we will give a brief overview of the different paradigms associated with 3D fibre deposition (i.e. extrusion-based techniques) in bioprinting; then, we will discuss the reasons that justify the interest for co-axial extrusion, along with the limitations associated with this approach. We will also describe the future perspective that arise from the integration of this class of extruders with microfluidic platforms for the simple production of heterogeneous living constructs and its potentialities as tools for the creation of vascularises engineered tissues.

2. Classic paradigms of extrusion-based bioprinting

Extrusion-based bioprinting produces 3D cellular constructs by depositing cellular aggregates, dissociated cells and/or supporting ECM in the form of filaments. The great popularity of this approach is easily justified by its technical similarity with the most popular 3D printing technique, fused deposition modelling. This simplifies its adoption in laboratories not necessarily specialised in additive-manufacturing, significantly fastening the advancements in the field of biofabrication [24, 25]. A thorough description of the pro and cons of the different extrusion-based bioprinting approaches is out of the scope of this work; for a deeper analysis of these strategies, we address the reader to recent dedicated reviews covering the subject [26, 27]. In the following, we will only give a concise description of the main trends in extrusion-based bioprinting, recapitulated in table 1, in order to compare them with co-axial extrusion later.

The most common technique embraced by researchers for extrusion-based 3D bioprinting processes is direct ink writing, which consists of tuning the rheological properties of bioinks to impart pronounced shear-thinning behaviours. In this way, bioinks can easily flow (liquid-like behaviour) under a pressure field and rapidly turn into a self-standing gel (solid-like behaviour) after extrusion when the force is removed (figure 1(a)). This strategy has been successfully tested with various bioink compositions and the printing accuracy and resolution, in few studies, have reached notable levels [31, 42]. However, tailoring the rheological behaviour of a polymeric solution is a time consuming procedure that requires several rheological measurements and printing optimisation experiments that have to be repeated each time the bioink composition is changed. Besides, meeting proper rheological properties for the deposition of a bioink does not necessarily imply meeting the cellular needs. In fact, a bioink that can be deposited with high accuracy and shape fidelity may not represent a proper matrix for cell spreading and differentiation, neo-ECM deposition, tissue maturation etc thus severely limiting its application in tissue engineering.

A different strategy for the 3D deposition of cellularized hydrogels consists in extruding a liquid bioink in a bath that can act as a coagulation bath for the rapid gelation of the bioink. The coagulation bath generally consists of a liquid that triggers a physical—e.g. ionotropic or thermoresponsive—gelation of the bioink

Table 1. Most common extrusion-based strategies for 3D bioprinting.

Technique	Mechanism of deposition	Suitable bioinks	Pros	Cons	References
Direct ink writing (DIW)	Pronounced shear-thinning behaviour of the bioink	• Highly concentrated solutions/pastes • Additional plasticizing components • Partial physical gels	• Simple process • Highly repeatable	• Dense gels and/or additional components may hamper cell spreading and migration • High dispensing pressures may induce cell damage and/or death • Needs time-expensive optimisation process for different bioink compositions	[28–31]
Coagulation baths	The bioinks is extruded inside a liquid bath, that physically/chemically triggers its gelification	Liquid bioinks with fast sol-gel transition	• Relative freedom in bioink composition	• Local turbulence of the bath can decrease printing accuracy • Small features are sensible to buoyancy • Often need templating agents (like alginate)	[32, 33]
Freeform reversible embedding	The bioink is extruded inside a pseudoplastic or granular bath, that holds it in place	• Liquid bioinks of arbitrary composition • Suspension of cells	• Great freedom in bioink composition	• Surface roughness • Possible complications for bath removal	[34–36]
Co-axial wet-spinning	The bioink and the crosslinking solution are dispensed using co-axial needles assemblies	Liquid bioinks with fast sol-gel transition	• Freedom in bioink composition	• Often need templating agents such as alginate	[37–41]

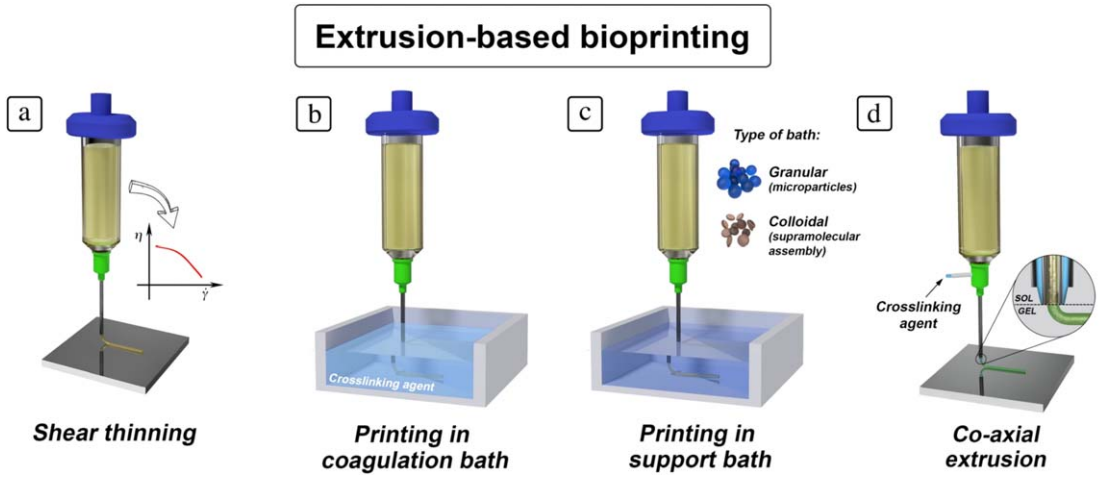

Figure 1. Different extrusion-based approaches for bioprinting cellularized constructs.

[32, 33]. This extrusion approach has been mostly employed in combination with alginate based bioinks: in such cases, the bioinks are deposited within a solution of calcium chloride (or vice versa) to prompt immediate ionic crosslinking of alginate-containing bioinks upon extrusion (figure 1(b)). A great advantage of this approach is the decoupling of bioink rheological properties from its printability. This, in turn, allows to expand the freedom in designing biomimetic inks and architectures. Despite this great advantage, this strategy suffers of few main drawbacks. In the first instance, issues related with clogging the needle are rather frequent due to the rapid diffusion of the bath solution inside the needle. Secondly, problems related with adhesion of consecutive layers, buoyancy of the deposited fibres and turbulence generated during printing are all concerns that generally limit the overall repeatability and precision of the printed construct.

To overcome these issues, a new deposition approach has been recently proposed, often addressed as freeform reversible embedding [34–36]. This consists in replacing the coagulation bath with a granular or colloidal bath which contains soft micro/nano-particles at very high volume fraction. In this case, the collective behaviour of the particles is exploited: when the printing material is extruded inside the granular medium, it locally fluidises around the deposition tip, allowing the embedding of ejected bioink, and then fast recovers a solid-like behaviour, holding the received material in place and giving structural support to the design of bioink 3D structures. This approach has been used to deposit living cells with [20, 34] or without supporting materials [36], and it holds great promises in biofabrication as it permits an 'omnidirectional' deposition of bioinks with arbitrary composition. The main limitation associated with this method regards the uncontrolled surface characteristics of the deposited materials, as the discrete nature of the bath imprints a rough surface to the deposited bioink, limiting the minimum achievable resolution to hundreds of micrometres [35]. Another concern regards with the elimination of the supporting material after the printing process, which can dictate some geometrical constrains (like full-open-pore geometry) to allow for its complete removal.

2.1. Co-axial wet-spinning: an emerging trend

Recently, a bioprinting paradigm that exploits the simultaneous and distinct delivery of the printing and crosslinking solutions in a core–shell fashion has been developed [37]. This system can be considered an unconventional wet-spinning process, in which the bioink and the coagulation bath are delivered simultaneously to the extruder, triggering the gelification of the bioink at the ending tip of the dispensing head. As the dispensing head moves, following the printing code, a hydrogel fibre is spun out and deposited in 3D. By adjusting the flows of the two solutions and speed of deposition, fibres of tuneable dimension can be deposited. The basic configuration of a co-axial extrusion system is depicted in figure 2. The two solutions of bioink and crosslinker are individually pumped through distinct needles assembled in a co-axial arrangement, permitting the deposition of either a bulk (bioink in the inner needle, figure 2(a)) or hollow fibre (bioink in the external needle, figure 2(b)). Co-axial extrusion/wet-pinning can be considered as an evolution of coagulation baths, in which the bioink and the crosslinking solution are co-ejected from a single depositing head in laminar flow conditions, ensuring a high repeatability of the deposition process.

The successful creation of living tissues with 3D bioprinting can be pursued through two different routes. The first one, based on a bottom-up approach, is based on the spontaneous self-organisation of cells, and relies on formulating a bioink that besides being biocompatible is also bioactive. In this case, the bioink is designed to be conductive of different types of

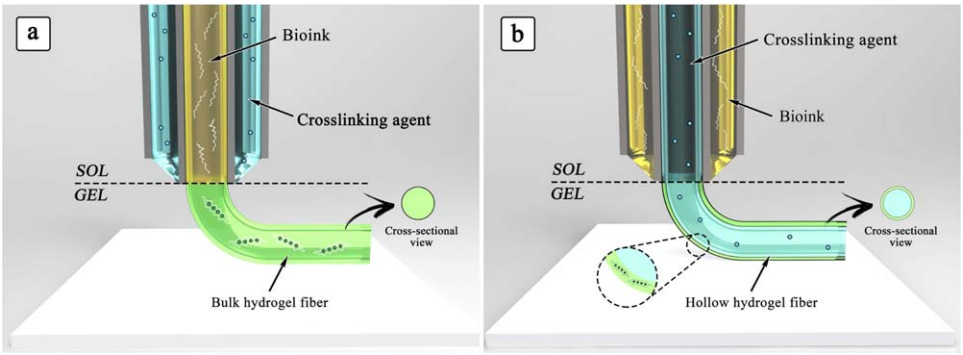

Figure 2. Schematics representation of a co-axial extruder: by inverting the deliver site of the bioink and crosslinking agent solution, either (a) bulk or (b) hollow hydrogel fibres can be laid down.

stimuli (e.g. chemical, electrical, mechanical), capable for example of guiding stem cell differentiation towards a desired cell phenotype, favouring cell spreading, motility and proliferation within the 3D hydrogel thus promoting cellular intrinsic capacity of organising into functional tissue-like structures. The second route, based on a top-down approach, deals with mimicking *a priori* the complexity of human tissue histo-architecture and relies on the constant evolution of bioprinting techniques to design living constructs of complex structures with high accuracy and resolution. However, it must be borne in mind that self-organised or predetermined cellular architectures are not separated options in tissue engineering, and the target of recapitulating the complexity of human tissues and organs will most probably involve both strategies.

In the next sections, we will highlight how the adoption of co-axial extrusion in 3D bioprinting presents important technological benefits with respect to these two approaches: (i) the achieved decoupling of printing accuracy from bioink rheological behaviour allow for the ease tuning of its composition (in terms of macromolecular composition or cellular density) to design specific and instructive cellular environments; (ii) the possibility to create heterogeneous structures that mimic defined histo-architectures can be obtained using a single extrusion head with potential integration with microfluidic platforms; (iii) the intrinsic capability of this system in creating hollow fibres can be used to overcome the great limitations associated with the vascularisation of thick tissue constructs.

2.2. Bioinks used in co-axial wet-spinning

In co-axial wet-spinning there is the necessity of achieving a fast and cell compatible gelation of the bioink during the deposition; nowadays, such a fast and mild gelation mechanisms has been demonstrated using mainly alginate-based bioinks crosslinked with calcium-chloride solutions [38–40, 43]. However, it must be considered that, beside its technological advantages and biocompatibility, alginate lacks binding sites for supporting cells adhesion and spreading which results in suboptimal functionality of the bioprinted constructs. At the scope of creating bioactive inks suitable for co-axial wet-spinning, alginate has been blended with more bioactive biopolymers. For instance, Colosi *et al* embedded human umbilical vein endothelial cells (HUVEC) in a blend of alginate and gelatin methacrylate (GelMA) to exploit the presence in gelatin of integrin binding sites that favour cell adhesion, spreading and motility (figures 3(a), (b)) [38]. Bio-constructs printed using alginate ionic gelation were then exposed to UV light to promote chemical crosslinking among methacrylic substituents of GelMA making the structure of the scaffold permanent. It was observed that the spreading behaviour of HUVEC was determined by the degree of crosslinking of gelatin-methacrylate network which could be tuned by the time of exposure to UV light, independently from the printing step. When the network density was correctly tuned, HUVEC migrated towards the periphery of the deposited fibres and organised into vascular-like structures. Cells at the borders developed tight junctions (figure 3(b)), forming a tubular-shaped endothelium templated on the hydrogel cylindrical fibres. At the junction areas among fibres belonging to adjacent layers, lumen-like structures emerged, revealing the capacity of endothelial cells to spontaneously organise in networks (figure 3(a)).

The same kind of bioink was used by Zhang *et al* in the engineering of endothelialized-myocardial tissues. In this study the bioink containing endothelial cells was printed according to an anisotropic fibres arrangement (high aspect ratio) in order to induce post-seeded primary cardiomyocytes to align and undergo spontaneous and synchronous contraction [43]. These bioprinted constructs were embedded in a microfluidic perfusion bioreactor, and the obtained endothelialized-myocardium-on-a-chip model might represent a useful platform for cardiovascular drug screening.

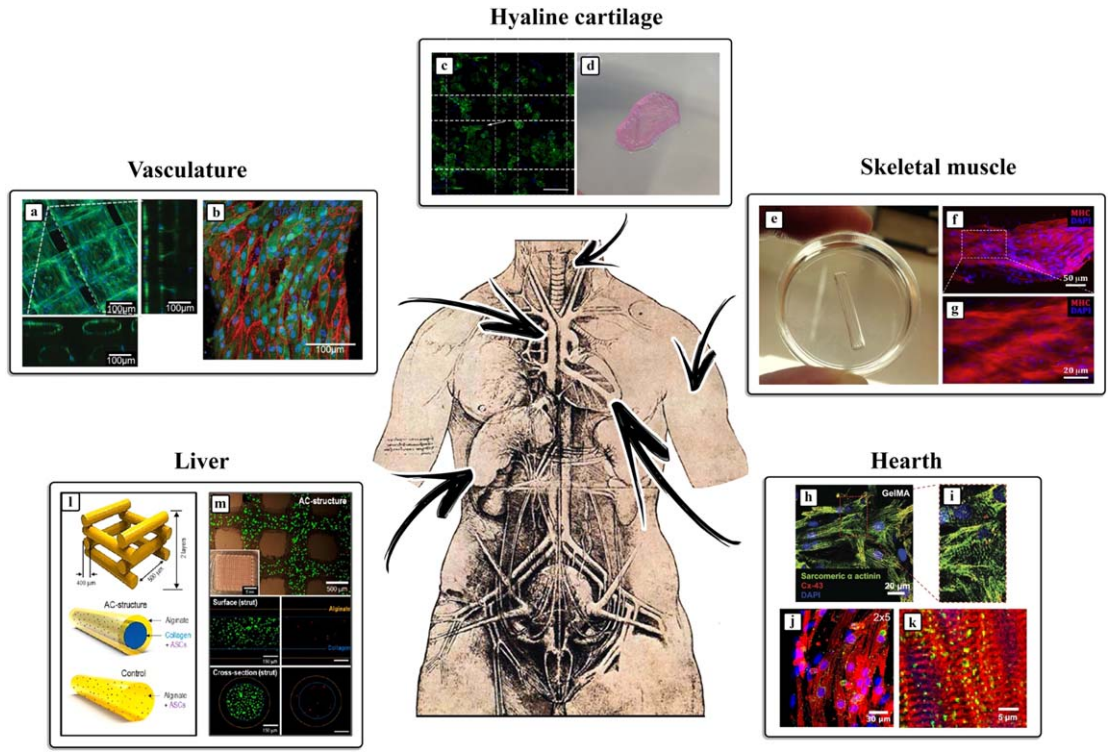

Figure 3. Various tissues engineered through co-axial extrusion approach: (a), (b) vasculature ([38] John Wiley & Sons. © 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim), (c), (d) hyaline cartilage (Reproduced from [39]. © IOP Publishing Ltd. All rights reserved.), (e)–(g) skeletal muscle tissue (Reprinted from [40], Copyright 2017, with permission from Elsevier. CC BY-NC-ND 4.0), (h)–(k) cardiac tissue ([43] John Wiley & Sons. © 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim), and (l), (m) liver tissue (Reprinted with permission from [45]. Copyright © 2016 American Chemical Society).

An example of how the formulation of the bioink permits to direct the differentiation of stem cells towards the desired phenotype is reported in the work of Costantini *et al* [39]. In this work, the Authors targeted articular cartilage as the tissue to be engineered. The formulation of the bioink was driven by the attempt to mimic the natural ECM of cartilage, a tissue rich of proteoglycans and collagen type II (figures 3(c), (d)). A series of photocurable bioinks of increasing complexity constituted by gelatin methacrylate (GelMA), gelatin methacrylate and chondroitin sulfate amino ethyl methacrylate (GelMA + CS-AEMA), and gelatin methacrylate, chondroitin sulfate amino ethyl methacrylate and hyaluronic acid methacrylate (GelMA + CS-AEMA + HAMA) were blended with alginate and human bone marrow-derived human mesenchymal stem cells (hBM-MSCs) to disclose the influence that each component exerted on the differentiation of hBM-MSCs. Interestingly, the Authors demonstrated that the aforementioned bioinks can be used not only to print complex geometrical structures but also to 3D bioprint with high accuracy anatomical parts such as neonatal-size ear (figure 3(d)). Among the formulated bioinks, the one composed of alginate, GelMA and CS-AEMA turned out to be the best candidate in neocartilage formation with the highest production of collagen type II/collagen type I and collagen type II/collagen type X ratios.

A great advantage of bioprinting using the co-axial extrusion method is that once a certain hydrogel has been proven particularly effective in inducing the differentiation of stem cells and their organisation into relevant tissue-like structures, the precursor components of the hydrogel can be readily bioprinted with alginate and cells according to a pattern mimicking the histo-architecture of the target tissue. This approach has been pursued with skeletal muscle tissue. In a previous report [46], Fuoco *et al* demonstrated that vessel-associated muscle progenitors, termed mesoangioblasts (Mabs), underwent myogenic differentiation in a hydrogel derived from photocurable PEG-Fibrinogen. Hence, C2C12 cells—a gold standard cell line for skeletal muscle tissue engineering—were suspended in a blend of alginate and PEG-Fibrinogen methacrylate and bioprinted in a compact array of highly aligned fibres (figure 3(e)) [40]. After 21 d of culture *in vitro*, C2C12 properly spread and fused forming highly aligned long-range multinucleated myotubes, with abundant and functional expression of myosin heavy chain and laminin (figures 3(f), (g)). The obtained myotubes showed high degree of alignment along the direction of hydrogel fibre deposition, further revealing maturation, sarcomerogenesis, and functionality.

Co-axial wet-spinning has been also employed in cardiac tissue engineering. A major problem commonly encountered in this field is the poor electrical

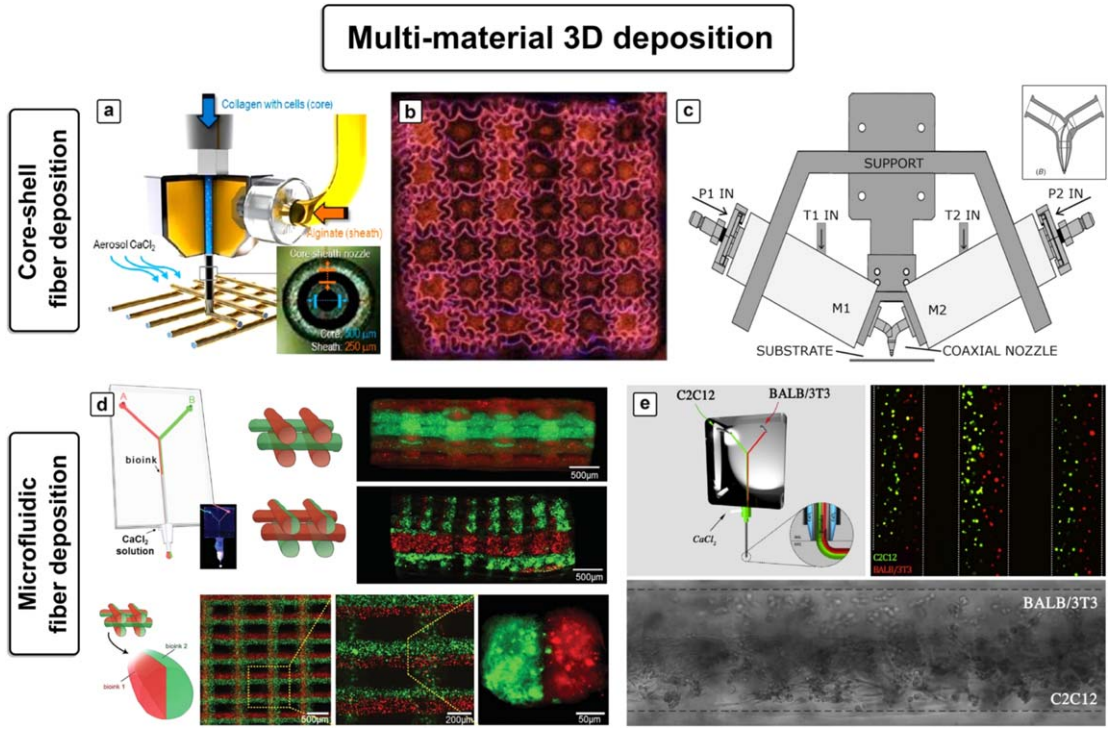

Figure 4. Multi-material 3D deposition of complex structures with co-axial nozzle extruders. In (a)–(c) the deposited fibres are characterized by a core–shell structure composed of two bioinks (a) reprinted with permission from [45]. Copyright © 2016 American Chemical Society, (b) reproduced from [41]. © IOP Publishing Ltd. All rights reserved and (c) reproduced from [48]. © IOP Publishing Ltd. All rights reserved. while in (d), (e) more complex constructs composed of alternate layers of different bioinks (and/or cells) and layers of Janus fibres are deposited through a microfluidic device (d) and (e) reprinted with permission, respectively, from [38] John Wiley & Sons. © 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim and [40], Copyright 2017, with permission from Elsevier. CC BY-NC-ND 4.0.

conductivity of the biomaterial that delay efficient electrical coupling between adjacent cardiac cells. In a recent article, Zhu *et al* formulated a bioink that beside alginate, comprised gelatin methacrylate and gold nanorods (GNR) [44]. The concentration of nanorods was optimized in order to guarantee low viscosity of the bioink, thus permitting the loading of high cell concentration and at the same time minimising the effect of shear stress field on cell viability. It was shown that cardiac cells in the printed GNR constructs performed better in term of adhesion and organisation when compared to the constructs without GNRs. Furthermore, the incorporated GNRs improved the electrical propagation between cardiac cells and promoted their functional improvement in the printed cardiac construct (figures 3(h), (i)).

2.3. Building multi-material or multicellular constructs with co-axial microfluidic extruders

Bioprinted 3D cellular constructs represent an important result in the realisation of functional tissues *in vitro*. The control over cellular disposition and the presence of hydrogel ECM equivalents that provide a 3D environment for cell growth and organisation can lead to the replication of essential structural and functional features of living tissues. However, a further improvement is represented by the simultaneous deposition of different types of ECM and cell populations so as to produce heterogeneous bio-constructs on demand, replicating the architecture of complex organs.

ECM components, collagen in the first instance, represent the ideal bioink for embedding cells, thanks to its bioactive property that favours cellular processes. Unfortunately, printability of collagen is challenging because of its low solubility, inadequate viscoelastic properties and mechanical weakness of the ensuing scaffolds. To circumvent such a problem, Yeo *et al*—by adapting an approach developed by Onoe *et al* [47]—3D printed core-sheath microfibers consisting of a collagen core embedding cells and an alginate shell crosslinked with calcium [45]. The use of alginate guarantees printability with good fidelity and protect cells from shear stress during deposition. As a result, when viability, spreading and hepatogenic differentiation of human adipose stem cells was assessed against a control scaffold constituted by alginate alone, the composite fibre-based scaffolds outperformed the alginate one (figure 4(a)).

Following the same approach, Liu *et al* confined a core of GelMA inside a shell of alginate serving as a fibre template [41]. In this way the polymeric network density of the cell embedding phase could be tuned by regulating GelMA concentration. It was observed that among the cell lines cultured in the laid scaffold, each

one has its optimal network density. The core–shell approach permitted the fabrication of cell-laden GelMA constructs at extremely low concentrations (<2.0%), a result not achievable using conventional bioprinting strategies (figure 4(b)). The major drawback of such core–shell approach is that alginate fibres are hold together only by ionic crosslinked junctions that have scarce endurance in cell culture medium greatly limiting cell culture times.

Scaffolds among other requirements, must also be capable of withstanding the mechanical environment of the native tissue to be replaced. This is particularly true for load bearing tissues such as bone and articular cartilage. To satisfy such a criterion, one strategy consists in the development of specialised scaffolds formed by combining two or more dissimilar materials. The result is a composite structure that possesses unique properties that cannot be replicated by a single material. Also in this respect, co-axial printing in combination with alginate, offers the opportunity to create composite structures in which the load bearing and the hydrophilic and bioactive phases are spatially segregated in a core–shell fashion.

Following this approach, Cornock *et al* manufactured a steel co-axial extruder using selective laser melting and refined it by electropolishing [48]. This extruder was used for the simultaneous deposition of a composite fibre composed of an alginate core and a poly(caprolactone) (PCL) shell (figure 4(c)). Process parameters were optimised to minimise the offset of the core fibre from the centre of the co-axial fibre. At the end of the scaffold deposition process, the alginate core was removed leaving a hollow fibre of PCL. Such scaffolds displayed excellent mechanical properties attributable almost exclusively to the PCL shell. Fibroblasts were either post injected inside the fibres of the scaffold or encapsulated in the alginate matrix during deposition. While in the first approach, cell viability was preserved at high level, in the second one, the heat stress caused the death of all cells. This co-extrusion process of both a synthetic, biocompatible and biodegradable polymer and a hydrogel while interesting does not hold great promises in the field of bioprinting. Even though the problem associated to heat stress could be overcome, the hydrogel core supporting cells is surrounded by impermeable hydrophobic shell that hampers the diffusion of nutrients and oxygen which can proceed only longitudinally to the fibres, starting from the ends. A similar solution completely based on the use of hydrogel materials was presented in the work of Mistry *et al* in which two different bioinks were delivered through the outer and inner needles of a co-axial extruder [49]. In particular, the core bioink containing collagen or Matrigel was loaded with HUVECs, while the shell fluid was composed by a mixture of alginate and poly(ethylene glycol) diacrylate (PEGDA) embedding hepatocytes. The interpenetrating network consisting of a dual crosslinking networks, namely covalently bonded PEGDA chains and a Ca^{2+} mediated alginate network, guaranteed good mechanical properties in particular with reference to gel shape and dimension recovery after deformation.

Co-axially bioprinted structures represent a useful platform on which investigate cellular mechanisms involved in tumours. Cellular spreading and cell–cell interactions are influenced by the surrounding matrix which should be at the same time supportive for cells and not hinder cell migration. Such a construct was developed by Dai *et al* by co-extruding an alginate solution sheet and a core of brain tumour cells and stroma MSCs suspended in a fibrinogen solution [50]. Fibres were deposited in a coagulating bath of $CaCl_2$ forming a 3D scaffold. The alginate sheet provided confinement and support to the cells that interacted with each other and self-assembled into multicellular heterogeneous brain tumour fibres. The alginate sheet was subsequently removed by complexing calcium ions with ETDA. The so obtained self-assembled heterogeneous tumour tissue-like fibres provided valuable 3D models for studying tumour microenvironment *in vitro*.

Human tissues are assemblies of multiple cell types arranged into definite histo-architecture. This implies that the cell-laden printing strategy has to provide the possibility of simultaneous bioprinting of different bioinks endowed with tailored mechanical, chemical properties and embedding different cell types, disposed in specific positions in the 3D space. The standard approach in extrusion-based bioprinters involve the use of multiple printing heads for the fabrication of complex and heterogeneous biological structures. However, an increased number of printing heads requires a more complex printing system and the deposition of the construct is rather slow. Co-axial needles printing heads in conjunction with alginate fibre templating offers new and straightforward opportunities in this respect. Two recent articles showed that, by exploiting microfluidic platforms, the bioinks delivered to the co-axial needle system can be changed on demand using a single printing head [38, 40]. The key of the system was based on a Y configured microfluidic chip mounted upstream the co-axial dispenser (figures 4(d), (e)). The inlets of the top channels are supplied with different bioinks that can be delivered to the co-axial printing head for instance in an alternate or simultaneous fashion, thus realising heterogeneous structures made up by differently composed layers or composite fibres (figure 4(d)). This strategy was implemented in the co-culturing of myoblasts with fibroblasts segregated in the two longitudinal half of the extruded fibres of alginate and PEG-MA-fibrinogen. It has been demonstrated that fibroblasts support myoblasts differentiation by secreting extracellular matrix components and growth factors (figure 4(e)).

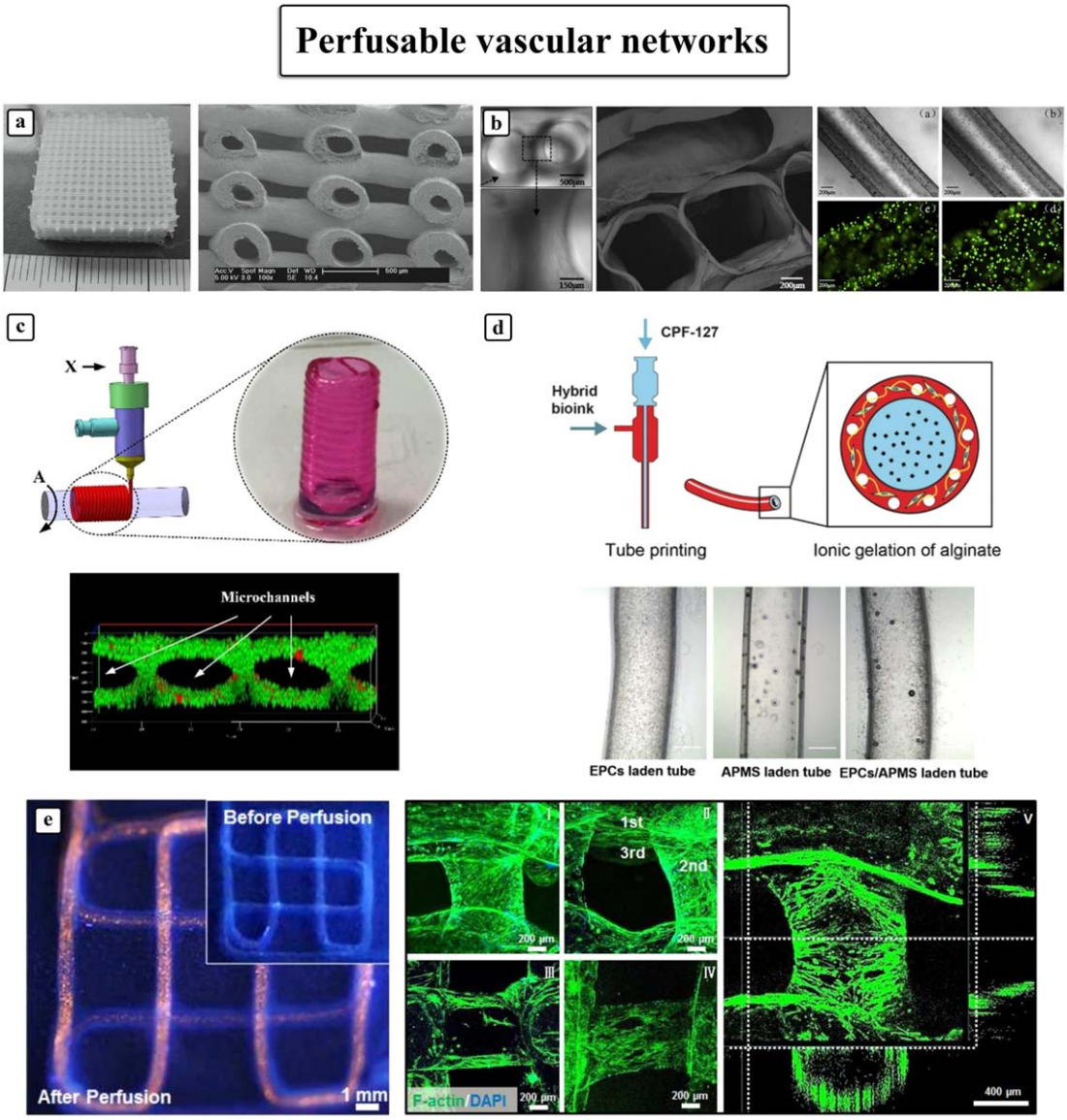

Figure 5. 3D deposition of hollow fibres trough co-axial extrusion systems for the assembly of perfusable vascular network. (a)–(e) reprinted with permission, respectively, from [28] John Wiley & Sons. Copyright © 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, [52], Copyright 2015, with permission from Elsevier. © 2015 Elsevier Ltd. All rights reserved, [53]. Copyright © 2017 American Chemical Society, [54]. CC BY 4.0, [55], Copyright 2016, with permission from Elsevier. © 2016 Elsevier Ltd. All rights reserved.

2.4. Co-axial extruders for the creation of perfusable vascular networks

The creation of a vascular network within engineered tissue constructs to promote the transport of oxygen, nutrients, and waste products, is a hot topic in tissue engineering and great efforts are being spent to achieve this goal. Co-axial extrusion is particularly promising for the creation of vascular-like structures. Given the configuration of the basic extruder composed of two co-axial needles, a quite intuitive development regarded the modification of the extruder for the deposition of hollow fibres (figure 2(b)) [51]. This development opens up the possibility of printing a blood vessel network that can guarantee the uniform supply of nutrients and oxygen to cells present in all compartments of the scaffold.

One of the first attempt to create a perfusable scaffold composed of hollow fibres using a shell/core nozzle tip was proposed by Luo *et al* [28]. These authors extruded a self-standing alginate/PVA paste through the hollow space of the extruding system. After deposition, the scaffold was soaked in a $CaCl_2$ solution. Young's moduli were of the order of few MPa, one or two order of magnitude higher than that characterizing conventional gels. At the same time, fibres walls were permeable to a dye simulating nutrients and oxygen (figure 5(a)). This article paved the way to the manufacturing of hollow fibres based scaffolds even though its applications in bioprinting are ruled out by the extremely high viscosity and network density of the extruded blend.

A completely perfusable scaffold was created by printing hollow alginate fibres using co-axial printing head. The concentrations of alginate and calcium chloride solutions were adjusted to allow the deposited fibres to fuse together [52]. The Z stage of the printer moved down across a $CaCl_2$ solution to keep the printed structure immersed in for complete crosslinking (figure 5(b)). The authors demonstrated that the crosslinked alginate walls were permeable to a model protein and as a consequence the embedded cells could be supplied with nutrient and oxygen delivered by the solution flowing through the hollow fibres network.

A design that allows producing and depositing on a collecting plane either solid or hollow fibres was described in the work of Li *et al* [56]. The key element for the obtainment of one or the other kind of fibre is the relative position of the tips of the core and external needle. When solid fibres are desired, the inner needle protrudes 500 μm out the outer one and the solution of alginate and calcium chloride are delivered through the core and external needles, respectively. This means that gelation starts from the exterior of the alginate flow and proceeds by diffusion of calcium ions towards the centre. When hollow fibres are the target of the extrusion process, the converse needle arrangement is exploited. In this case, gelation of the shell alginate solution starts from the interior and proceeds outwards. The locus of the beginning of the physical gelation has important implications as far as adhesion among laid fibres is concerned. When gelation starts from the outer shell of the printed fibres, as in the case of solid gel fibres, the alginate solution is completely gelled and fibres belonging to adjacent layers, glue to each other only weakly at the points of mutual contact. As a consequence, scaffolds suffer of poor stability especially in the presence of interfering ions (e.g. in cell culture medium). On the contrary, if gelation started from the internal layers of the printed hollow fibres, the surface layers being still in a sol-gel transition state could effectively glue one to each other at the points of mutual contact. This concept was exploited by Gao *et al* in the fabrication of hollow vessels-like structures [53]. By using in tandem two spinnerets, hollow fibres embedding either smooth muscle cells or fibroblast were collected around a rotating mandrel (figure 5(c)). Since the outer shell of the alginate hollow fibres was still gelling after deposition, contacting fibres fused to each other creating an unicum construct. This approach permitted to arrange according to a multilevel order, cells making up blood vessels. Thus, smooth muscle cells were deposited within the first fibre layer, on top of which a second layer of fibre supporting fibroblasts was laid down. The inner walls of the vessel-like structure were coated with collagen to favour endothelial cell adhesion. The printed structures with multilevel fluidic channels have structural similarity to blood vessels, have sufficient mechanical strength to support loading, and exhibit biocompatibility. A foreseeable drawback of this approach is related to the creation of groves perpendicular to flow direction that may cause local turbulence on vessels walls. This, *in vivo* applications can bring about to the formation of deposits on walls which on long terms may cause the clogging of the vessel.

The regeneration of heart tissue damaged by ischaemic events is a goal of great impact in medicine due to the high rate of incidence of this pathology worldwide. The treatment of ischaemic tissues involves the replacement of the tract of the blood vessel clogged. The achievement of this goal is not trivial since the hostile ischaemic conditions (e.g., low nutrition, oxidative stress, inflammation, reactive oxygen species) limit survival and differentiation of transplanted endothelial progenitor cells (EPCs). In a recent publication Gao *et al* used as bioink vascular-tissue derived decellularized extracellular matrix (VdECM) blended with alginate, EPCs and PLGA microparticles loaded with atorvastatin, a molecule that enhances angiogenesis by reducing subdural haematoma, and promotes endothelial cells function [54]. As stressed previously, the co-axial extrusion system is particularly well-suited for the straightway formation of hollow structures. The gel inducing solution is constituted by Pluronic F127 dissolved in a solution of $CaCl_2$ that helps avoiding the deformation of the tubular structure before the thermal induced gelation of VdECM (figure 5(d)). These devices once implanted in a mouse ischaemic model, increased the rate of neovascularization and salvage of ischaemic limbs.

A major drawback of most blood vessel bioprinting strategies, is the weak mechanical strength of the constructs that hampers its surgical anastomosis with the host blood vessel in the implantation process. The use of co-axial-needles extruders offers the opportunity of printing mechanically robust and perfusable constructs. In the work by Jia *et al* [55], tri-axial nozzle was used to create perfusable hollow fibres of various diameters and wall thickness in a single step. In this approach, the bioink was delivered through one or both of the two external co-axial needles while the calcium chloride solution was pumped through the innermost needle (figure 5(e)). In this way either a single or double walled concentric hollow fibre were produced permitting to modulate its mechanical property. The bioink was a blend of gelatin methacrylate, alginate and 4-arm poly(ethylene glycol) acrylate PEGTA that conferred to the bioprinted hollow constructs improved mechanical properties by increasing the crosslinking density. The co-axial needles printing method possesses distinct advantages over conventional complicated microfabrication and sacrificial-templating approaches where a templating bioink is firstly deposited and embedded in a hydrogel matrix, followed by removal of the bioink to obtain the hollow vessel-like structures.

3. Ongoing challenges

The described literature suggests that using co-axial wet-spinning in 3D bioprinting represents a promising and interesting tool in tissue engineering. However, this technique suffers from some limitations connected with the defining characteristics of all extrusion-based printers, and with the almost unavoidable use of calcium-alginate hydrogels.

Indeed, alginate gels often hinder the spreading and migration of embedded cells. This makes the use of chelating agents (like EDTA) or alginate-lyase enzymes necessary, in order to dissolve or digest the alginate network in sacrificial-templating approaches. This may preclude the successful bioprinting of particularly sensitive cell populations, as the post-treatment of bioprinted samples exposes cells to varying chemical and mechanical stimuli that may negatively affect their survival. Another criticality concerns with the use of calcium chloride as crosslinking agent, which somehow limits the possibility to use phosphate buffered solutions. Phosphate salts interacting with calcium ions can yield to the formation of calcium-phosphate precipitate and a subsequent acidification of the bioink, leading to cell death. In this respect, other types of buffering agents, such as HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) or TRIS (tris(hydroxymethyl)aminomethane), have been used in saline solutions or mediums to maintain the pH of calcium-crosslinked bioinks in the 7.2–7.4 range. However, phosphate buffered solutions are the most commonly used in cell culture, and their substitution can be critical when dealing with sensitive cell populations, whose culture is optimised with standard (e.g. phosphate buffered), commercially available mediums. This limitation will be overcome with the emergence in the market of products specifically designed for such bioprinting processes. In addition, the use of abundant divalent calcium ions as crosslinking agent can directly affect in a negative manner cell behaviour/interaction. It is well known, for instance, that calcium ions are important for cellular signalling playing a key role in signal transduction through the activation of ion channels or as a second messenger regulating a multitude of fundamental physiological functions such as muscle contraction, neuronal transmission, cellular adhesion etc. In this regard, a possible solution could be found in the use of different divalent ions—such as Mg^{2+}, Sr^{2+} or Zn^{2+}—as crosslinkers for alginate gelation [57]. Replacing calcium ions with other divalent ions could also allow researchers to tune the stability of the ionotropically crosslinked network as alginate has a specific affinity toward each of them [58].

The described limitations associated with the use of calcium-alginate gels could be bypassed with the development of new types of bioinks suitable for co-axial wet-spinning. Among polysaccharides, a certain number undergo gelation upon cooling or exposure to ions. These properties have already been exploited in shear-thinning-based extrusion bioprinting and can be potentially translated into co-axial bioprinting. As illustrative examples, Wilson *et al*, exploited the ability of kappa-carrageenan (kCA, a biocompatible polysaccharide) to undergo both ionic and thermos-reversible gelation [59]. When kCA is heated and dissolved in water, it presents a random coil structure; upon subsequent cooling double helices junctions are formed due to hydrogen bonding between galactose units of the polymer backbone. This thermoreversible gelation can be further stabilised by ionic crosslinking through positive ions—such as potassium—with kCA's negatively charged sulfate groups. To tune kCA gelation temperature in the range of physiological one and improve the mechanical properties of the printed constructs, kA was blended with two dimensional nanosilicates.

Another bioink component widely exploited is gellan [60]. Gellan is a linear anionic polysaccharide composed of tetrasaccharide repeating units (1,3-β-D-glucose, 1,4-β-D-glucuronic acid, 1,4-β-D-glucose, 1,4-α-L-rhamnose). The carboxyl side group on the glucuronic acid is responsible for the gelation behaviour of the molecule. Upon cooling, the coiled polymer forms double-helices (coil-helix transition) while upon addition of mono-, di- or trivalent cations, gelation a sol-gel transition occurs as the helices aggregates into junction zones which are linked into a three dimensional network via the coiled part of the molecule. Also in this case, the gelation temperature can be handled by blending gellan with other biopolymers [61] or by introducing substituents (e.g. benzyl groups, peptides) onto the polymer backbone [62, 63]. The use of these biopolymers within co-axial bioprinting approach would follow the same scheme employed with calcium-alginate gels: the bioink and the ion solutions will be delivered separately in the characteristic core/sheath fashion with the formation of gel fibre at the tip of the co-axial extruder. The feasibility of such an approach has been demonstrated in a recent article [64].

Chemical derivatization of biopolymers is another feasible approach applicable virtually to all biopolymers. As an illustrative example, Shi *et al* [65], introduced both phosphate calcium binding and methacrylic groups on hyaluronic acid chains. This approach, that in principle can be extended to many bioactive biopolymers, permits to avoid the necessity of blending alginate with other biopolymers and the alginate removal step since ionic and chemical crosslinking occur within the same macromolecule. Along the same line, Highley *et al* [18], coupled separately adamantine and β-cyclodextrin moieties to hyaluronic acid to create two hydrogel-precursors that formed a supramolecular assembly upon mixing. To further enhance the structural integrity of the printed constructs, photocrosslinkable methacrylate groups were introduced onto the macronomers. Also these

approaches and related ones could in principle be translated into co-axial technology.

As mentioned, the co-axial extrusion shares some limitations with all the extrusion based bioprinting approaches. Being a 3D fibre-deposition process, it creates 3D structures by assembling micron-scale fibres. If this can be an advantage for the creation of cylindrically symmetric tissue, like blood vessels, it may represent a limit for the production of those tissues that do not present repeated, microscopic functional units. Indeed, it is possible that the single, discrete cell-laden hydrogel strut will not efficiently fuse with the others and, even when structurally adhered, will represent a distinct cylindrical-shaped 3D microenvironment, surrounded by other identical but virtually independent micro-tissue compartments. This issue can be irrelevant or negligible for the production of cellular *in vitro* testing platforms, where the local replication of tissue functions at the micrometric scale is sufficient to obtain reliable experimental results. On the contrary, it may be a critical drawback for regenerative medicine applications, where the engineered tissues need to integrate functionally and spatially with the surrounding macroscopic, heterogeneous, bulk tissues of the receiving patient.

Finally, it should be kept in mind that cell-laden fibres are appropriate for the growth of soft tissues and organs, given the range of mechanical stiffness attributable to hydrogel materials, but are unlikely applicable for the creation of hard tissues, like bones or ligaments. The overcoming of this constraints may come from the successful integration of different biofabrication approaches, that would allow the deposition of more complex constructs made of soft (e.g. hydrogel) and hard (e.g. thermoplastic) materials.

4. Conclusions and future directions

The ambitious goal of producing functional human tissues *in vitro* pushed forward the research field of tissue engineering in the last decades. The experience gained so far, revealed how a key factor for the successful production of lab-grown tissues and organs will be the convergence of different disciplines, ranging from biology, nanotechnology, material science, bioengineering, physics and many others. This paradigm of multidisciplinarity echoes also in the development of the technological tools associated with tissue engineering. 3D bioprinting is a perfect example of that, where digital fabrication, 3D cell culture, microfluidics, biomaterials science and microfabrication are all basic elements of the technology.

As a recently developed technique, 3D bioprinting is still in an explorative phase of its evolution, where many different approaches are tested, evaluated, refined, integrated or abandoned. In this review, we focused our attention on a particular deposition strategy based on co-axial extrusion systems. It is our opinion that this technique enjoys unique advantages in terms of simplicity, versatility and performances. It is an extrusion-based approach, the class of 3D-printers that is generally considered more easily implementable, faster and cost-effective with respect with more sophisticated techniques. This could determine a diffused use of such a technology, favouring the investigations and fastening the sightings based on 3D-bioprinted tissues. Further, the system allowed for the embedding of cells into many different ECM analogues using a templating approach, enabling the deposition of materials otherwise difficult to be 3D-printed, like permissive low-density gels. This can shorten the time needed for the optimisation of bioprinting protocols addressed to maximise the biological activity of the bioink. Also, the possibility to handle liquid, low viscous bioinks consents the integration of microfluidic platforms in the bioink-handling apparatus and deposition system of wet-spinning-based bioprinters, simplifying the creation of biomimetic, heterogeneous tissue structures. These considerations suggest that, in convergence with other biofabrication technologies, 3D bioprinting based on co-axial-extruder wet-spinning processes can give great contributions in making the creation of functional lab-grown tissues a closer reality.

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