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Biofabrication of engineered blood vessels for biomedical applications

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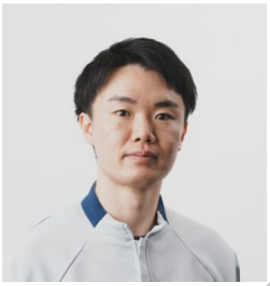
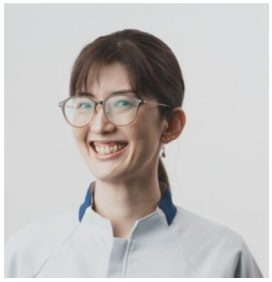


Kentaro Uchida received his master's degree from Graduate School of Engineering, Nagoya University in 2019. After that, he has worked as an analytical scientist in Panasonic Holdings Corporation since April 2019. He has been participating in joint research with Prof. Matsusaki's laboratory since 2020. His research interests focus on the application of 3D tissue model to analysis of beauty appliances.



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Michiya Matsusaki was born in Kagoshima, Japan in 1976. He received his Ph.D. degree in 2003 from Kagoshima University. He started his academic career as a Postdoctoral fellow at Osaka University in 2003. He was a visiting scientist at Lund University in 2004. In 2006, he joined the Department of Applied Chemistry in the Graduate School of Engineering at Osaka University as an Assistant Professor. He was promoted to Associate Professor in 2015 and to full Professor in 2019. He was a JST-PRESTO researcher (Concurrent position) from 2008 to 2011 and from 2015 to 2019. He was awarded 20 awards including the Young Scientist's Prize by the Minister of Education, Culture, Sports, Science, and Technology. His research interest is biomaterials and tissue engineering for regenerative medicine and pharmaceutical applications.

Biofabrication of engineered blood vessels for biomedical applications

To successfully engineer large-sized tissues, establishing vascular structures is essential for providing oxygen, nutrients, growth factors and cells to prevent necrosis at the core of the tissue. The diameter scale of the biofabricated vasculatures should range from 100 to 1,000 μm to support the mm-size tissue while being controllably aligned and spaced within the diffusion limit of oxygen. In this review, insights regarding biofabrication considerations and techniques for engineered blood vessels will be presented. Initially, polymers of natural and synthetic origins can be selected, modified, and combined with each other to support maturation of vascular tissue while also being biocompatible. After they are shaped into scaffold structures by different fabrication techniques, surface properties such as physical topography, stiffness, and surface chemistry play a major role in the endothelialization process after transplantation. Furthermore, biological cues such as growth factors (GFs) and endothelial cells (ECs) can be incorporated into the fabricated structures. As variously reported, fabrication techniques, especially 3D printing by extrusion and 3D printing by photopolymerization, allow the construction of vessels at a high resolution with diameters in the desired range. Strategies to fabricate of stable tubular structures with defined channels will also be discussed. This paper provides an overview of the many advances in blood vessel engineering and combinations of different fabrication techniques up to the present time.

Impact statement: This review covers several aspects and advancements of engineered blood vessel biofabrication, which are essential for establishment of large-sized tissues in different areas of biomedical applications.

Keywords: blood vessel engineering; biofabrication; endothelialization; 3D printing; large-sized tissues

1. Introduction

The circulatory system comprises complicated vasculature networks of differing vessel types with different diameters, including the aorta and vena cava (25-30 mm), arteries and veins (0.6-16 mm), arterioles and venules (20-25 μm), and capillaries (~ 9 μm), totaling more than 100,000 km in length inside the human body [1–3]. Blood vessels play an important role in the delivery of oxygen, nutrients, growth factors (GFs), and cells to functional tissues, as well as removing metabolic waste products, to prevent necrosis and cellular death [4,5]. The structure, function, and cell composition of each type of vasculature varies greatly throughout the size scale, where the vascular wall becomes thinner in smaller vascular constructs [5,6]. The first generations of blood vessel grafts from the 1970s were mostly based on synthetic materials such as poly (ethylene terephthalate) (Dacron) or poly(tetrafluoroethylene) with diameters larger than 6 mm, limiting their use for the treatment of large-diameter vessels such as aortic, iliac, and femoral arteries [6,7]. However, there are requirements for fabrication technologies that allow the construction of blood vessels with smaller diameters to thoroughly supply essential molecules within the volume of tissue at the implanted site. Decreasing blood vessel size also makes it easier to control the vascular alignment within a tissue so that the spacing does not exceed 100-200 μm from each other, due to the diffusion limitation of oxygen [4,8,9]. Development of fabrication techniques for small-diameter vessels could satisfy the demand for large-scale tissue construction in different applications including clinical transplantation, pathological models, drug testing, and *in vitro* cultivated meat.

Although they have already reached the commercialized stage, clinical applications of synthetic grafts can result in several complications including undesirable immune responses, mechanical mismatch to host vasculature, and disruption of blood flow that eventually leads to

thrombosis [6,7]. In later generations of vascular replacement research, biomimetic constructs were developed by incorporating cells and/or pro-angiogenetic GFs into 3D scaffolds, where the interaction of these components was thoroughly investigated [10]. This is when the discipline of tissue engineering, which focuses on the involvement of scaffold, signaling, and cells in tissue regeneration has started to contribute to this area of research [11]. Endothelial cells (ECs) and pericytes can be integrated into the fabricated structure by mixing cells with a pre-solution of scaffold materials and/or seeding cells into the fabricated tubular scaffolds [8]. To date, several technologies have been developed to fabricate highly organized, precisely controlled microenvironments for ECs to attach, proliferate, secrete native vascular extracellular matrix (ECM), and eventually develop into engineered blood vessel constructs. Nano-scaled aspects of scaffolds such as surface roughness and pore size can also be adjusted to control the endothelial barrier functionality of the engineered vessel constructs [8]. Furthermore, the resolution of the fabrication techniques should be in the scale of 100 – 1,000 μm to mimic the size of mesoscale vasculatures including arterioles and small arteries to control the orientation of capillary branches throughout the target tissue, where the diffusion of gases and transport of molecules take place [6]. Hydrogels of both synthetic and natural polymers are widely used as a component of vascular scaffolds due to their high water content and viscoelastic characteristics which are similar to the physiological vessel extracellular environments. Protein-based hydrogels possess cell instructive features but still need chemical modification or blending with polysaccharide-based and/or synthetic hydrogels for better control of the mechanical properties [12]. There have been several strategies to enhance the rigidity of hydrogels but demonstration of these materials' capability to construct a high- resolution tubular scaffold with a complex architecture as well as testing their functionality after *in vivo* implantation is still a work in progress [8].

In this review, several micro-scaled blood vasculature fabrication techniques, strategies, and challenges will be compared and discussed. Considerations in different areas including selection of the main components and optimal characteristics of fabricated vessel scaffolds will be thoroughly explained (**Scheme 1**). Furthermore, recent advances in several blood vessel fabrication strategies will be discussed. It is anticipated that this work will provide insights for the development of blood vessel engineering to support large-scaled tissue engineering in the future.

2. Materials

Several vessel fabrication strategies require scaffolding to provide support during vessel formation and maturation even though scaffold-free strategies including spheroid or cell-sheet technologies are also available [12]. Hydrogels have been widely used as a material for vasculature scaffold fabrication because they share similarities with native ECM including high biocompatibility, high water retention capacity, high permeability, and have viscoelastic properties [8,12]. They can be formed by physically and/or chemically crosslinking hydrophilic polymers from natural and synthetic sources, which advantages, disadvantages, and possible modifications of different hydrogel materials are summarized in **Table 1.** However, some hydrogel materials possess relatively weak mechanical properties, leading to difficulties when processing into a defined structure, especially fabrication of hollow channels [4]. Hydrogels of both natural and synthetic origin can be combined to maintain cell-instructive and biocompatible properties while adequate mechanical properties are achieved [12]. Furthermore, to ease the fabrication procedure for a blood vessel structure, the suitable material should also be selected based on rheological parameters such as viscosity [13]. In case of using bioinks, which is composed of hydrogel material and cells, lower viscosity is preferable for maintaining cell

viability as it lowers the shear stress occurring during fabrication [14].

2.1. Protein-based hydrogels

Hydrogels containing ECM and basement membrane (BM) proteins such as collagen, gelatin, fibrinogen, laminin, elastin, fibronectin, and Matrigel® possess intrinsic bioactivity and cell adhesion ligands to enhance cell adhesion, proliferation and differentiation [4,5,12]. They can also influence the endothelial barrier function and tissue remodeling including activation of αvβ3 integrin signaling during migration of ECs to form lumenized structures [5,15]. Collagen and elastin, which are the main components in ECM, are responsible for elasticity and stiffness of the tissue and can provide strength and elasticity in fabricated vascular constructs [8]. Homogenization and sonication of crosslinked collagen sponges result in high water -dispersible collagen microfibers, which is reported to guide cell alignment inside hydrogels and on scaffolds by acting as a micro-sized scaffold for cell adhesion. This alignment can be easily controlled by shear stress compared to a 6 mg/mL collagen hydrogel that requires manipulation of the casting mold strain [16,17]. Furthermore, ECM and BM-derived proteins can provide binding sites for several GFs responsible for the formation of new blood vessels during angiogenic sprouting and for BM remodeling [6,12]. Even though ECM protein-based material is present with proteolytic degradable sites, their degradation rate is difficult to control, resulting in an inability to withstand high stress and pressure while maintaining the lumen structure of blood vessels. [8,12]. During *in vitro* culture, highly glycosylated adhesive molecules in natural ECM can interact with bioactive molecules and protein components in the cell culture medium, affecting the cell function and microenvironment. In some experiment settings, the mentioned interaction can hinder the analysis of target parameters [15]. To overcome these limitations, plastic compression, chemical modifications, and fabrication of hybrid scaffolds with polysaccharides or synthetic components

can be performed [8,12,18]. In the case of synthetic polymers, poly (ethylene glycol) (PEG) is usually incorporated with ECM proteins such as fibrin and collagen [19,20]. Furthermore, hydrolysis of collagen results in gelatin, which is also a widely used ECM-based material that still possesses many characteristics of collagen such as providing cell adhesion sites and matrix-metalloproteinase (MMP) degradation sites. However, phase transition of gelatin hydrogels occurs at 37 °C so it cannot form gels in *in vitro* and *in vivo* conditions [12,21]. Methacrylated gelatin (GelMA) has become popular due to its excellent biocompatibility and biodegradability, and ability to tune its mechanical properties by changing crosslinking conditions. The crosslinking mechanism, which occurs when exposed to light irradiation in the presence of photoinitiators, allows a wide range of applications on several 3D printing platforms. There have been several publications regarding GelMA-based composite materials to date, where their light-induced crosslinking ability complements the characteristics of other materials [21]. Additionally, tropoelastin (Tro), which is the form of elastin before native enzymatic crosslinking, can be modified with methacryloyl groups to synthesize photo-crosslinkable MeTro [22]. Lee *et al.* mixed human-recombinant-derived MeTro with GelMA as a new biomaterial approach for 3D bioprinting of vascularized soft tissues. Incorporation of MeTro in the widely-used GelMA improved elasticity and stability of the printed hydrogel structures. It was demonstrated that the degradation of printed GelMA constructs is faster than printed MeTro constructs due to the greater MMP-sensitiveness of GelMA [23]. These examples demonstrate that several ECM-derived molecules can be modified for vascular construct fabrication by photo-crosslinking.

2.2. Polysaccharide-based hydrogels

Apart from proteins, ECM also contains glycosaminoglycans (GAGs) such as hyaluronic

acid and heparin. These polysaccharide molecules can bind to GFs, interact with cell-surface receptors, and are degradable by enzymes. However, they require additional modification with biologically functional molecules such as small peptides or protein domains to encourage cell adhesion and microenvironment remodeling [12]. Hyaluronic acid (HA) is a non-sulfated GAG that can form receptor-ligand interactions with ECs via CD44 to promote cell proliferation and migration but has difficulty forming gels and exhibits fast degradation. [13,24]. It has been reported that HA oligosaccharides exhibit anti-coagulation and endothelialization properties, allowing their emerging application by immobilization on vascular graft surfaces [25,26]. HA can also be used with other materials such as gellan gum (GG) and collagen to enhance cell adhesion properties and gain more control of the mechanical properties and degradation rate [24,27]. Furthermore, HA can be acrylated, modified with arginine–glycine–aspartic acid (RGD) moieties, and polymerized using a thiol crosslinker with matrix metalloproteinase (MMP)-sensitive peptides to increase its bioactivity [12,28]. On the other hand, heparin contains many sulfate groups which contribute to the overall negative charge available for binding of GFs. Thus, heparin is widely used for incorporation of biological cues, and also for the enhancement of hemocompatibility on several vascular tissue engineering scaffolds [12,29,30]. Polysaccharides with origins other than ECM are also used in tissue engineering applications due to their sustainable sources and unique characteristics. Sodium alginate (SA), an anionic polysaccharide derived from brown algae, is widely used due to its ability to form hydrogels in the presence of bivalent cations such as Ca^{2+} [4,31]. Although it is a biocompatible material, SA is bioinert in that it does not promote effective human cell adhesion, cell proliferation, and only exhibits partial biodegradation inside the human body. Hence, sodium alginate is mostly used along with ECM derived materials such as gelatin [9,32]. Furthermore, oxidation of SA with sodium periodate to generate aldehyde groups on the chains, allows control of the degradation

rate according to their oxidation percentages [33,34]. In contrast to SA, chitosan possesses an overall cationic charge and provides several amino groups which can react with multiple aldehydes through Schiff's base reaction [4]. Chemical modification of chitosan side groups including amino and hydroxyl groups can improve its solubility in water, although chitosan is originally more soluble in weak acidic conditions [35]. However, chitosan exhibits low angiogenic potential which can be overcome by the addition of ECM components such as fibrin [36,37]. Other than SA and chitosan, GG, which is a water-soluble polysaccharide of microbial origin, is also widely used in tissue engineering applications due to its thermoreversible gelation and shear-thinning properties. GG bioinks can be easily extruded while forming a gel by ionic crosslinking with multivalent cations, even though they possess a high molecular weight [38,39]. Application of GG as a sacrificial material for fabrication of pseudo blood vessels was demonstrated by Matsusaki *et al.*, where GG hydrogels can be removed by cytocompatible Tris-HCl buffer [40]. Most of the polysaccharide-based hydrogels cannot be used by themselves and need further chemical modification and incorporation of ECM components to obtain bio-instructive and mechanically stable hydrogels for vascular tissue engineering.

2.3. Decellularized Extracellular Matrix (dECM)

The whole ECM can be decellularized (dECM) and utilized as a biomaterial, which is applicable for fabrication techniques of engineered blood vessels such as 3D printing [41]. Compared to proteins and polysaccharides, dECM possesses intrinsic bioactivity and can provide specific tissue and organ microenvironments, which is beneficial for stem-cell based vascular engineering strategies [42,43]. It was demonstrated by Lee *et al.* that dECM material prepared from porcine blood vessel promoted GFs expression, and fibroblast migration and proliferation compared to atelocollagen [44]. This is due to mixtures of different collagen, proteoglycans,

glycoproteins, and GFs present in dECM with unique spatial distribution in each type of tissues and organs that can provide synergistic effect to cultured cells [45,46]. However, protein and proteomic analysis of human vascular dECM has not been reported yet. Most of the studies only reported analysis of porcine-derived dECM materials from different organs such as heart, liver, skin, and cornea up to date [42,47]. To utilize dECM as a bioink, it can be solubilized to acquire the printable form by enzymatic acidic digestion. At the same time, optimization of solubilization process is needed to preserve ECM components including GFs and low MW peptides [44,45,48]. Collagen fibrils and BM assembly mainly contribute to the gelation of solubilized ECM with some contribution from other ECM components especially heparin [45,49]. Nevertheless, pure dECM possesses weak mechanical properties and slow gelation time [45,50]. Hence, further chemical modification and incorporation with other materials can be done to enhance printability. For example, Gao *et al.* incorporated porcine vessel-derived dECM bioinks, with alginate hydrogel to enhance mechanical properties and balance matrix stiffness. This dECM-based bioink formulation was successfully used in fabrication of tissue engineered blood vessel by 3D coaxial bioprinting [51,52]. In the recent work of Isik *et al.*, they orthogonally crosslinked bovine aorta dECM with sodium alginate and tyramine-modified hyaluronic acid to further enhance mechanical properties of the bioink [53]. Even though dECM is an attractive biomaterial, its availability is limited especially for human donors [45]. Obtaining dECM from sustainable animal sources is possible, especially pigs that share anatomical and physiological similarities to humans [47]. Even though porcine dECM is possible to cause immune rejection, its safety and feasibility in a clinical setting has already been proven by a study from Traverse *et al.* [45,54].

2.4. Synthetic polymer-based hydrogels and scaffolds

Hydrogels of synthetic origin have advantages in terms of improved mechanical properties and controllable matrix adhesiveness, stiffness, and degradability to mimic the native vessel microenvironment [15]. Several types of synthetic polymers have been shown to be biocompatible and provide a suitable degradation rate and by-products for tissue regeneration [55]. The implanted synthetic vascular grafts are expected to last for more than 2 months to allow complete organization of endothelial cells, especially at the anastomosis site [55,56]. Modification of PEG into the form of poly (ethylene glycol) diacrylate (PEGDA), and poly(ethylene glycol)-tetra-acrylate (PEGTA) allows the polymers to form hydrogels by photocrosslinking, making them applicable for UV/visible light assisted printing techniques. To compare, PEGDA is a better candidate than PEGTA for vascular scaffold fabrication due to its availability of multiple crosslinking sites [57]. Another interesting candidate is Pluronic®, which is a polyoxyethylene–polyoxypropylene–polyoxyethylene (PEO–PPO–PEO) amphiphilic triblock copolymer with a unique thermoreversible gelation characteristic at 4 °C. It is usually used as a sacrificial material for fabrication of vascular channels, but its low liquid transition temperature limits its application to 3D printing techniques other than extrusion-based printing [57]. Recently, poly(*N*-acryloyl glycinamide) (PNAGA)-supramolecular hydrogels have also been introduced as candidates for bioink formulation due to their favorable mechanical properties and stable swelling characteristics [58,59]. It was reported that PNAGA synthesized from 25 wt% *N* -acryloyl glycinamide (NAGA) possess high fracture energy of over 1000 J.m $^{-2}$ and remained stable in shape even after immersion in deionized water for 24 hours [58]. In a study by Liang *et al.*, PNAGA based hydrogel ink has been prepared via incorporation of reversible hydrogen bonds of *N*-acryloyl 2-glycine (ACG) into GelMA hydrogel networks and

mixed with nanoclay to demonstrate fabrication of tubular structures with tunable diameters from 0.5-3.0 mm [60]. On the other hand, degradable synthetic polymers such as poly (lactide-co-glycolide) (PLGA), polylactide (PLA), poly-L-lactic acid (PLLA), polycaprolactone (PCL), and polyglycolic acid (PGA) can be electrospun, cast, patterned, or 3D printed as scaffolds with high mechanical durability for vascular engineering [6,61,62]. PGA is especially noted for its elasticity and ability to promote neovascularization [18,63]. Composite scaffolds can be prepared by blending synthetic polymer with natural polymer prior to electrospinning, or coating and grafting electrospun constructs with natural polymers to improve their bioactivity [27,29,64,65]. Even though synthetic polymers possess tunable crosslinking, some points on crosslinking design should be considered. Especially, to maintain structural integrity when swelling occurs and maintain desirable degradation rate in presence of hydrolytic crosslink cleavage. Physical and mechanical properties should also be characterized during the swollen state to prevent obtaining inaccurate results [4]. Synthetic hydrogels and scaffolds are mostly modified with cell-adhesion moieties or incorporated with ECM-based hydrogels to improve ECs viability, spreading and proliferation that eventually results in the successful endothelialization of fabricated 3D tissue constructs.

3. Physical and chemical properties, and incorporation of biological cues

To ensure successful vascular engineering, several physical and chemical parameters affecting the vasculature's function need to be optimized. Biological cues including GFs and ECs can also be incorporated in the scaffolds as they play a major role in vascular modeling, especially after implantation *in vivo*. In this section, these parameters will be discussed as a guideline for designing and characterizing engineered blood vessels.

3.1. Mechanical properties

Burst pressure, elastic modulus, and stiffness. Optimal mechanical properties in macro-scale, microscale, and nanoscale are essential for maintaining blood vessel structure, cell attachment, and cell signaling. Burst pressure, compressive modulus, tensile strength, and elastic modulus of the fabricated structure should be adequate to prevent shrinkage or narrowing of blood vessels in the presence of blood flow pressure (80-120 mmHg at physiological condition) and the presence of surrounding cells [13,66]. Perfusion of vascular grafts can generate internal pressure, where the highest pressure that the structure can withstand before failure is defined as burst strength: $P_{burst} = \sigma_y \times t/r$, where σ_y is yield stress, t is wall thickness, and r is radius of the graft [13]. Furthermore, when the tubular engineered vasculature is embedded inside the tissue, there will be an external force that acts towards the vessel wall that may result in scaffold collapse if the scaffold strength is insufficient [41]. Uniaxial compressive modulus, which is correlated to elastic modulus of native vascular structures, is mostly in the MPa range. However, hydrogel-based structures usually possess lower compressive modulus values in the kPa range [67]. Reinforcement strategies for hydrogels such as the incorporation of nanomaterials and microporous scaffolds make it possible to overcome these limitations [8]. For example, Wu *et al.* incorporated montmorillonite (MMT) nanocomposite in a gelatin-alginate bioink that achieved an elastic modulus of 25.8 MPa, which is similar to the native mammary artery, and a burst strength of 3,196 ± 1,264 mmHg, which is greater than physiological blood flow pressure [68]. To develop vascular scaffolds with an elastic modulus and stiffness similar to native vessels, which range from 1 to 4 MPa and from around 10 kPa to 1.5 MPa respectively, the optimal concentration of material compositions as well as crosslinking strategies need to be investigated [41,66]. Mechanical properties of vessel equivalents should be designed to match the physiological values because they can affect ECs survival, proliferation, and their specific

characteristics at the microenvironmental level [5]. For example, stiffness of vascular scaffolds can be adjusted by introduction of different crosslinking strategies. Duan *et al.* developed a pH-responsive double network HA hydrogel consisting of vinyl double bond and Schiff base crosslinking. The change of pH from 7.4 to 5 can decrease crosslinking degree by degradation of Schiff base crosslinking, causing the compression modulus to decrease from 15.77 ± 3.96 kPa to 4.8 ± 1.35 kPa [69]. Nevertheless, precaution should be taken to avoid the loss of the endothelial phenotype through endothelial-to-mesenchymal transition in the presence of material with a high stiffness, which is correlated to elastic modulus [70]. It was demonstrated by Zhang *et al.* that ECs could not attached on poly(L-lysine)/hyaluronate acid polyelectrolyte film with lower stiffness of 196 ± 41 kPa but can maintain ECs phenotype at elastic modulus of 317 ± 30 to 431 ± 39 kPa. However, when substrate elastic modulus reaches 491 ± 63 kPa, ECs lose expression of CD31 endothelial markers [71]. Endothelial to mesenchymal transition can also be mediated through TGF-β cytokine, while the sensitivity and signaling can be adjusted by several mechanical contributors including lumen wall stiffness, strain, and flow shear stress [72]. Vascular BM, which is the structure underlying the vascular endothelium, provides several biophysical cues to the ECs [66]. The response of ECs to substrates are mediated by their integrins, where endothelial permeability, cell morphology, and cell migration are enhanced depending on substrate stiffness. On stiff substrates, intracellular contractile forces are promoted through a Rho-associated signaling pathway, causing cell-cell junctions to be pulled apart and increase cell permeability [7]. On the other hand, increased expression of MMP-2, 9, 13 and 14 reduces the ECM stiffness to allow microvessel sprouting during angiogenesis [73]. It has been reported in the literature that ECs form more focal adhesion and exhibited a more spread morphology on stiff substrates (20 kPa- 2 MPa) than soft substrates (1-5 kPa) in 2D conditions [66,72,73]. However, when ECs complete the establishment of cell-cell contact, the difference

between cell morphology on substrates with low and high stiffness disappeared [72]. Interestingly, ECs exhibit a more spread morphology in the presence of softer 3D substrates, which is the opposite to the presence of 2D substrates [66]. This happens due to the ability of ECs to migrate and assemble into microvascular structures [66,73,74]. To gain control of cell adhesion, cell migration, and vessel permeability on the engineered blood vessels, scaffold matrix stiffness can be adjusted by varying polymer or crosslinker concentrations of their components. Other factors that influence ECs behavior including scaffold density and pore size may also be changed accordingly [6].

Surface topographies. Other than stiffness, the BM can also influence the formation of endothelium through its topography [66]. ECs can sense topographical features in micro and nanoscale including basement membrane roughness, thickness, pore architecture, and fiber alignment then change their cellular behavior through mechanosignaling [70]. Thus, different behaviors such as cell elongation, cell alignment, chemokine secretion, ECM production, and tissue remodeling capacity can be regulated through adjustments of scaffold topography [6]. In native blood vasculatures, ECM proteins and vascular membranes provide surface topographies through fibrous and porous structures where collagen I fibrils with diameters of 20-200 nm are assembled into larger collagen bundles with diameters of 1-20 μm [73]. The topography of vessel BM is also a complex meshwork of pores and fibers within the dimensional range of 100-1000 nm that can influence cellular behavior without depending on external biochemical factors [73,75–77]. In biomimetic blood vessel engineering, scaffolds with designed fibrous and porous structures can control cytoskeletal reorganization of ECs by regulating integrin binding and focal adhesion complex formation [73]. For example, layer-by-layer assembled electrospun nanofilms with greater roughness present inhibiting effects on adhesion of human umbilical vessel endothelial cells (HUVECs) [70]. In the case of porous structures, the pore size of vascular

support scaffolds can be adjusted to control vessel invasion to adjacent tissue constructs [18]. It was demonstrated by Yu *et al.* that PEG hydrogels with a larger pore size (50-150 μm) allow vascular ingrowth within the material volume while hydrogels with a smaller pore size (25-50 μm) only support vascularization at the surface [78]. Furthermore, scaffold pore architecture and fiber orientation could provide micropatterns for ECs alignment during regeneration of vascular structures. Uniaxially aligned and highly interconnected pores have been shown to promote cell-cell interactions, when compared to randomly oriented pores (**Figure 1(a)** [6,79]. Different strategies such as electrospinning of aligned fibers and introduction of collagen fibers to cells have been applied to guide ECs alignment and their interaction to form capillary-like structures so far [80–82]. Control on ECs orientation is especially necessary for fabrication of tissue-specific structures with highly organized vasculatures such as in skeletal and smooth muscles [16]. For example, as mentioned in section ***2.1. Protein-based hydrogels***, Liu *et al.* used aligned collagen microfibers (CMF) with length of 20 μm to control ECs orientation, induce luminal capillary formation, and achieve aligned capillary network (**Figure 1(b)**). Because the microfibers are purely made of collagen, ECs can interact and adhere to CMF via integrin β1 molecules as shown in the immunostaining images of **Figure 1(b)**. Interestingly, significant aggregation of CMF with longer length of 200 μm occurred, which leaded to less capillary formation and no interconnection compared to their shorter counterparts [16]. On the other hand, melt-electrospun networks of PCL needed fibronectin coating to facilitate ECs adhesion. In the study conducted by Bertlein *et al.*, PCL networks with different pore sizes all guided newly forming microvasculature orientation at day 3 **(Figure 1(c)).** However, the microvessel became non-oriented and migrated towards the pore volume in case of larger pore size at day 7 **(Figure 1(c))** [83]. Independent control on collagen fiber anisotropy and directionality can also be achieved by utilization of a non-uniform microfluidic channel network, as demonstrated by

Ahmed *et al.* in their collagen microengineering attempt (**Figure 1(d)**). This presents a potential production method for manufacturing directional defined 3D gels applicable as flow channels such as vascular microenvironment models [84]. Control of scaffold surface topography is also possible by other fabrication techniques including soft lithography and laser photolithography. However, this approach is mostly utilized for the purpose of microvascular network formation and guidance [73].

Shear stress. Intraluminal shear stress on ECs is a key mechanical stimulus for vascular adaptation and maintaining vascular homeostasis from microvessel sprouting, cell-cell interactions, cell alignment, endothelium permeability, and vessel maturation [6,72]. The shear stress participates in vasculature remodeling where high shear stress increases wall thickness and diameter and low shear stress reduces vessel diameter to maintain the luminal shear force at a physiological level [13]. Definition of blood vessel wall shear stress is the frictional force per unit area, which can be explained through the Hagen-Poiseuille equation: shear stress (τ) = $32\eta Q/\pi d^3$, where η is mean viscosity, Q is mean blood flow rate, and d is the vessel diameter [13]. In physiological conditions, the normal level of time-averaged shear stress is ~1 Pa in the aorta, ~5 Pa in arterioles, ~2 Pa in venules, and ~0.1 Pa in the vena cava [66]. A healthy wall shear stress of ~1.2 Pa produced by laminar flow is reported to resist accumulation of lipids, which is associated with atherosclerosis [72]. However, shear stress throughout the same vessel can be varied markedly according to geometric features such as vessel curvature and branching [66]. At the same time, branching of vessels according to Murray's law occurs to maintain homogeneous wall shear stress during hemostasis [9,85]. Increasing shear stress results in capillary sprouting, while reduction of shear force results in capillary retraction [7]. When the flow shear stress is at a non-physiological level, it will result in cell cytoskeleton disassembly, leaky vessel walls, and inflammatory responses [6]. A multi-complex unit of cytoskeletal

remodeling and adjustment of barrier functions has been activated through the NOTCH1-mediated mechanosensitive pathway in ECs, taking place at the flow sensing complex located at EC junctions including VE-cadherin, VEGFR2, and CD31 [7,72]. Recognized by the flow sensing complex, laminar flow encourages expression of eNOS and production of nitric oxide (NO), while disturbed flow results in oxidative stress and inhibits NO production [72]. Other than expression of eNOS, shear stress from blood flow and electrochemical stimulation also manages the expression of VEGF-A and angiopoietin in ECs and pericytes that stimulate survival, proliferation, and migration of ECs [73,86–88]. Furthermore, laminar and steady blood flow influences the morphology and alignment, where ECs are elongated in the direction of flow and control flow shear stress by spreading to increase cell surface area [66,73]. The effect of flow on cell morphology is also dependent on cell stiffness sensing. ECs alignment required only 0.6 Pa shear stress on 10 kPa substrates, while 2.2 Pa shear was required on 100 Pa substrates. On the other hand, the spreading of ECs only slightly increased with flow shear on 10 kPa substrates, while the cell spreading markedly increased in the presence of 100 Pa substrates at shear stress above 1.2 Pa [72,89]. In a study of perfusable 3D microvascular networks on a chip, it was demonstrated that constant blood flow with shear stress below 10 dyn. cm^{-2} influences NO synthesis and ECs cytoskeleton remodeling [5,90]. However, shear stress of >10 $dyn.cm^{-2}$ (1 Pa) stimulates microvessel sprouting towards the interstitial fluid flow through activation of MMP-1. The resulting higher density of microvasculature then reduces the flow stress as a part of the angiogenic negative feedback mechanism [7,91]. In addition, interstitial flow was reported to participate in controlling morphogenesis and sprout formation where physiological interstitial flow (0.1-10 $\mu m.s^{-1}$) could weaken spatial VEGF gradients to provide αvβ3 integrin-mediated directional cues against the interstitial flow for microvascular sprouting [7,92,93]. Furthermore, it was demonstrated that shear stress of both luminal and transmural blood flow induced

angiogenic sprouting when a threshold of 10 dyn.cm^{-2} (1 Pa) is surpassed [91]. Other than steady flow, the effect of non-reversing, reversing, and oscillatory blood pulsatile flow on cell signaling have also been demonstrated in some studies [41,66].

Other than intrinsic mechanical properties of vascular scaffolds, external mechanical stimulation can also be recognized by the ECs and vascular smooth muscle cells (vSMCs) and act as a mechanosensitive barrier. These cells are responsible for adaptive modeling during anastomosis of vascular grafts to host vessels, which results in a state of mechanical homeostasis [7]. Mechanical stimulations such as cyclic strain can result in upregulation of integrin αvβ3/αvβ5 expression in ECs to promote branching of blood vessels [73,94]. Notably, external cyclic stretch of physiological range (5-10 %) in the uniaxial direction was reported to markedly promote vascular growth in a particular direction [6,95]. For example, muscle induced mechanical stimulation can enhance MMP expression responsible for EC sprouting and was mimicked in mechanically constrained hydrogels to improve microvascular alignment [73,96,97]. Overall, mechanical properties in the larger scale including elastic modulus and burst pressure, as well as mechanical properties in the microenvironmental scale including stiffness, topography, and shear stress of vascular scaffold, can be tailored to obtain endothelialized, functional, and stable vascular structures.

3.2. Chemical properties

The chemical properties of vascular scaffolds including degradation time, crosslinking degree, and swelling, can be controlled by optimization of polymer concentration and adjustment of molecular interactions within the network [12]. In the case of scaffold material degradation rate, it should match the ECM synthesis rate of ECs to provide enough stability for the engineered vessels, while the degradation product should not cause cytotoxicity nor impede

blood vasculature tissue regeneration [8]. Furthermore, there will be a change in mechanical properties during degradation that can influence maturation and organization of the microvasculature that needs to be considered during the design and fabrication of scaffolds [73]. The ability of ECs to migrate and the remodeling of the matrix to form lumen structures is highly dependent on the degradation of scaffolds including hydrogels [15]. However, materials with high degradability result in single-cell migration rather than multicellular migration, which hinders the tubular structure formation [15]. In hydrogels, the mechanism behind their degradation is mainly ion exchange, hydrolysis, breaking down of crosslinked networks, and enzyme-induced digestion [8]. When vascular cells are in the presence of ECM-derived hydrogels, enzymatic activities are induced to break down the matrix, leaving biocompatible by-products including glycolic acid, glucose, and lactic acid [8]. Interestingly, it has been reported that degraded hyaluronan could block shear stress-driven spreading of cells on soft hydrogels (100 Pa) up to 2.2 Pa of shear force [72,89]. In the case of ionic crosslinked hydrogels, degradation activities are difficult to control due to the reversible gelation mechanism. For example, cations in a cell culture medium can contribute to the exchange of divalent cations and dissociation of alginate hydrogel crosslinks [8]. On the other hand, the hydrolysable linkages in synthetic materials such as ester and amide bonds can be easily designed, and adjustments including grafting and copolymerization with degradable macromers are possible to achieve the desired degradation rate [4,8]. However, the hydrolytic degradation of synthetic hydrogels such as polyethylene glycol (PEG) hydrogels from photopolymerization of poly (ethylene glycol) diacrylate (PEGDA) is relatively slow *in vitro* and *in vivo* due to limited bioactivity to cellular signals and enzymes [8]. To increase the degradation rate, MMP-sensitive crosslinkers can be used to increase sensitivity towards physiological enzymes [4]. This modification allows matrix metalloproteases (MMP) and plasmin to break down ECM proteins for the release of angiogenic

GFs, and for ECM turnover during vessel formation [12]. On the other hand, equilibrium between degradation and stability of matrices can be controlled by enzyme counterparts such as alpha 2-antiplasmin and tissue inhibitors of metalloproteinases [12].

Different crosslinking strategies including ionic and covalent crosslinking can be used on hydrogels to achieve suitable stiffness, pore size, swelling ratio, and degradation. Non-covalent crosslinking provides viscoelastic bonding due to reversible organization of breaking and reforming interactions between polymer chains, while covalent bonding provides rubber-like elasticity on hydrogels [4]. The crosslinking strength can be controlled by changing crosslinking agent concentration and modification of crosslinking sites on polymer chains, where the density of crosslinking bonds markedly affects the mechanical properties of hydrogels [4,8]. However, an excessive or insufficient crosslinking degree can hinder cell migration and cell adhesion [73]. To overcome the challenge of balancing hydrogel crosslinking strength, several studies on the development of double-network hydrogels have been published to date [4]. For example, a SA and GelMA solution blend were ionically crosslinked, and then exposed to UV for 30 seconds to achieve an elastic modulus between 15 and 25 kPa. After 10 days of cell culture, this alginate-GelMA hydrogel group showed the highest cell viability and spreading compared to other experimental groups that were exposed to UV irradiation for more than or less than 30 seconds [14]. Furthermore, incorporating nanomaterials that are not participating in crosslinking is also possible to strengthen the hydrogel and increase the elastic modulus [4,8].

After fabrication of scaffolds to support blood vessel engineering, surface modification by physical modification, surface adsorption, plasma treatment, and chemical modification can be performed to further improve the surface characteristics of biomaterials such as hydrophilicity/hydrophobicity and hemocompatibility [13,55,98]. The surface of a scaffold

material should be smooth and possess hydrophilicity to avoid adhesion and accumulation of platelets, while be able to interact with vascular cells [70,99,100]. Cell adhesion on the scaffold surface is also greatly influenced by hydrophilicity where very highly hydrophilic surfaces have no affinity to protein and resulting in poor cell adhesion [41]. Precautions should be taken during surface modification since incorporation of some highly hydrophilic functional groups may cause challenges in controlling swelling ratio, resulting in reduced mechanical strength [98].

To achieve vascular tissue engineered structures with optimal chemical properties both as a bulk construct and locally at the surface, several strategies can be applied to the available materials before or after the printing process. Furthermore, pro-angiogenic cues can be incorporated into the fabricated structures to enhance ECs growth, ECM secretion, and maturation of blood vessel tissue in a shorter period of time. Following this, requirements related to the printing process and the final structure are also essential during the design of vascular scaffold chemical characteristics to ensure successful fabrication.

3.3 Incorporation of biological cues

Although the fabricated vessel hydrogel scaffolds possess excellent chemical characteristics, they may still need angiogenic signaling cues for local environment remodeling as occurs in the native vessel basement membrane [12]. Main vascular angiogenetic GFs including vascular endothelial GFs (VEGF), platelet-derived GFs (PDGF), fibroblast GFs (FGF), and angiopoietins are selectively affinitive at distinct ECM locations, mostly on fibrin and fibronectin [6,12]. However, fibrin hydrogels have a relatively fast degradation rate that results in a limited time of GFs exposure to surrounding vascular cells. For example, fibrin hydrogels that were covalently linked to VEGF had almost all disappeared within 12 days *in vivo* based on experimental results from Largo *et al.* [12,101]. To overcome this limitation, fibrinolysis inhibitors such as α2-

antiplasmin have been applied to slow down degradation of fibrin biomaterials and release of (VEGF)-A and (PDGF)-BB according to Liu *et al.* [102]. Furthermore, several mechanisms to improve sustained release on different hydrogel systems have thus far been developed and studied including covalent bonding, use of linker molecules, physical adsorption, and loading into microparticles [18]. In 2013, prominin-1 derived peptide (PR1P), which is also known as CD133, was investigated to directly interact and stabilize with VEGF in endothelial progenitor cells and was applied to several biomaterials by covalent conjugation to prolong VEGF release [103–105]. Nevertheless, VEGF structural integrity can be compromised during the scaffold encapsulation process, which can be overcome by the use of *de novo* engineered VEGF-mimic peptide alternatives [106]. Another possible strategy is the introduction of collagen-mimetic peptide tethers (CMP)-mediated GFs gene delivery to control VEGF activity, which has been demonstrated to modulate ECs vascular network formation with a larger diameter (up to around 70 μm) and larger volume [107].

Even though different incorporation strategies have been introduced to achieve controlled release of GFs, they can become instable over time [108]. However, the required amount of GFs in the microenvironment can be replenished by secretion from ECs, which can be added to materials for engineered blood vessel fabrication [109]. The selection of ECs needs consideration on heterogeneity among different types of blood vessels in different types of organs, and possible immune rejection [8,73,110]. Different types of ECs including HUVECs, human microvascular ECs (HMVECs), endothelial progenitor cells (EPCs), and embryonic stem cells have been utilized in vascular tissue engineering with HUVECs being the most prevalent choice [8]. HUVECs are used in most of the vascular engineering studies due to their abundant source, non-invasive harvesting method, and can facilitate capillary remodeling and connect to host vasculature in an *in vivo* study [56,111]. Because of vascular heterogeneity in different types of

organs as mentioned in section ***3.1. Mechanical properties*** **(Surface topographies)**, usage of stem cells and progenitor cells are beneficial for recapitulation of specific microenvironments by multipotent differentiation [110,111]. For example, Gao *et al.* stated the importance of EPCs on treatment of ischemic diseases and developed engineered blood vessels to enhance the survival and differentiation capability of the cells [52]. Furthermore, perivascular cells including pericytes, smooth muscle cells (SMCs), and fibroblasts are usually cocultured with ECs to assist in stabilizing the newly formed vasculature by interaction with ECs and secretion of GFs, MMPs, and ECM secretion [1,16,112–114]. This is especially beneficial for vascular tissue engineering for applications in specific types of tissue, where particular type of fibroblasts were co-cultured with ECs [79,115]. However, there are still some challenges on optimization of cell ratio and appropriate cell culture medium during cocultures [5].

4. Fabrication techniques on 3D engineered blood vessels

Up to date, there have been several fabrication techniques and strategies with purpose ranging from controlling cellular orientation to providing scaffolding structures for ECs. For example, microfibers are mostly utilized to facilitate the orientation of ECs as mentioned in section ***3.1***, while microparticles are used as encapsulating and adherent platform for ECs with ability to assemble into larger structures [16,116–118]. Utilization of microparticles was demonstrated by Twal *et al.*, where HUVECs were cocultured with human aortic smooth muscle cells (HASMCs) inside gelatin microcarriers, then were assembled into tubular tissue constructs. However, this approach is limited to construction of tubular tissues with inner diameter above 2 mm [117]. Encapsulation of ECs or other types of cells inside microcarriers can provide spacing for nutrient delivery or introduction of angiogenesis to maintain cell viability and function after implantation as demonstrated by Correia *et al.* and Zhao *et al.* [116,118]. In the larger scale,

there are several challenges to overcome in creating lumen structures within the vessel architecture. Templating strategies and needle-based micromolding allow casting of polymer solutions into a desired architecture, but it is quite difficult to maintain the same 3D structure after removal from the mold [67,119–123]. Subtractive techniques, where a sacrificial material template is embedded inside a bulk matrix is easier to remove or dissolve away by temperature variations or solvents [5,33]. These materials can act as a support to prevent the collapse of channels because of the surrounding hydrogel weight [4]. Furthermore, utilization of sacrificial materials with several fabrication techniques including 3D printing and electrospinning is possible to acquire luminal structures with complex architectures. In this section, different fabrication strategies to achieve engineered blood vessels with diameter ranging from 100 to 1000 μm is going to be discussed.

4.1. 3D printing

There are two main categories of 3D printing according to the incorporation method of cells in the printing material. In conventional printing, ECs are seeded onto a pre-shaped structure post-printing. In 3D bioprinting, the bioinks consisting of cells and printing material are mixed and printed together to fabricate a vessel structure [41,124,125]. In bioinks, the printing material should be able to protect the encapsulated cells from mechanical forces and other external factors during the printing process, while at the same time be printable and possess shape retention [13]. There are four main methods of 3D printing that are commonly utilized in the literature regarding the fabrication of blood vessels: inkjet printing, extrusion-based printing, 3D printing by photopolymerization, and laser-assisted printing [5]. These 3D printing techniques have been utilized in fabrication of blood vessels targeting application in different types of tissues up to date, which has been summarized in **Table 2**.

Inkjet-based printing, especially the commonly used drop-on-demand type, allows accurate positioning of the droplets into structures with complex architecture [57]. During fabrication, the ink with or without cells is deposited dropwise on the crosslinking substrate or reservoir using of thermal or piezoelectric actuators [6,41]. This fabrication method allows low cost and high-resolution (50 μm) printing but is limited to bioinks with a low viscosity (around 10 mPa·s and below) and low cell densities to prevent the clogging of nozzles [5,6,9]. Furthermore, high working temperatures and mechanical stress during the depositing of bioink droplets can compromise the viability of cells [5]. Application of inkjet printing allows both patterning of ECs deposition and construction of 3D tubular structures. For example, bioprinting by patterning of ECs within multilayered 3D human liver tissue chips was conducted by Matsusaki *et al.* in 2013 [126]. In addition, it was demonstrated that droplet-based bioprinting of alginate hydrogels allows free-form fabrication of complex constructs such as structures with bifurcations on a crosslinking and supporting calcium chloride bath [127]. However, process-induced deformation decreased the resolution of printed structures. There have been several attempts to improve the resolution of droplet-based printing including improvement of viscosity of $CaCl_2$ bath with hyaluronan or PVA, and positioning of droplets on substrates such as gelatin-Ca^{2+} hydrogel and a fibrinogen surface (**Figure 2(a)**) [128–130]. Nevertheless, inkjet-based printing is not widely used for vascular tissue engineering yet because of difficulties to fabricate thick constructs and it requires more time to create larger vascular network structures [33,73].

Extrusion-based printing is another type of 3D printing and uses pneumatic pressure, mechanical force, or thermal extrusion rotation to deposit the bioink loaded in cartridges into a columnar or rod-like structure [131]. Several parameters such as dispensing force and pressure, extrusion rate, extrusion volume, and nozzle diameter can be adjusted to control the printing resolution [6]. However, using small nozzle diameters as well as highly viscous printing

materials can cause high shear stress that results in low cell viability retention, which is a limitation for high-resolution bioprinting. The viscosity of the bioink should be carefully balanced since a lower viscosity can result in structures with poor shape retention [13]. For example, bioprinted cell-laden strands with 5% GelMA concentration exhibited a lower elastic modulus (<1 kPa) and resulted in non-uniform structures compared to constructs printed with 7-15% polymer concentrations [132]. However, it is still challenging to fabricate a structure that does not collapse without supporting materials. Use of suspension baths or a supporting bath allows the bioink to be wrapped and held by the surrounding material to prevent gravity-induced deformation. The supporting reservoir can be a crosslinking solution, sacrificial ink, or soft granular gels. There are emerging trends in the development of granular gel baths due to their ability to support ECM-based printing, which is fragile and requires a longer gelation time, into highly complex large-scale structures (**Figure 2(b)**) [4,61,133–135]. Furthermore, core-shell bioprinting or coaxial deposition can be implemented with extrusion-based printing to design tubular structures with controllable diameters [61]. This approach allows instantaneous crosslinking during the extrusion process and simultaneous printing of core and shell structures applicable for fabrication of multi-layered vascular structures [5]. For example, SA-based hydrogels can either be extruded in a partially crosslinked form as a shell structure or extruded from an inner nozzle in the presence of a $CaCl_2$ solution flow sheath from the outer nozzle [136,137]. Co-axial extrusion-based 3D bioprinting also allows fabrication of self-standing tube constructs without support and have been reported in several publications [138–141].

To avoid direct contact with the printing material during the printing process, photopolymerization has been widely implemented with 3D printing platforms. This 3D printing approach can overcome the limitations of soft lithography molding for microchannel fabrication, which allows tubes and branched channels to be fabricated in the scale of 50-200 μm but results

in non-physiological rectangular channels [4,6,73]. A similar limitation was also found in the use of photodegradation or photoablation-based 3D printing, where subtractive fabrication of complex structures using focused laser beams to create channels mainly resulted in an oval or rectangular cross-section due to the difficulties in controlling the z-axis resolution on the light path [4,61]. 3D printing techniques such as digital light processing (DLP), stereolithography (SLA), and two-photon polymerization (2PP) allow printing of high spatial resolution structures via curing photo-sensitive synthetic polymers in the presence of a liquid-based supporting phase [61,142]. DLP-based 3D printing can pattern the photo-sensitive material using a digital micro-mirror device (DMD) in a layer-by-layer manner, allowing fast fabrication of 3D constructs with high spatial resolution up to micrometer scales [33,142,143]. On the other hand, SLA is carried out by using a laser beam to trace line profiles or projecting light in continuous small pixels on the specified layer to induce crosslinking of the printing material (**Figure 2(c)**) [6,61,142]. Compared to DLP and SLA, the 2PP fabrication technique provides a markedly higher spatial printing resolution but there are still some limitations on printing structure size, printing speed and 2PP-compatible bioinks [61]. Nevertheless, this type of 3D printing has been applied for patterning of RGD moieties for regulating ECs migration, organization, and micro-vessel growth in several studies [73]. Overall, 3D printing by photocrosslinking eliminates the mechanical stress generated from the extrusion presented in droplet-based and extrusion-based 3D printing strategies. However, there have been some reports that oxidative stress from light sensitive moieties and exposure to light sources can compromise cell viability and proliferation [5].

There has been recent emerging interest in the use of laser-assisted bioprinting (LAB) for fabrication of vascular constructs or vascular channels. The mechanism of this technology is based on projecting a pulsed laser beam on the donor slide on top of an energy-absorbing layer, while the resulting shockwave allows the bioink underneath to be deposited dropwise on the

collector slide [5]. The ability to print of printing highly viscous bioinks, availability of nozzle-free strategies, and high precision (5 - 10 μm resolution) are the attractive factors of this approach [5,57]. This method has been mostly used in the organization of capillary vascularization in a defined pattern [144–146]. For example, Koch *et al.* used LAB techniques to print ECs as a 2D pattern of a grid or lines on Matrigel® where capillaries with a lumenized structure of ~30 to 100 μm diameters were formed after 24 h (**Figure 2(d)**) [145]. Furthermore, *in situ* patterning of ECs within a mouse calvaria defect was shown to be possible using this printing technique [146]. LAB can also be used implementation to construct mouse fibroblast cell-laden 3D alginate tubular structures with a diameter of 5mm without any supporting structure by the drop-on-demand approach, as demonstrated by Xiong *et al.* in 2015. However, the post-printing cell viability inside Y-shaped tubes was only 68.1% immediately after printing and 70.8% after 24 h of incubation [147]. Better cell viability should be achieved for this fabrication technique to be further applicable to ECs printing and for ECs proliferation and vascular ECM secretion. Photonic cell damage, possible cell sedimentation, high cost of printing equipment, and limited scalability can explain the lack of demonstration of this method for implantable vascular constructs to date [5,57].

Nevertheless, there are still some challenges on fabrication of 3D printed tubular structures with complex architectures, including deformations or collapse without supporting materials. To overcome this limitation, combination of this 3D printing technique with sacrificial materials such as carbohydrate-glass lattice, PVA, gelatin, and Pluronic F127 allows fabrication of perfusable and stable channels [148–152]. The sacrificial materials should be easily removed by dissolving or temperature-dependent phase change. For example, Ryma *et al.* demonstrated the use of thermoresponsive poly(2-cyclopropyl-2-oxazoline) (PcycloPrOx) for melt-electrowriting of physiological-like branching structures within different types of hydrogels [85].

Most of the sacrificially fabricated vascular channels are seeded with ECs afterwards, where SMCs or fibroblasts can be incorporated in the bulk volume of surrounding materials to mimic the *in vivo* vascular microenvironment [1,153]. Compared to other types of 3D-printing, sacrificial materials are mostly combined with extrusion printing techniques (**Table 2**).

4.2. Electrospinning

Fabrication of vascular scaffolds by electrospinning polymer solutions results in native ECM-like fibrous and porous nanoenvironments which are essential for maturation of vascular tissues [5]. Dispensing polymer solutions through a strong electrical field allows nano-to microscale fibers to be drawn, where pore size, fiber diameter, and fiber alignment can be precisely controlled [4]. A wide range of synthetic and natural materials are applicable to this technique and can be dispensed together to form composites using several tailored strategies such as co-electrospinning, coaxial electrospinning, and sequential electrospinning [6,154]. Synthetic polymers provide higher mechanical durability to constructs, while electrospun fibers could be functionalized or grafted with different moieties such as peptides, GFs or drugs to increase bioactivity and provide therapeutic capabilities [5,6]. Moreover, coaxial electrospinning allows fabrication of tubular structures with thicker walls and with several layers [5,155–157]. In 2020, Yan *et al.* fabricated double layered tubular constructs with an inner and outer diameter of 4 mm and 6 mm by electrospinning of eggshell membrane (ESM) and thermoplastic polyurethane (TPU). ESM, which is a fibrous structure similar to ECM, was functionalized with dopamine and heparin, and enhanced the adhesion and proliferation rate of HUVECs [158]. Combining electrospinning constructs with hydrogels such as nanofiber/ hydrogel core/shell scaffold is also possible to provide a moisturized microenvironment and allow infiltration of perivascular cells [29,64]. However, this fabrication technique requires more time to fabricate

long vascular constructs and the resulting pore size and fiber density may impede cell infiltration inside the structure. Several modifications of electrospun scaffolds such as surface treatments and coupling with a laser cutting fabrication method can overcome the aforementioned issues regarding porosity [5,159]. Apart from solution electrospinning, there is another variation of electrospinning called melt electrowriting [64]. In this electrospinning approach, polymer melts were used instead of solutions, which improves the degree of precision on fiber deposition due to lower electrical conductivity and higher viscosity. However, the choice of material applicable to this technique is limited to synthetic thermoplastics [160]. It is also possible to combine electrospinning with other fabrication strategies such as sacrificial methods and melt electrowriting. It was demonstrated that dual electrospinning with sacrificial poly(ethylene oxide) (PEO) fibers and electrospraying of sacrificial PEO microparticles can increase cell infiltration within electrospun scaffolds [161,162]. Furthermore, combination of solution electrospinning with electrowriting makes it possible to adjust the electrospun scaffold mechanical properties to match native vessels from different organs. The recent publications regarding electrospinning vascular scaffolds from 2022 onwards mostly consider attempts to optimize endothelialization and *in vivo* model studies [163,164]. Nevertheless, the lumen diameter of tubular constructs fabricated by electrospinning in general are limited to larger (mm) scales. One possible direction for fabrication of multi-scale engineered vasculatures is to combine 3D printing with electrospinning techniques.

4.3. Decellularization of blood vessels from donors

Compared to other fabrication strategies, decellularization of blood vessels from different allogeneic or xenogeneic sources can provide a scaffold structure full of native biomolecules, GFs and topography readily for ECs cell seeding [46]. It is important to emphasize that the

obtained dECM will not be solubilized for this purpose, which is different to what was mentioned in section ***2.3. Decellularized Extracellular Matrix (dECM).*** The objective of decellularization is to prepare a scaffold with compatible shape for specific types of blood vessels including carotid artery, aorta artery, internal mammary artery, umbilical artery, saphenous vein, coronary artery, femoral vein, and the vena cava without immunological responses [165–174]. During decellularization, donor cells and DNA were removed from the ECM by a variety of techniques and then replaced by autologous ECs before transplantation [175]. Several publications have reported different decellularization protocols including use of ionic and non-ionic detergents, osmotic pressure gradients, enzymes, chelating agents, and dehydration [160]. The criteria of tissue decellularization is that the tissue samples must contain less than 50 ng dsDNA per mg of dry ECM and the residual DNA fragment lengths must be less than 200 base pairs. Moreover, DAPI or H&E staining of sample sections should not show any visible nuclei [160,176]. At the same time, the decellularization protocols should allow the preservation of native ECM characteristics such as composition, architecture, topography, and mechanical properties which contribute greatly to enhancing vascular cell proliferation and vessel maturation [6]. A widely used protocol of vascular decellularization by treatment with (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate) (CHAPS) buffer and sodium dodecyl sulfate (SDS) was established by Gui *et al.*, where decellularized umbilical arteries could support endothelialization and remained functional *in vivo* for up to 8 weeks [177]. Moreover, vascular cells could be grown on an engineered scaffold to form ECM available for implantation in patients, then decellularized to eliminate possible immune responses. For example, human SMCs were seeded on PGA scaffolds to produce vascular ECM before the structure was subjected to decellularization. This product is currently commercially available for treatment of chronic hemodialysis in end-stage renal disease [178]. Decellularized blood vessels

derived from different organs of different species are currently available for clinical use as summarized by Wang *et al.* [179]. However, the limitation of this scaffold fabrication technique is lack of sustainable resources, difficulties in establishing a reproducible protocol, batch-to-batch variations, and possible alterations of ECM characteristics [6]. Most of the resulting vessels from this approach are also limited to mm-scale inner diameters, where constructs with the smallest dimensions have inner diameters of around 1 mm [180,181]. Nevertheless, there are continuous developments in the areas of decellularized vascular grafts such as comparison of scaffolds from different origins and further clinical investigation of long-term endothelialization [182,183].

To conclude, 3D printing strategies especially extrusion-based and photo-crosslinking based techniques, enable fabrication of microvascular structures within the scale of 100-1,000 μm, that are crucial for establishing cell viability within large-sized (mm-scale) tissue constructs. Droplet-based 3D printing still faces limitations of long and complex fabrication procedures for larger vessel networks, and printing resolution that it is not very widely applied compared to the other two 3D printing techniques. Electrospinning and decellularization techniques are also widely used, but they are only available for fabrication of vascular scaffolds with an inner diameter of 1 mm or larger.

5. Conclusion and future perspectives

It is still challenging to fabricate vascular scaffolds using ECM- based hydrogels with optimal biological functions and mechanical properties at the same time, where the implanted engineered vascular constructs need to withstand the pressure from surrounding cells [9]. Several strategies such as incorporation of nano-spun fibers, porous scaffolds, and nanomaterials are

required for fabrication of load bearing, durable scaffolds. To optimize endothelialization and vessel maturation of vascular grafts, precise control of the topography and surface chemistry are essential, especially to the remodeling process by cells after implantation. Then, mechanical properties of both macro and micro scale including compressive modulus, elasticity, shear stress, and stiffness should be thoroughly examined to ensure physiological endothelial cell biomechanics and stability of the overall structure. Sometimes, bioactivity of the engineered vascular scaffolds of synthetic and natural origins is insufficient and immobilization of GFs is required for neovascularization induction. To establish a mm-size engineered tissue without necrosis within the structure, the scale of the vascular structure should be in the range of 100 - 1,000 μm, which has thus far been mostly achieved by extrusion-based 3D printing and 3D printing by photocrosslinking. As the native small vasculature branches into smaller capillaries, fabrication strategies of multi-scale vasculature are also necessary [184]. Within the engineered blood vessel, the luminal structure can be achieved using fabrication strategies such as co-axial sacrificial 3D printing. Furthermore, important blood vessel characteristics such as molecular size exclusion properties, contribution to surrounding cell viability, and hemocompatibility should be confirmed to assess the functionality of the resulted engineered vasculature.

Several studies regarding fabrication of engineered blood vessels are still focused on demonstrating the advantages of the newly developed techniques and *in vivo* implantation to confirm biocompatibility and tissue integration but lack thorough characterization of the functionality. Moreover, most of the fabricated vascular systems co-cultured with organ-specific cells *in vitro* require complicated perfusion systems with high maintenance costs to support nutrient distribution within the construct. Development of 3D-printed artificial blood vessel fibers that can perform the functional responsibilities of native blood vessels is a promising concept to overcome the addressed limitations of perfusion systems. For example, the

supplementation of oxygen, nutrients, as well as GFs of blood vessel-mimic constructs as well as their tissue remodeling capabilities and scaffold degradation profiles should be carefully monitored for more than 2 weeks to ensure their applicability as biomedical models or implants [185–187].

To date, most of the tissue-engineered grafts are still under research or in clinical trials and have not been commercialized yet, resulting in a lack of coverage of characterization standards for cell-based products in the ISO 7198:2016 regulations regarding vascular prostheses [188]. Because of that, most of the commercialized products are synthetic grafts and decellularized blood vessels, which are mostly limited to constructs with larger diameters [13,179]. Further establishment of standards for cell-based graft products from the production process to clinical or industrial applications is still a work in progress.

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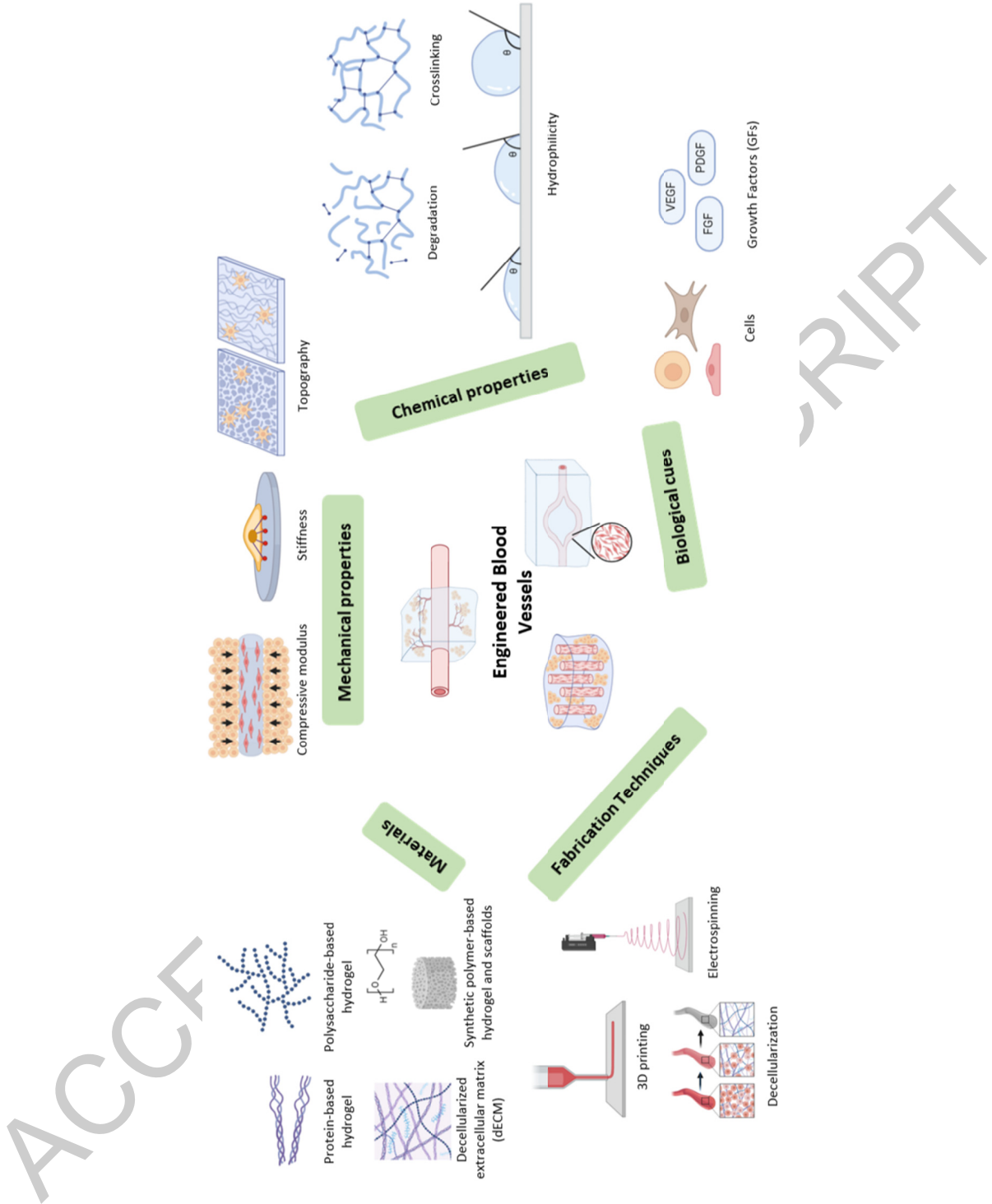


Scheme 1 Different areas of considerations for biofabrication of engineered blood vessels (Created with BioRender.com)

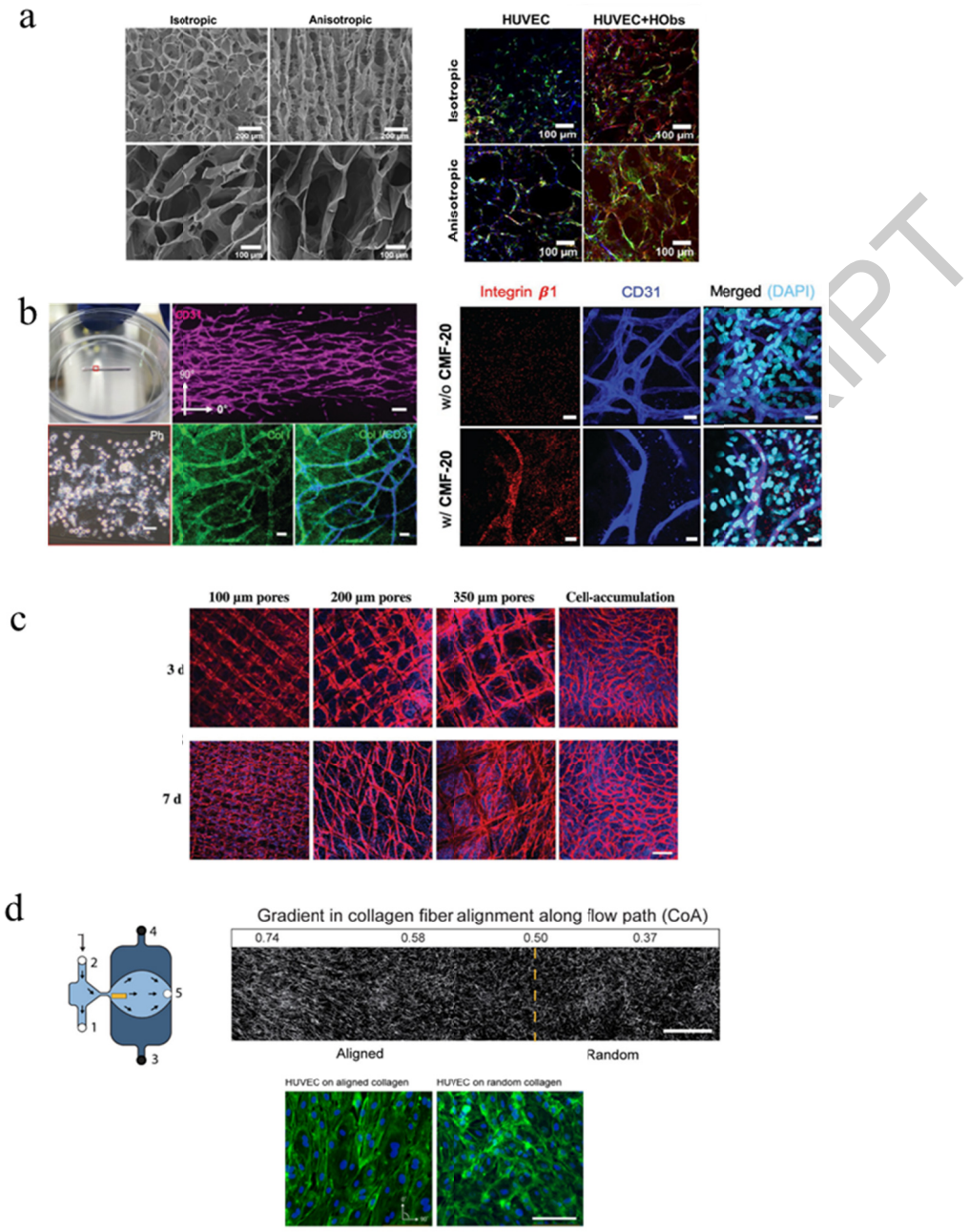


Figure 1 Effect of scaffold surface topologies on ECs cellular orientation (a) SEM images showing cross-sections of collagen scaffolds with different pore orientations at lower and higher magnifications (left), fluorescence images of HUVECs, both in mono- and co-culture with osteoblasts (HObs), seeded on porous collagen scaffold with different pore architectures. endothelial cell marker CD31 is shown in green, actin in red and cell nuclei in blue (right),

reprinted with permission from ref. [79]. Copyright 2019, Elsevier (b) Observation of 3D fabricated tissues presented alignment of capillary networks and orientation of ECs to the same direction with CMFs, scale bars are 200 μm in Ph and top right fluorescence image, 50 μm in bottom right fluorescence images (left), immunostaining images of integrin β1 suggested that there was an interaction of ECs to CMFs during microvascular formation, scale bars are 20 μm (right), reprinted with permission from ref. [16]. Copyright 2020, John Wiley and Sons (c) Fibronectin-coated PCL scaffold fibers with different pore sizes serves as orientation guide for ECs at the beginning of cell culture, while non-oriented microvascular formation to fill in pore volume can be observed at day 7, scale bars are 200 μm, reprinted with permission from ref. [83]. Copyright 2018, John Wiley and Sons (d) Anisotropy of collagen fibers were tuned by a microfluidic platform, which affect actin filament alignment of HUVECs, scale bars are 100 μm, reprinted with permission from ref. [84]. Copyright 2021, John Wiley and Sons.

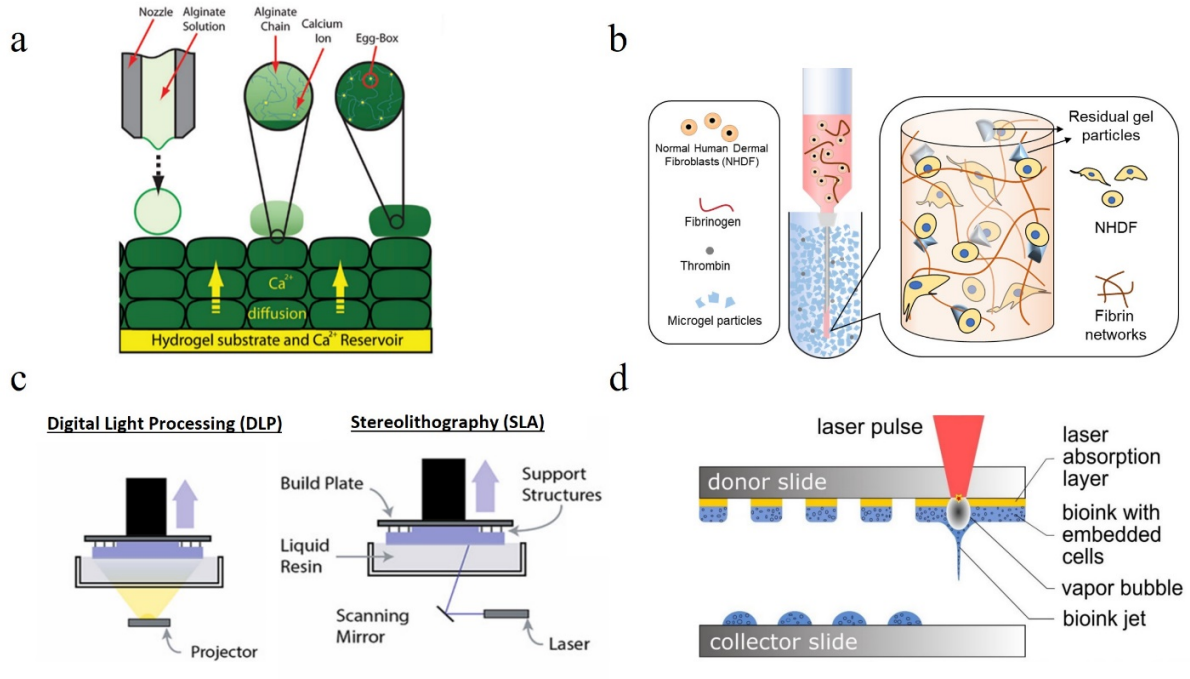


Figure 2 Schematics of 3D printing techniques for fabrication of engineered blood vessels (a) Droplet-based printing of alginate ink on a gelatin hydrogel substrate loaded with $CaCl_2$, reprinted with permission from ref. [129]. Copyright 2011, John Wiley and Sons (b) Cell-embedded extrusion printing of fibrinogen in granular gel bath loaded thrombin solution to form fibrin hydrogel, reprinted with permission from ref. [135]. Copyright 2023, John Wiley and Sons (c) Working mechanism of DLP- based and SLA- based printing, adapted with permission form ref. [142] (d) Schematic representation of LAB technique, which was used for patterning of ECs, reprinted with permission from ref. [145].

Table 1 Different materials used for engineered blood vessel fabrication.

Category	Examples	Advantages	Disadvantages	Possible modifications	References
Protein	Collagen	Can control ECs alignment, can bind to GFs, facilitate tissue remodeling, native-like stiffness and porosity, biocompatible, gelation at 37 °C	Difficult to control degradation, weak mechanical properties (E < 200 Pa), slow gelation, non-reversible polymerization at 25 °C	DNA interlocking, plastic compression, chemical modifications, incorporation of polysaccharides or synthetic polymers	[4,8,12,16,17, 116,189,190]
	Elastin	Intrinsic elasticity and resilience, provide cell binding motifs and cell signaling pathways	Difficult to control degradation, weak mechanical properties, coacervation can occur, non-homogeneous cell penetration	Methacrylation, incorporation of GelMA, incorporation of PCL, using high pressure CO_2	[22,23, 191,192]
	Fibrinogen	Assist cell attachment and growth, can be autologously isolated, biocompatible, completely biodegradable, enzyme-catalyzed gelation, biodegradation can be controlled, angiogenetic, can bind to GFs	Rapid degradation, weak mechanical properties, gel shrinkage	Control of crosslinking by Ca^{2+} ions, usage of degradation inhibitors, chemical modifications, incorporation of polysaccharides or synthetic polymers	[193–200]
	Laminin	Encourages iPSCs endothelial differentiation, induce ECs adhesion and tubule formation, provides cell signaling cues, present in native vessel BM	There is no report of using laminin as a main component of vascular scaffolds up to date	Used for increasing cell adhesion and signaling in other materials	[15, 201–203]
	Gelatin	Lower antigenicity than collagen, reversible temperature dependent gelation, can be used as sacrificial material	Cannot form gels at physiological temperature (37 °C)	Methacrylation, incorporation of polysaccharides or synthetic polymers	[4,12,21, 148]
	Fibronectin	Provides several cell-binding peptide sequences, biodegradable, can control ECs alignment, angiogenetic, promote deposition of ECM	There is no report of using fibronectin as a main component of vascular scaffolds up to date	Used for increasing cell adhesion and signaling in other materials	[204–207]

Polysaccharides	Animal-based	Hyaluronic acid	Interact with cell-surface receptors, biocompatible and biodegradable, anticoagulation and endothelialization ability, angiogenetic in oligosaccharide form	Lack of cell adhesion sites and does not facilitate tissue remodeling	Modification with cell-adhesion and MMP-sensitive peptides, chemical modification, double crosslinking, incorporation with other polysaccharides, protein components, and/or synthetic polymers	[24–28, 53, 69, 208–210]
		Heparin	Can bind to various GFs, biocompatible and biodegradable, hemocompatible, is an anticoagulant, anti-inflammatory, angiogenic regulatory	Mostly need incorporation of other materials to fabricate vascular scaffolds	Incorporation with self-assembling peptides, widely used for functionalization on other materials	[12,29,30, 211,212]
	Derived from Algae, crustacean, fungi, and plants	Sodium Alginate	Sustainable source, biocompatible, forms hydrogel by physical crosslinking (chelation) with divalent ions, can be used as sacrificial material	Bioinert, only exhibits partial degradation	Oxidation with sodium periodate, double crosslinking design, modification with cell-adhesion peptides and MMP-sensitive peptides, incorporation with other polysaccharides, protein components, and/or synthetic polymers	[4,8,9, 31–34, 213–216]
		Chitosan	Sustainable source, biocompatible. biodegradable, nontoxic, nonimmunogenic, can bind growth factors in sulfated form, antibacterial	Insolubility at physiological pH, low angiogenic potential, possess coagulation promoting abilities	Chemical modification, incorporation with other polysaccharides, protein components, and/or synthetic polymers	[4,35–37, 217–220]
		Gellan Gum	Sustainable source, biocompatible, thermoreversible gelation, shear-thinning properties, forms hydrogel by physical crosslinking with divalent ions, can be used as sacrificial material	Bioinert, low degradation rate	Oxidation, methacrylation, functionalizaion with proteins, incorporation with other polysaccharides, protein components, and/or synthetic polymers	[24,38–40, 221,222]
	dECM		Allow fabrication of autologous material, low antigenecity and immunogenecity	Poor mechanical properties, limited resources, batch viability, slow gelation time	Crosslinking, incorporation of polysaccharides or synthetic polymers	[45,51–53]

Synthetic polymers	PEGDA, PEGTA	Applicable for photocrosslinking-related fabrication techniques, does not compromise cell viability, can be crosslinked rapidly	Bioinert, resist protein adsorption and nonspecific cell adhesion, low degradation rate	Immobilization with ECM proteins, incorporation with protein components, polysaccharides, and/or other synthetic polymers	[57,202,208, 223,224]
	Pluronic®	Gelation can be controlled by temperature, unique micellar-packing gelation allows great printability, can be used as sacrificial material	Bioinert, poor mechanical strength, low liquid transition temperature limits its application	Modification with photocrosslinkable acrylate groups, modification into monocarboxylate form, incorporation with protein components, polysaccharides, and/or other synthetic polymers	[5,57, 225–227]
	PNAGA	Stable swelling characteristics, high mechanical strength, biocompatible, unique strong physical crosslinking	High softening temperature at high concentrations limits its application, bioinert, lack of long-term studies on vascular engineering	Incorporation with GelMA and nanoclay, copolymerization with other monomers	[58–60,191]
	PGA	Biodegradable, high elasticity, can promote neovascularization, high mechanical strength, already in clinical trial	Bioinert, produces late inflammatory reaction, leave residual fragments, relatively fast degradation (around 6-8 weeks)	Incorporation with protein components, polysaccharides, and/or other synthetic polymers	[18,63, 165,204, 228–230]
	PLA, PLLA	Biodegradable, decomposes into lactic acid, non-immunogenic, high mechanical strength, already in clinical trial	Bioinert, slow biodegradation (2 months), hydrophobic structure, poor cell affinity	Functionalization with heparin, incorporation with protein components, polysaccharides, and/or other synthetic polymers	[62,165, 229,231]
	PCL	Degradable, less acidic decomposition products, high mechanical strength, already in clinical trial	Bioinert, slow biodegradation (more than 2 years), challenges on long-term patency	Immobilization with ECM proteins, incorporation with protein components, polysaccharides, and/or other synthetic polymers	[62,83,156, 163,164, 229,232]

BM, Basement membrane; dECM, Decellularized extracellular matrix; E, Young's modulus; ECs, Endothelial cells; ECM, Extracellular matrix; GelMA, Gelatin methacrylate; GFs, Growth factors; iPSCs, induced pluripotent stem cells, MMP, Matrix metalloproteinase, PCL, polycaprolactone; PEGDA, poly(ethylene glycol) diacrylate; PEGTA, poly(ethylene glycol)-tetra-acrylate; PGA, polyglycolic acid; PLA, polylactide; PLGA, poly(lactide-co-glycolide); PLLA, poly-L-lactic acid; PNAGA, poly(*N*-acryloyl glycinamide).

Comparison of materials used in fabrication of engineered blood vessels from each category, advantages, disadvantages, and their possible modifications.

Table 2 3D printing techniques and their applications in several target tissues.

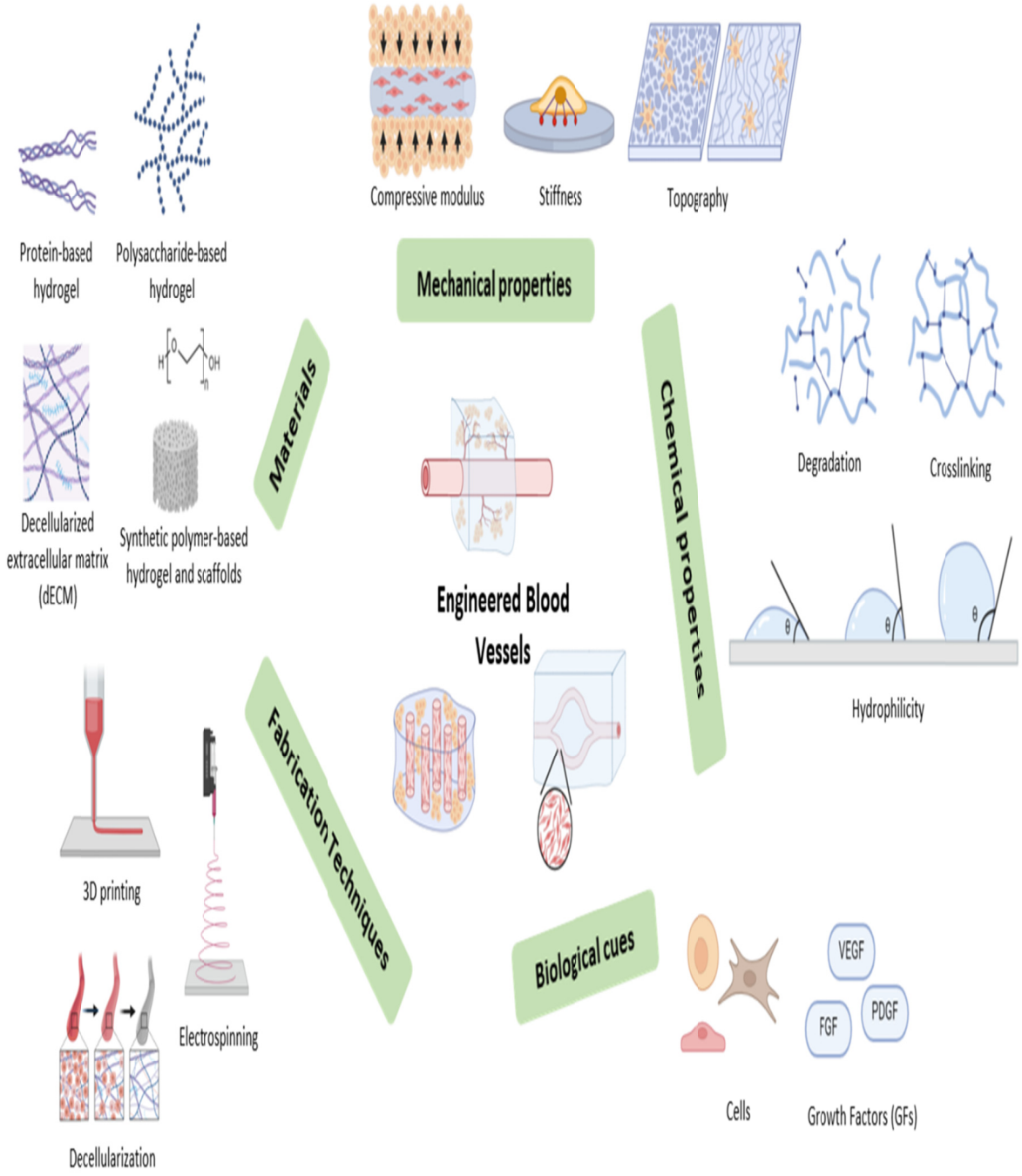
Methods		Ink material/ Main material	Sacrificial material	Cell type	Blood vessel diameter	Crosslinking	Target tissue/ applications	References
Inkjet 3D printing	Piezo-electronic inkjet printing	Fibronectin and gelatin solution	None	Human HCC cell line (HepG2) and HUVECs	~500 µm	Spontaneous drying	Human liver tissue chips	Matsusaki *et al.* [126]
	Drop-on-demand bioprinting	Fibrinogen	Gelatin	SMCs and HUVECs	1000 µm	Enzymatic crosslinking, ionic crosslinking	Biofunctional multilayered vessel model	Schöneberg *et al.* [1]
Extrusion-based 3D printing		Human omental tissue-derived collagen	Gelatin	iPSC- derived CMs and iPSC-derived ECs	300 µm	Incubation at 37 °C	Vascularized cardiac patch	Noor *et al.* [153]
		Acidified collagen ink	None	None	From ~5 mm down to ~100 µm	pH-driven gelation	Multiscale vasculatures of adult human left ventricle	Lee *et al.* [233]
		Gelatin and fibrin	Pluronic and PEO	None	410 µm	Enzymatic crosslinking	Vascularized proximal tubule (kidney-on-chip) model	Lin *et al.* [152]
		HMW PCL in HFIP solution (by coating and solvent evaporation)	3D- printed melted sucrose template and salt leaching	None	500 µm	Polymer evaporation	Epicardial implantation	Lei *et al.* [234]

Laser assisted 3D printing		Cells in culture media	None	HUVECs	N/A	None	*in situ* endothelial cells organization in a mouse calvaria bone defect	Kérourédan *et al.* [146]
3D printing by photopolymerization	Projection stereolithography	PEGDA (6 kDa) and GelMA	None	None	~1000 µm	photopolymerization	Rodent model of chronic liver injury	Grigoryan *et al.* [235]
		PEGDA (6 kDa)	None	None	350 µm	photopolymerization	Alveolar model topology	
		PEGDA (6 kDa) and GelMA	None	Lung fibroblast (IMR-90)	400 µm	photopolymerization	Cellular alveolar model	
	Laser direct writing stereolithography	GelMA	Microneedle-based subtractive technique was performed to obtain a lumen structure	None	500 µm	photopolymerization	Vascularized tissue model for investigating breast cancer metastasis to bone	Cui *et al.* [236]
	Two-photon laser scanning photo-polymerization	PEGDA (700 Da), PETA	None	None	50 µm	photopolymerization	Long term perfusion of cerebral organoids and liver tissue constructs	Grebenyuk *et al.* [237]

CMs, Cardiomyocytes; ECs, Endothelial cells; GelMA, Methacrylated gelatin; HCC, Hepatocellular carcinoma; HFIP, Hexafluoroisopropanol; HMW, High molecular weight; HUVECs, Human umbilical vein endothelial cells; iPSC, induced pluripotent stem cells; PCL, Polycaprolactone; PEGDA, poly(ethylene glycol) diacrylate; PEO, poly(ethylene oxide); PETA, Pentaerythritol triacrylate; SMCs, Smooth muscle cells.

Comparison of ink material, printing diameter, and crosslinking mechanisms of different 3D printing techniques that have been utilized in fabrication of engineered blood vessel for applications in specific types of tissue.

[[Graphical Abstract]]



Impact statement: This review covers several aspects and advancements of engineered blood vessel biofabrication, which are essential for establishment of large-sized tissues in different areas of biomedical applications.