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REVIEW

Ink-structing the future of vascular tissue engineering: a review of the physiological bioink design

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Abstract

Three-dimensional (3D) printing and bioprinting have come into view for a plannable and standardizable generation of implantable tissue-engineered constructs that can substitute native tissues and organs. These tissue-engineered structures are intended to integrate with the patient's body. Vascular tissue engineering (TE) is relevant in TE because it supports the sustained oxygenization and nutrition of all tissue-engineered constructs. Bioinks have a specific role, representing the necessary medium for printability and vascular cell growth. This review aims to understand the requirements for the design of vascular bioinks. First, an in-depth analysis of vascular cell interaction with their native environment must be gained. A physiological bioink suitable for a tissue-engineered vascular graft (TEVG) must not only ensure good printability but also induce cells to behave like in a native vascular vessel, including self-regenerative and growth functions. This review describes the general structure of vascular walls with wall-specific cell and extracellular matrix (ECM) components and biomechanical properties and functions. Furthermore, the physiological role of vascular ECM components for their interaction with vascular cells and the mode of interaction is introduced. Diverse currently available or imaginable bioinks are described from physiological matrix proteins to nonphysiologically occurring but natural chemical compounds useful for vascular bioprinting. The physiological performance of these bioinks is evaluated with regard to biomechanical properties postprinting, with a view to current animal studies of 3D printed vascular structures. Finally, the main challenges for further bioink development, suitable bioink components to create a self-assembly bioink concept, and future bioprinting strategies are outlined. These concepts are discussed in terms of their suitability to be part of a TEVG with a high potential for later clinical use.

Keywords Vascular wall histology · Vascular cells · Microenvironment · Extracellular matrix · Cell–matrix interaction · Bioink · Printability

Abbreviations

AFs	Adventitial fibroblasts
bFGF	Basic fibroblast growth factor
CAD	Computer-aided design
DDR	Discoidin domain receptor
ECM	Extracellular matrix
ECs	Endothelial cells
dECM	Decellularized ECM
F-actin	Filamentous actin
GAGs	Glycosaminoglycans
GIA	Amino acid sequence GPQGIAGQ
HA	Hyaluronic acid
HUVECs	Human umbilical vascular endothelial cells
PCL	Polycaprolactone
PEGDA	Polyethylene glycol diacrylate
PDGF	Platelet-derived growth factor
RGD	Arginine–glycine–aspartic acid
SF	Silk fibroin
SM	Smooth muscle
TE	Tissue engineering
TEVG	Tissue-engineered vascular graft
TAZ	Transcriptional coactivator
YAP	Yes-associated protein
VEGF	Vascular endothelial growth factor
VSMCs	Vascular smooth muscle cells

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Introduction

Additive manufacturing with three-dimensional (3D) printing and bioprinting methods of cell suspensions is very attractive for future tissue-engineered product development. These methods mimic the complexity of native tissues and organs. Therefore, additive manufacturing has been recognized as one of the most promising biofabrication techniques in the past decade. Bioprinting inherently requires a bioink, a cell-laden hydrogel composition suitable for the automated homogenous distribution of cells with a bioprinter. Before printing, a computer-aided design (CAD) model is created. The term "bioink" is derived from a publication of Mironov, who first described in 2003 the possibilities of rapid prototyping in tissue engineering (TE). He created the term "inkjet printing" in mind, a method altered and adapted for bioprinting [1]. The precise automated spatial positioning of cells allows much more complex structures and much higher reproducibility than the manual seeding of cells onto a scaffold. Bioinks are the necessary medium for living cells during printing and can be functionalized with diverse supporting molecules that enhance complex tissue generation. These bioinks must also provide a suitable microenvironment to lift the cell activity and support the cell secretion of extracellular matrix (ECM).

The most feasible efforts to create artificial tissues by 3D printing for TE can be seen in the sections of skin generation [2]. Skin constructs are created as multilayered tissue constructs with keratinocytes and fibroblasts, and such skin patches are supposed to be used as a cover for a wound (e.g., in patients with severe burns) [3]. An interesting application for form-stable 3D printing is represented by creating a 3D bioprinted human ear [4] as a very individual and highly complex structure for plastic surgery in injured patients. Bone healing can be supported using a polymeric hydrogel with incorporated growth factors. Such hydrogels are currently prepared for 3D in situ bioprinting in bone defects [5]. In all these applications, clinical transferability is near. In that context, vascular TE is considered a very relevant sector in TE, as all tissues require sustainable oxygenation and perfusion with nutrients that can only be provided by a functioning vasculature. Therefore, this review especially deals with suitable bioinks for 3D printing of scaffolds coated with cell-laden bioinks or for direct bioprinting of form-stable vascular structures as a new method in the field. Vascular bioinks must meet specific criteria to develop into sustainable vascular tissues.

To develop a deeper understanding of vascular tissue, the specific structure of the vascular wall will be outlined, specific cell types will be described, and vascular ECM and cell–matrix interactions will be elucidated in detail. Then, the relevant characteristics of printable bioinks for vascular TE will be described, and the spectra of diverse bioinks available will be sorted by their suitability as a vehicle for diverse vascular cells in bioprinting. Later, challenges for vascular substitutes developed by bioprinting in TE will be discussed.

The design of materials and compositions for bioinks and bioprinting techniques for a tissue-engineered vascular graft (TEVG) has expanded greatly in the past five years. Most reviews on this topic have focused on the requirements of a bioink to ensure good printability while maintaining cell viability. This review also aims to define the optimal physiological properties that support printed vascular cells in growth and intercalation. Furthermore, mechanical properties give the printed bioink cell suspension a sustainable form stability postprinting. Generally, in TEVG design, there are two possibilities: a TEVG can consist of a scaffold with a hydrogel cell suspension laden on top, and there could be a scaffold-free TEVG, where the bioink itself is so stable that a sustainable vessel remains. This review deals with these different advanced 3D printing and bioprinting strategies and bioink compositions and checks whether they are useful for tissue-engineered blood vessels. This review aims to identify ECM cues that can and must be mimicked by a bioink for vascular cells to behave like in native tissue.

General structure of the vascular wall and the role of ECM

The structure of arterial vessels comprises three principal layers: tunica intima, media, and adventitia, as illustrated in Fig. 1. In contrast, venous vessels consist of only two layers, tunica intima and adventitia, and do not possess a contractile media. This absence is because venous vessels do not bear the arterial blood pressure and do not serve as resistance vessels. Instead, they function as capacity vessels, redirecting blood flow to lung circulation for oxygenation.

Tunica intima

Tunica intima represents the inner luminal surface of a vessel. It consists of a monolayer of endothelial cells (ECs) on a basement membrane, and ECs are in direct contact with blood. Thus, tunica intima plays an important role in the counterplay between blood cells and the interstitial room behind the vascular wall as a barrier. It reacts to the near-wall shear stress induced by blood flow, and ECs provide diverse signal cascades for regulating the vasomotor tone [6]. A functional monolayer of ECs is required to resist thrombosis due to platelet accumulation. With age, tunica intima can become thicker and multilayered. This neointima formation can lead to vascular occlusion as the basis for many cardiovascular events [7].

Tunica media

Tunica media is structured by ECM proteins such as elastin and collagen. Vascular smooth muscle (SM) cells (VSMCs) are located in tunica media. VSMCs are crucial for the resistance of an arterial vessel against the relatively high blood pressure compared to veins. In that function, VSMCs react to contractile stimuli, playing a role in vasoreactivity. A contraction of VSMCs with their contractile cytoskeleton is provoked by a sympathetic nervous impulse or local or systemic contractile mediators. VSMCs can change their phenotype in response to local microenvironmental signals. The contractile phenotype is characterized by myosin heavy chains or calponin [8], but VSMCs can also switch to a secretory phenotype upon vascular injury.

Between the medial layer and the tunica intima and adventitia in arteries, concentric elastin lamellae can be found, which lessen cell migration between the layers.

Tunica adventitia

The outermost tunica adventitia is a collagen-rich protective, supportive layer of adventitial fibroblasts (AFs). Tunica adventitia is more rigid than the other layer and gives vessels an external support. Further, it anchors blood vessels to adjacent tissues. In bigger vessels with a wall strength of more than 1 mm, such as the aorta, this region also contains vasa vasorum. Without those, the comparatively thick aortal wall would get necrotic due to a barrier problem for oxygen and nutrients. This principle is important when developing a TEVG with bigger dimensions by TE, and one would have to provide tiny little vasa vasorum inside the printed wall to sustain the structure alive [9].

Specific functions of vascular cells in the vascular wall

Different phenotypes of VSMCs

As outlined before, VSMCs play a mediating role in vasoreactivity. They appear in a quiescent or differentiated contractile phenotype. The differentiation process can be detected by early markers (e.g., SM α-actin, SM22α, indicating the quiescent type), intermediate markers (h-caldesmon and calponin, playing a role for contractility), or late markers (SM myosins and smoothelin, reflecting high contractile power). Furthermore, the phenotypic spectrum contains proliferating VSMCs referred to as synthetic [10]. When VSMCs are used in a bioprinting approach, VSMCs should show a synthetic phenotype with synthetic organelles for the recreation of ECM before they switch to a contractile phenotype and build myofilaments to yield a functional tunica media. VSMCs seeded densely with a quick reach of confluency quickly return to their contractile spindle shape, but sparsely seeded VSMCs may permanently appear in synthetic type [11].

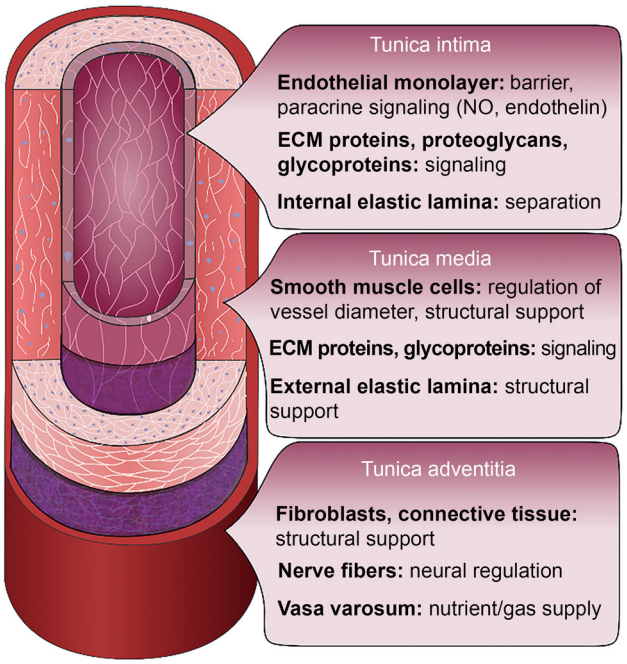

Fig. 1 Arterial wall structure. Structure of an arterial vascular wall with its three components (tunica intima, media, and adventitia) and their specific main functions

Vascular cell cross talk and remodeling

All vascular cells mentioned so far (i.e., ECs, VSMCs, and AFs) can produce matrix proteins that impact not only their function but also the cross talk with neighboring cells and a phenomenon of vessels called "remodeling" [12]. The cross talk between cells requires the paracrine production of bioactive molecules elicited directly to neighboring cells through cell–cell junctions or transported in extracellular vesicles (EVs) or adjacent to ECM [12]. An example of a direct cell–cell junction molecule is N-cadherin, expressed in layers of ECs and VSMCs beneath the internal elastic membrane [13]. Another is given by connexins, which are transmembrane gap junction proteins enabling the exchange of small molecules [10], and posttranslational modifications of connexins promote vasculoreactivity [14].

Remodeling is physiological as a completing part of natural vasculogenesis [15], but it is also part of a pathological cascade induced by stress factors such as vascular injuries or microinflammation. Furthermore, pericytes play an essential role in remodeling, as they can boost ECM production. These cells occur in the end route of the vascular system—the microvasculature, including terminal arterioles, precapillary

Table 1 Selection of intercellular cross talk inside the vascular wall under physiological conditions

Cell type	Type of interaction	Nature of interaction	Effect of interaction	Reference
EC-VSMCs	Contact-dependent signaling	Myoendothelial gap junctions (connexins)	Feedback pathways to control constriction–relaxation, phenotype switch, and inflammation	[163–165]
		Notch signaling	Capillary organization, VSMC phenotype switch, and EC monolayer integrity	[166]
		Ephrin-B2	Adhesion, motility, and vascular remodeling	[167]
	Paracrine signaling	Angiopoietin-1	Regulating vascular tone and permeability, endothelial sprouting	[168]
		Angiopoietin-2	Destabilization of quiescent endothelium	[169]
		Transforming growth factor β (TGF-β)	Vessel diameter, proliferation, angiogenesis, and VSMC phenotype	[170]
		Nitric oxide, prostacyclin	Vasorelaxation	[171]
		Heparan sulfate proteoglycans	Inhibition of VSMC proliferation upon the mechanical strain of vessel	[172]
		PDGF	VSMC recruitment, proliferation, and migration	[173]
	Parenchyma players (interaction via ECM)	Secretion of collagen type I, fibronectin	Inducing synthetic VSMC phenotype	[174]
		Secretion of collagen type IV	Inducing contractile VSMC phenotype	[174, 175]
Macrophages-EC	Paracrine signaling	PDGF	Regulating vascular inflammation and remodeling, transendothelial immunocyte migration	[176, 177]
Platelets-EC	Contact-dependent signaling	Von Willebrandt factor	Regulating binding to ECs for clot formation and vessel repair	[12, 178]
	Paracrine signaling	Inflammatory cytokines, e.g., IL-1 β	Inflammatory reaction	[178]
		Angiogenic factors: VEGF, PDGF, bFGF	Contributing to angiogenesis	[178]
		miRNAs	Regulation of synthesis of angiogenic factors and endothelial proteins	[178]
		CXCL12	Progenitor cell migration to vascular lesions, adherence	[178]
Pericytes-EC	Contact-dependent signaling	Notch	Pericytes survival and apoptosis, EC proliferation, and differentiation of arteries and veins	[179, 180]
	Paracrine signaling	TGF-β-pathway	Integrity and homeostasis of microvasculature	[181]

Table 1 (continued)

Cell type	Type of interaction	Nature of interaction	Effect of interaction	Reference
		Angiopoietin-Tie2	Injury response, pericyte migration, and endothelial sprouting	[182,183]

Different interactions of specific vascular cell types and their effects are summarized

venules, and capillaries. Here, pericytes are a source of regeneration. They can differentiate into vascular cells such as VSMCs and AFs. Pericytes accumulate on ECs and communicate with those cells by paracrine signaling. Pericytes induce increased EC proliferation and maturation, promoting angiogenesis. They contribute to vasoreactivity as they possess contractile elements. The following events are induced by pericytes: triggering of immunological defense such as the invasion of leukocytes, or triggering of cell debris removal by phagocytosis [16]. A deeper insight into the vascular cell cross talk and its impact on remodeling is given in Table 1.

Angiogenesis and sprouting

Vessel sprouting is a complicated process in which ECs are heavily involved [17]. Unmatured endothelial progenitor cells play a role. These cells are initiated by angiogenic stimuli and extend filopodia out of their primary vessel before cell migration out of the parent vessel occurs, whereas contact with neighboring cells is still kept. The initiation cascade is often provoked by a state of hypoxia and the hypoxia-inducible factor (HIF) responsible for increased synthesis, e.g., vascular endothelial growth factor (VEGF). Shear stress resulting out of blood flow can also be involved, inducing mechanical stimulation [18]. Those sprouting ECs are called "tip cells" as they migrate to the tip of the new vascular shoot. The migration move is paralleled by certain biological signals, such as Notch [19], that lead cells into a cell arrest. The arrested tip cell guides the new sprout according to the angiogenic gradient, followed by proliferating cells. The sprout forms a lumen, which is connected to the lumen of the parent vessel. As a result, a new vessel network arises [17].

In addition to ECs, SM cells (SMCs) contribute to new vessel formation. As SMCs react to blood flow, hemodynamic forces trigger short-term vasodilation (involving vasodilators such as nitric oxide (NO)) and vascular remodeling. The latter can be mediated by fluid shear stress that can lead to microinflammation with the induction of chemokines and adhesion molecules. Microinflammation also involves the entry of monocytes that transmigrate through the endothelium and mature into secreting macrophages. These macrophages provide a microenvironment consisting of factors such as transforming growth factor-β, tumor necrosis factor-α, epidermal growth factor (EGF), or fibroblast growth factor (FGF), which also occur in scarring. These growth factors have a strong impact on SMCs. SMCs get into a state characterized by increased cell proliferation and invasion into tunica intima and increased matrix production. The result is a pathogenic wall status of the vessel called "neointima," with a substantial narrowing of the arterial diameter [20]. This is a relevant pathological element of high blood pressure. It is assumed that these events can also be triggered by fatty acids or chronically elevated blood glucose, as it is often observed in the "metabolic syndrome" of patients with obesity and/or diabetes. Not all of these signal cascades have been fully explored [21] but they can provoke an SMC phenotypic switching and dedifferentiation into a proliferative, migratory, or synthetic phenotype [22].

The sprouting of new vessels can also be induced by fibronectin-1, synthesized by neural crest cells. This occurs within early embryogenesis, where neural crest cells arise from stem cells in a period when the neural tube closes. The fibronectin-1 effect is mediated by integrin $\alpha_5\beta_1$ adhesion receptors. Neural crest cells can also differentiate into VSMCs [23], and this signal cascade can surely be mimicked in future SMC differentiation scenarios for TE [10].

Vascular ECM

Components of vascular ECM

Vascular ECM, as a specialized microenvironment, provides many structural proteins such as collagens. There are 28 collagen types that occur tissue-specifically. For vessels, collagen type I is the most frequent compared to collagen types III, VI, VIII (the latter one predominantly in tunica media), XIII, and XXI (the latter one secreted by VSMCs). Other structural proteins are elastin, diverse laminins, lecticans, fibronectin, diverse fibrillins, and unbranched, typically anionic polysaccharide chains known as glycosaminoglycans (GAGs) covalently attached to protein cores, namely proteoglycans [9]. More than 100 different proteins have been identified in ECM of the human aorta [24]. One discriminates between instructive and canonical proteins, as they were once defined by Bornstein [25]. Whereas canonical proteins of ECM just represent connective tissue elements and

have a mechanical relevance, instructive proteins can interact with cells and provoke new matrix proteins such as collagen and elastin, often observed in tumor tissues [26]. One of these "matricellular proteins" (a term introduced by Bornstein) can also regulate the coagulation cascade by induction of fibrinogen and later fibrin, such as thrombospondin, and platelet activation. Tenascin-C can dock to integrins of the cell membrane on the one hand and proteoglycans and fibronectin of ECM on the other. In this way, it regulates the interaction between the cells and the matrix fibers of ECM [27]. In summary, the regulatory effects of matricellular proteins may be useful in vascular engineering strategies.

Differentiation and function of vascular ECM

The formation of a vascular functional ECM results from the secretory function of all contributing vascular cells, i.e., ECs, VSMCs, and AFs [9, 13]. This means that all these cell types are capable of producing ECM. For a matured artery, the VSMC-derived matrix has the greatest relevance. This media matrix has a contractile character and is responsible for avoiding vascular injury induced by high-pressure peaks [9, 28]. As mentioned earlier, the major protein in vascular ECM that impacts elasticity is elastin [9]. The elastin precursor peptide tropoelastin can be secreted by VSMCs, ECs, and AFs. Elastic fibers play a key role in helping arteries reflect the pulse wave, thereby driving blood flow to spare the heart pump and conserve energy. Thus, elastin represents an important component for constructing TEVGs [29].

Mediation of vascular stiffness and cell proliferation stimuli exerted by ECM

The stiffness of the common carotid artery in normal adults typically exhibits an elastic modulus of about 1.5 MPa [30, 31]. The elasticity or stiffness of the scaffold tissue is important for the vascular cell spreading and fiber secretion function of vascular cells such as fibroblasts and ECs. This is because it determines a certain grade of mechanical stimulation. The signal cascade behind that phenomenon is given by factors such as the WW domain-containing transcription regulator protein 1 (TAZ) and the Yes-associated protein (YAP) as part of the Hippo-pathway [32]. TAZ and YAP are induced by mechanical forces above 5 kPa, and their increase leads to an exaggerated unfolding of talin [33]. Talin directly links integrins such as vinculin to actin and activates other integrins such as β_1-integrin [34]. As a result, the adhesion of cells to the matrix is favored. In addition, TAZ and YAP can favor cell proliferation and differentiation [35]. This is because β_1-integrin can induce transcriptional programs. The β_1-integrin-induced effect is crucial. This has been shown by an animal experiment that crossed a tamoxifen-inducible SM22α-Cre-mouse line to a floxed β_1-integrin transgenic mouse line. In this adult mouse model, SM22α was inducible by tamoxifen, but β_1-integrin was knocked out. The lack of β_1-integrin was associated with a very limited survival of these mice only over 10 weeks postinduction. This was due to a loss of vasomotor control, and increased SMC apoptosis could be observed, although the VSMC proliferation factor SM22α was induced. In addition, there was increased ECM deposition [36]. β_1-integrin is also important for ECs because they get apoptotic when detached from ECM (a phenomenon called anoikis, a certain form of programmed cell death when cells dissolve from ECM and die) [37]. The process could be interrupted by adding immobilized β_1-integrin antibodies. The integrin-mediated signal here is required for EC survival [38].

For VSMCs, a matrix stiffness range of 10 to 30 kPa has been suggested to mimic the native ECM stiffness of arterial walls [39]. For vascular ECs, the stiffness ranges from 2.5 to as much as 70 kPa in the natural model of the blood vessel basement membrane. The response of ECs is very variable, and this might depend on their concrete localization (in large or small vessels) and the hemodynamic impact of blood flow or the functional activity [40, 41]. Interestingly, the stiffness dependence of vascular cells is not present when ECs are confluent or during intense cell–cell contact of AFs. This might be because cell–cell communication using cadherin as a junction molecule has a comparably stronger impact on cell activity than cell–matrix interaction [35, 42].

ECM promoting cell migration

Focal adhesions, where the dynamic filamentous actin (F-actin) cytoskeleton is coupled to ECM, drive the cellular behavior not only concerning adhesion and proliferation but also migration [43, 44]. As outlined earlier, the ability of vascular cells to migrate is essential during angiogenesis, a process in which ECs migrate to the site of injury and form new vessels. Additionally, the ability of VSMCs to migrate is important for arteriogenesis, in which collateral vessels develop to bypass blocked or narrowed arterial segments. The morphological changes and physical forces that occur during migration are largely generated by the F-actin cytoskeleton [44]. Fibronectin has an important mediating role because it provides binding domains for $\alpha_5\beta_1$ integrin as a cell adhesive molecule and VEGF [45]. Upon binding of VEGF and $\alpha_5\beta_1$ integrin, EC migration was observed to increase enormously. In parallel, ECs proliferated, presumably due to the activation of extracellular signal-regulated kinase and the activation of the VEGF receptor through phosphorylation. Both binding events might represent a synergism important for enhanced angiogenesis. Vice versa, analysis of integrin receptors and their ligand specificity on the vascular endothelium led to the finding that inhibition of integrin-dependent adhesions in sprouting ECs inhibits angiogenesis [46].

Bioinks in vascular TE

Bioprinting of living cells requires a specific set of conditions only met by a certain class of materials—hydrogels. These hydrogels can be imagined as 3D polymer networks. Hydrophilic groups in the respective hydrogel can adsorb large amounts of water, which is important for cell survival [28]. In addition, hydrogels have elastic and shear-thinning properties that might be useful for cellular matrix production and cell migration but also for later form stability after printing. However, a huge variety of hydrogels have been used for bioprinting, and not all of them might be suitable for the specific requirements of vascular cells.

An ideal bioink for soft-tissue generation, such as vascular tissue, should be printable and biocompatible. The term "biocompatibility" comprises two features: favored cell adherence and maintained cell viability. The bioink should act as a natural ECM, which means that the ink also gives space for cell migration and supports cells to differentiate into a final phenotype (e.g., in stem or progenitor cells or within processes of transdifferentiation). This is why there are considerations to dissolve natural ECM as a physiological material to create a specific microenvironment and use it as a milieu-specific bioink [47, 48].

The optimal rheological properties enabling high-resolution printing depend on the printing technique so that the bioink can be positioned as desired to allow cells to distribute optimally within the printed structure or on a shaping scaffold. Developing dense cell–cell contacts between cells of the engineered vascular wall is a critically decisive aspect of their longevity. Thus, cells must be able to spread and stretch through the ink. Diffusion of gas, nutrition, certain growth factors, or signal proteins must be allowed by the hydrogel [49].

According to the vision of van Lith and Ameer, for a biohybrid strategy of a vascular graft, the degradation rate of the scaffold or ink material used ideally matches the tissue-building rate [50]. Furthermore, the material must be printable and sterilizable with low production effort and cost.

Relevance of cell–matrix interactions in the vascular wall for bioinks

Knowing how cells interfere with their surrounding matrix is necessary to provide them with a physiological environment in a bioink. As outlined before, ECM has distinct effects on the cells and vice versa. Here, specific binding signals of cells are important to react to characteristics of the respective matrix and transport that "matrix information" to the cell. One possibility of such a reaction is the switch into a secretory cell phenotype with increased matrix production in "remodeling." A comprehensive summary of those signal cascades between matrix and vascular cell types and the corresponding ligands is given in Table 2.

Adhesive features of vascular ECM proteins as bioinks

Cell–matrix interactions are predominantly regulated by discoidin domain receptors (DDRs) and focal adhesions, which are transmembrane attachment sites positioned at the F-actin cytoskeleton. These adhesion points are offered by ECM proteins such as fibronectin and collagen via their extracellular domains, and a 3D printed scaffold likely made up of these proteins has a strong adhesive effect on cells [51]. As outlined before, integrins are components of focal adhesions. They can be influenced by mechanical forces through dynamic cultivation [52, 53]. The adhesion grade can be increased from single-cell attachment to focal cell complexes up to strong fibrillary adhesions [44], the latter one being characterized by nanoclusters of up to 100 ligand-bound integrins [34]. Regarding peptides, the critical binding element in fibronectin and many other ECM proteins is the short amino acid sequence Arg-Gly-Asp (RGD) [54]. One has to pay attention to the structural organization around this peptide motif, as cells recognize and choose the appropriate ligands surprisingly specific when given a choice between different substrates with respect to structural layout, ligand density, and stiffness [55]. Because nanofibers have an optimized mass–surface quotient and a 3D structure comparable to natural ECM, they are very useful for bioinks, as they support cell adherence and a physiologically relevant microenvironment. They are naturally formed by various autoassembling ECM materials, such as collagen, fibrinogen, or elastin, but can also be generated artificially from synthetic polymers, such as polycaprolactone (PCL), through advanced techniques like electrospinning. Inspired by the natural model of cell–matrix interactions in the vascular wall, Fig. 2 attempts to summarize the essential features of an ideal vascular bioink. These elements are intended to replicate the micromilieu of each specific wall layer of a printed vessel to give cells the greatest possible development for differentiation, migration, growth, and function (Fig. 2).

Different kinds of basic materials for bioinks

As stated earlier, bioinks are mostly represented by hydrogels. These can be divided into three groups: (i) natural hydrogels (unmodified or modified), (ii) synthetic hydrogels, and (iii) semisynthetic. Natural hydrogels can be tuned by diverse cross-linking methods (e.g., gelatin methacryloyl (GelMA)); these chemically modified natural hydrogels are defined as semisynthetic.

Table 2 Cell–matrix interactions inside the vascular wall

Cell	Matrix receptor	Ligand	Function	Reference
ECs, VSMCs, AFs	Integrin $\alpha_5\beta_3$	Vitronectin, fibronectin, osteopontin, fibrinogen, and thrombospondin	Promoting cell survival, migration, angiogenesis, and regulating inflammation	[55, 184, 185]
ECs, VSMCs, AFs	Integrin $\alpha_5\beta_1$	Fibronectin, fibrinogen, and tenascin-C	Stiffness sensing, focal adhesion, cell spreading, migration, and proliferation	[35]
ECs	Integrin $\alpha_6\beta_1$	Laminin, collagen type IV	Cell adhesion and cell survival	[35]
ECs, AFs	Integrin $\alpha_5\beta_5$	TGF, fibronectin, vitronectin, and osteopontin	Angiogenesis, cell migration, and cell proliferation	[35]
VSMCs	Integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$	Fibrillar collagen and laminin	Cell adhesion, proliferation, and differentiation	[35]
ECs, VSMCs, AFs	Syndecan-1, Syndecan-4	Fibronectin, VEGF, bFGF, PDGF, thrombospondin-1, tenascin-C, and vascular cell adhesion molecule 1 (VCAM-1)	Cell adhesion, migration, differentiation, proliferation, and cytoskeletal organization	[186]
ECs, VSMCs, macrophages, leukocytes	CD44	Hyaluronan, fibronectin, osteopontin, and collagens	Cell adhesion, migration, proliferation, differentiation, and apoptosis	[187, 188]
ECs, VSMCs, macrophages	Receptor for advanced glycation end products	Glycated ECM proteins, elastin, HMGB1, S100 proteins, laminin, collagens, thrombospondin, tenascin-C, and VCAM-1	Inflammation, cell survival, proliferation, and migration	[189]
ECs, VSMCs	Discoidin domain receptor 1 (DDR1)	Collagen types I, II, III, IV, and V	Remodeling, cell adhesion, migration, differentiation, and proliferation	[190]
ECs, VSMCs, AFs	Discoidin domain receptor 2 (DDR2)	Collagen types I, II, III, and V	Remodeling, cell adhesion, migration, differentiation, and proliferation	[190, 191]
ECs, VSMCs, AFs	Lysyl oxidase-like 2, (LoxL2) receptor	Elastin, collagen types I, III, and IV, fibronectin, decorin, and lumican	ECM remodeling, cell adhesion, migration, proliferation, and differentiation	[192]
VSMCs	Elastin receptor S-galactosidase	Elastin	ECM remodeling	[193]
VSMCs, AFs	Receptor for hyaluronan-mediated motility	Hyaluronan, small leucine-rich proteoglycans, and growth factors (TGF-b/PDGF)	Cell motility, migration, and adhesion	[188, 194]

Matrix binding receptors of vascular wall cells with their respective ligands and functions involved in cell–matrix interactions in the vascular system

Natural hydrogels

Natural polymers are broadly classified as protein- or polysaccharide-based biopolymers, with another class to note being decellularized ECM (dECM). A detailed description of the pros and cons of commonly used biopolymers is given in the following paragraphs, with a shorter side-by-side comparison in Table 3.

Protein-based bioinks Among natural sources, protein-based hydrogels are highly biocompatible, biodegradable, and tunable and provide a high binding ability for cells and

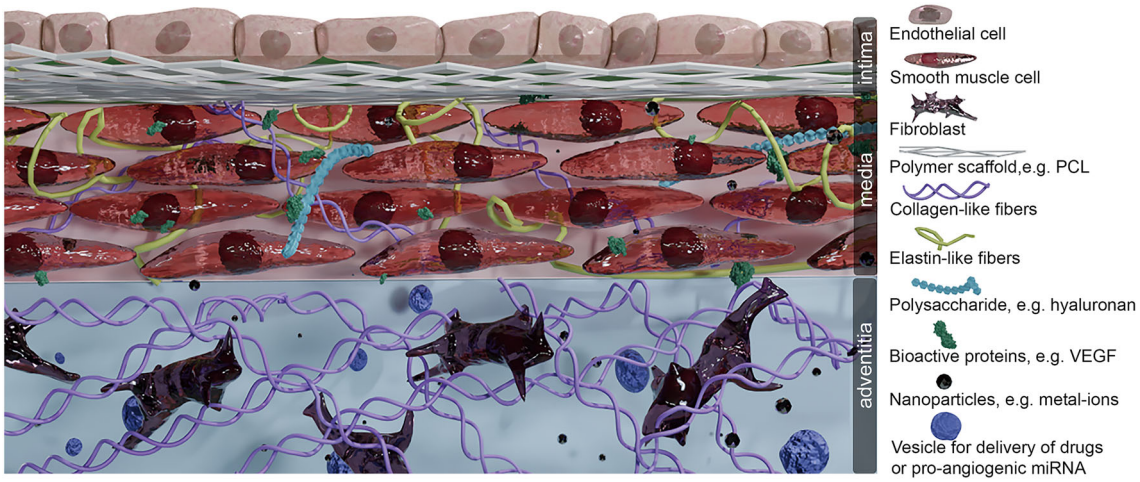

Fig. 2 Specific composition of a bioink for vascular wall segments: tunica intima, media, and adventitia (as indicated). Bioinks are a vehicle to print vascular cells with various artificial fibers and factors to mimic the native ECM in a specific vascular wall segment. Bioink and cells can interact by signaling between cell-specific receptors for different matrix proteins. The bioink must favor cell proliferation and correct phenotypic differentiation, which are further influenced by segment-specific cell organization in each layer and the respective matrix stiffness/elasticity. The schematic shows an exemplary state-of-the-art vascular bioink. An artificial tunica intima needs a dense fiber network to which ECs can attach to form a monolayer. This could be provided by 3D printed, for example, melt electrospun or electrowritten polymer fiber constructs out of biodegradable polymers such as PCL or others, decorated with a hydrogel coating such as the adhesiveness-favoring fibrin. A bioink for the media will contain collagen- and elastin-like fibers that allow SMCs to attach to and provide the elasticity needed for vasoreactivity. Tunica adventitia provides mechanical stability; therefore, collagen-like fibers and enhanced ECM production are crucial. The bioactivity of bioinks can further be tuned with stimulants, such as growth or differentiation factors. Extracellular vehicles incorporated into the ink could provide segment-specific microenvironments. Nanoparticles could carry specific features (e.g., metal ions for antiinfective effects). ECM: extracellular matrix; ECs: endothelial cells; PCL: polycaprolactone; SMCs: smooth muscle cells; VEGF: vascular endothelial growth factor; miRNA: microRNA

many bioactive properties [56]. Protein-based bioinks usually have a protein content of 1% to 15% (0.01 to 0.15 g/mL).

Collagen Collagen is a major protein component of native ECM. In arteries, collagen makes up to 50% of the dry weight [57]. It is dominantly circumferentially aligned [58]. The frequent occurrence of collagen as a matrix component supports its suitability for biomedical applications and bioprinting. In addition, collagen can relatively easily be prepared from animal sources [59]. Collagen hydrogels show superior biocompatibility with excellent cell loading capacity and low immunogenicity. Types I, III, IV, XV, and XVIII collagens have proven angiogenic properties [60]. Comparable to fibronectin or fibrin, collagen provides suitable binding domains for ECs. In particular, integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ play a role. Again, matrix binding induces the activation and/or suppression of various signaling pathways involved in angiogenesis and cell survival by suppressed apoptosis. This induction may be mediated by the activation of mitogen-activated protein kinase pathway [60]. In addition, integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ binding to collagen I lead to a suppressed activity of the protein kinase A via cyclic adenosine monophosphate (AMP)—signaling. This is followed by a reorganization of the cytoskeleton, favoring cell migration [61]. Collagen I also initiates the formation of a lumen in newly arising vessels by merging pinocytic intracellular vacuoles [62]. Among collagen-like ECM glycoproteins, laminins appear during the late phase of angiogenesis and terminate EC proliferation. Laminins also recruit pericytes and activate Notch signaling within the sprouting process [63]. Therefore, laminins may be interesting for bioink functionalization. From a biological point of view, collagen is a valuable constituent for a TEVG-associated bioink, although printing is difficult due to its low rigidity. That is why collagen must be mechanically supported by other materials or must undergo additional cross-linking in or during bioprinting [64]. Attempts to bioprint collagen by microextrusion, inkjet, and laser-assisted bioprinting [65] are limited by this material weakness. In addition, collagen is difficult to sterilize (e.g., due to its heat sensitivity and degradation upon heating), and collagen scaffolds tend to shrink (contract) in response to cellular activity [65]. Shape fidelity is typically not given due to a slow, hard-to-control polymerization process. Thus, collagen must be thickened for bioprinting by adding alginate or semi-cross-linking before printing [66]. An example of such an approach was given by Bosch-Rué et al., who recently published the results of a triple concentric microextrusion printing generating small-diameter collagen-based artificial blood vessels with a burst pressure of at least 620 (1 mmHg=133 Pa) [67]. This level of burst pressure was

Table 3 Natural materials as bioinks. Physiological ECM proteins, alternate but also naturally occurring substances from algae or crustaceans, and proteins out of the blood coagulation cascade, skin, bones, or connective tissue possess different biocompatibility properties and can be gelled using various methods

	Compound	Concentration (in media)	Gelation trigger	Biocompatibility	Reference
Physiological ECM	Collagen	1%–5%	Physical (*T*, pH); chemical cross-linker	RGD, bioinstructive, and proangiogenic	[67, 81, 195]
	Hyaluronan	1%–3%	UV + photo-initiator; chemical cross-linker	Adhesion, proliferation, differentiation, and ECM organization	[74, 196, 197]
	Elastin	1%–5%	UV + photo-initiator; chemical cross-linker; enzymatic	Supporting adhesion, spreading, EC network formation, SMC contraction, and angiogenesis	[74, 75]
	dECM	1%–10%	UV + photo-initiator; chemical cross-linker	Supporting adhesion, spreading, SMC differentiation, and ECM synthesis	[104, 198]
Nonphysiological ECM	Alginate	1%–4%	Physical (divalent cations)	Bioinert	[199]
	Fibrin	2%–5%	Thrombin-induced fibrinogen cleavage	Supporting adhesion, proliferation, differentiation, and ECM production; inducing angiogenesis	[72, 81]
	Gelatin	5%–15%	Physical (*T*, pH); chemical glutaraldehyde, methacrylate	RGD signal, bioinstructive, and proangiogenic	[71]
	Chitosan*	0.5%–5%	Ionic, chemical	High biocompatibility and antibacterial properties	[200]
	Silk fibroin**	2%–8%	Chemical	Cell adhesion, survival, and proliferation	[201]

*Chitosan is dissolved in acidic solutions
**SF is commonly dissolved in aggressive solvents, such as hexafluoroisopropanol or LiBr solution, and dialyzed against water for solvent removal

higher than that of previously reported collagen TEVG but corresponded to only 25% of the burst pressure of human arteries [68].

Gelatin Hydrolyzed collagen forms gelatin. Gelatin also contains bioactive sequences such as the aforementioned RGD motif and exhibits even superior biocompatibility and degradability than collagen [69] but similar weak mechanical attributes. Gelatin can be easily functionalized via chemical modification, being endowed with self-healing [70] properties or controllable photo-cross-linking capacity [71]. Due to its thermoresponsiveness, gelatin can also be used as a sacrificial material for coaxial bioprinting to create microchannels. Encapsulating cells in the sacrificial material may be an effective method for seeding of ECs onto the inner wall surface of such microvessels [72]. To summarize, gelatin could be a promising material when modifications increase its biomechanical stability, especially after printing.

Elastin As mentioned before, elastin is crucial for the resilience of vessels, whereas collagen fibers are responsible for their resistive character against elevated blood pressure [73]. Histological studies show an alternate pattern of elastic fibers and SMCs within an arterial tunica media. This pattern connects fibers and cells, enabling mechanical force transduction [73]. Elastin can be produced with biological techniques to form hydrogels with elastic properties that support the adhesion, spreading, and growth of human umbilical vascular ECs (HUVECs) and their formation of tubular structures within the gel [74, 75]. Considering the high influence of the elastic modulus, elastin is underrated as a bioink constituent. In contrast to collagen fibers produced during

scarring after vascular injury, elastic fibers do not regenerate. Their production within embryonic vasculogenesis is almost completely stopped after birth, and the slightly ongoing elastin fiber replacement is confined after puberty at the latest [76]. To create new vessels by TE, comparable to "young" vessels in the human body, elastic fibers are an interesting element of vascular bioinks.

Fibrin Fibrin is a fibrous polymer that stimulates coagulation in vivo. It is produced by an enzymatic process out of fibrinogen and has an inducing effect on ECM [77–79]. Collagen synthesis is favored by fibrin in AFs and SMCs [80]. Similar to collagen, fibrinogen and fibrin provide only poor printability despite good gel stability, quick gelation time. Schöneberg et al. created a fibrin-TEVG by jetting thrombin onto an SMC-loaded fibrinogen layer [72].

Fibrin is also a useful additive for other hydrogel systems to induce angiogenesis in the tissue-repairing process [81] and has been used as a scaffold material to form a vascular graft by molding [79, 82] and as a composite by electrospinning [83]: a coculture of HUVECs and human dermal fibroblasts successfully led to vascular network formation on fibrin scaffolds [84].

Silk fibroin (SF) SF is produced by silkworms or spiders. This protein is considered a universal biomaterial platform for TE and drug delivery. Also, TEVGs were produced out of SF, as SF is highly biocompatible, has been widely investigated for vascular graft development due to its good biocompatibility, and shows a controllable biodegradability up to one year in vivo and sufficient mechanical properties. In addition, SF has a low immunogenicity [78, 85]. SF can be dissolved in highly concentrated salt solutions such as lithium bromide and is transferred to aqueous solutions after salt removal with dialysis. SF can be fabricated to form hydrogels, scaffolds, films, coatings, and electrospun fibers and has also been used as an adhesive material on top of other scaffolds [6]. In the literature concerning TEVG applications, SF is more often electrospun and seeded with cells than bioprinted as a hydrogel. Alessandrino et al. showed that SF favors adhesion, survival, and proliferation of ECs, SMCs, and AFs on their three-layered tubular electrospun scaffold [86].

Polysaccharide-based bioinks Polysaccharides are considered elements of a vascular bioink because there is no resource problem and they can be easily synthesized or isolated from natural sources. In addition, polysaccharides allow appropriately tailored chemical modifications to tune the self-assembly out of two divergent components to reach form stability [87]. They can easily be functionalized to give them more cross-linking options like methacrylation for photo-polymerization, self-healing properties, or instructive sequences for adhesion. Some polysaccharides (e.g., GAGs) are present in the natural ECM and, in general, are molecularly similar to other ECM components, namely glycoproteins and glycolipids [88]. Some polysaccharides, such as alginate, are unfortunately bioinert due to the lack of cell-adhesive motifs. The cell undergoes anoikis when printed in alginate [89]. The polymer content of polysaccharide-based bioinks is generally lower than that of protein-based bioinks and is about 0.3% (3 g/L) to 5% (0.05 g/mL), which may be due to the impermeability of higher concentrated polysaccharide bioinks for oxygen and nutrients, lowering the cell survival likelihood.

Alginates Alginates are naturally derived polysaccharides isolated from marine algae. Alginate production is cheap and shows good biocompatibility, water retention, and nonantigenicity. Alginates have carboxylate groups that interact with divalent cations such as calcium, strontium, and zinc to rapidly form hydrogels after the introduction of cations [90]. Alginates dissolve well in water and show a short gelation time. The good prospect for use as a bioink is somewhat blurred by severe disadvantages. The necessary form stability can only be reached by a calcium treatment postprinting [91]. Moreover, even calcium-treated alginate-hydrogel prints redissolve in physiological, sodium-containing buffer or medium. This prevents the mechanical stability of printed structures for in vitro tissue modeling [92]. Furthermore, nonbonded alginate polymers cannot be degraded enzymatically by the enzymatic tools of mammalian cells; the biological decay thus is not controllable [93]. One approach for modified alginate degradation would be the application of alginate lyases that occur in algae but cannot be easily integrated into a TE product [89]. Successful alginate-containing bioinks bear rather low concentrations. This is probably because alginate is a great thickener for printing, but higher amounts create a robust gel with low cell compatibility.

Hyaluronan or hyaluronic acid (HA) HA belongs to the large family of GAGs that occur within the natural ECM. It is a versatile biomolecule used for clinical purposes for a long decade now [94]. HA binds cell surface receptors and promotes cell proliferation and differentiation, wound repair, morphogenesis, and matrix organization [95]. Initially, HA is a component solely occurring in the connective tissue. More recent findings showed that HA is a metabolite in many tissues. Its biological effect is strongly connected to its respective molecular weight. Whereas a high molecular weight HA acts as a genoprotective [96] and cytoprotective [94, 97] factor, short HA oligosaccharides with up to 25 units have a proangiogenic effect [98]. Vascular TE strategies can take advantage of the angiogenic potential of HA fragments that could be generated using hyaluronidases [99], with tissue half-lives ranging from hours to days [95]. Further, HA

oligomers are proinflammatory, as they bind to the cell surface marker CD44 on ECs, thus activating wound healing, the attraction of fibroblasts, and consequently a process comparable to scarring. It remains to be elucidated whether or not this CD44 interaction is favorable when using HA as a bioink or provokes rapid inflammation and decay of the later TE product [100]. Another disadvantage of HA may be a quick degradation in vivo. Various strategies, such as blending and chemical modification, try to overcome the short-timed fragmentation of hyaluronan; thus, in situ cross-linking strategies have been tested that fit well, at least with TE of osteogenic structures up to now [95]. Whether low molecular weight HA would be appropriate as an element of a vascular bioink is not clear yet.

Chitosan Chitosan is an abundant cationic polysaccharide. It can be isolated from crustacean exoskeleton, insect cuticles, and algae and out of fungal cell walls. Further, chitosan derivatives can be technically obtained through deprotonation–demineralization–decolorization–deacetylation of chitin-containing structures. Single-chitosan derivatives may also be synthesized in a chemical pure manner. Chitosans possess intrinsic biocompatibility and biodegradability, mediated by enzymes such as lysozyme. Because of its marine origin, there is a lower risk of transmitting infectious diseases compared to mammalian polysaccharides, but a limitation for transplantation is the presence of endotoxins in raw natural chitosans, which are difficult to remove and vary in content [101]. Chitosans trigger an immune response in humans that is not clearly understood but used with chitosan as a vehicle for vaccines [101]. The rate of degradation, viscosity, and solubility may depend on the degree of acetylation, and high-polymer chitosans that do not enter cells have a lower immunogenic potential than low-polymer chitosans with a higher rate of cell entry. A recent study showed a satisfying biomechanical robustness of small (6 mm) chitosan conduit tubes ranging up to Young's modulus of about 5 to 7 MPa [102]. In this approach, TEVGs are generated by fiber-based knitting, and the resulting chitosan tubes are additionally coated with chitosan and gelatin. These TEVGs could withstand a burst pressure of about 4,000 mmHg with high suture retention and could be successfully seeded with SMCs [103]. In vitro studies also showed that chitosan hydrogels support human endothelial progenitor cell adhesion and proliferation. TEVGs with chitosan or chitosan/PCL hybrid scaffolds were tested in sheep [104], dogs [105], and rats [106]. A big disadvantage may be the degradation time of pure chitosan of only several days after implantation [90, 107]. In comparison, chitosan–PCL blends show the highest potential as further TEVG material.

To summarize, polysaccharides are another particularly interesting platform for developing novel bioinks due to the ease of derivatization/functionalization, high diversity of chemical structures, adequate rheological and mechanical properties, and intrinsic biocompatibility and biodegradability.

dECM ECM is present within all tissues and consists of water, proteins, and polysaccharides. dECM is attractive to be used as a bioink because each tissue has a unique ECM composition with physiological properties for tissue-specific cells [108]. Recently, soluble dECM was developed through decellularization, lyophilization, and enzymatic digestion processes, which remained the main complex ECM compositions but lost native hierarchical microstructures [109]. A dECM can form hydrogels at body temperature, which simulates the composition of the complex biophysical environment but is rapidly degraded, restraining its application [90]. Furthermore, the preparation process is costly and work-intensive, and the product shows high batch-to-batch variations. However, modified ECM hydrogels could promote angiogenesis [110]. Gao et al. reported that a dECM bioink for TEVG bioprinting promotes cell proliferation and favors differentiation as reflected by distinct VSMC-specific markers, and dECM bioink leads to a comparatively increased ECM synthesis compared to a collagen hydrogel [111].

Going back to the topic of this review, bioprinted dECM would probably meet all clinical challenges as it represents a naturally originated protein composition [112]. However, gaining this material is a resource problem, and decellularization is not always complete, leaving residual cell detritus with an immunogenic risk for later rejection processes.

Synthetic hydrogels for 3D bioprinting

Natural polymers produced by biotechnological production processes present some drawbacks, such as low reproducibility and difficulties of functionalization compared to synthetic counterparts. Compared to some natural bioinks isolated from natural sources mentioned earlier, these polymers also comprise an immune reactivity with severe safety concerns. One example of this issue is the matrix mixture given by Matrigel™, which is produced by murine cancerous cell lines. It was successfully used in bioprinting but has no prospect to be clinically used due to the aforementioned reason [56].

It is attractive to tailor synthetic polymers as chemically engineered hydrogels to target and significantly improve a certain attribute, such as adhesion or degradation qualities. However, as found in the literature so far, these synthetic polymers play only a single note in the metaphorical orchestra of signals in ECM. Tailoring synthetic polymers may enhance specific cell instruments, but the complexity of ECM makes chemical mimicry an unreasonable task compared to using other biomaterials as bioink substrates.

Enzyme-sensitive peptide sequences must be incorporated into synthetic hydrogel networks to enhance biodegradation.

To give an example, Zhu et al. incorporated a collagenase-sensitive GPQGIAGQ (GIA) sequence (derived from collagen type I) in the polyethylene glycol (PEG) diacrylate (PEGDA) chain [113]. An increase in collagenase sensitivity was observed, and seeded HUVECs formed capillary-like networks on top of this modified hydrogel. Anyway, this proves that both cell adhesion and the biodegradability of scaffolds are crucial features in inducing vessel formation [84].

However, a downside of synthetic hydrogels for 3D bioprinting is often poor biocompatibility, the occurrence of nonnatural degradation products, and a loss of mechanical properties during degradation [114]. For this reason, fully synthetic hydrogels are candidates for additives to compensate for specific missing properties of a natural bioink or should be based on natural materials to enable proper cell—cell and cell–matrix interactions, which are vital for vascular cells. Currently, no examples are found in the literature where vascular cells are cultivated in solely synthetic hydrogels.

Semisynthetic hydrogels for 3D bioprinting

As outlined earlier, conventional hydrogels usually suffer from weak mechanical properties and are easily deformed or damaged when subjected to a mechanical force. The resulting printed hydrogel will exhibit minor defects after implantation; due to the irreversibility of destruction, those will gradually increase and merge into cracks in vivo due to the complex dynamic physiological and mechanical environment inside the body [115]. Polymers of natural and synthetic origin are chemically modified to enhance their properties. Particularly, photo-cross-linkable moieties, such as methacrylate, acrylate, vinyl, and allyl, are used as linkers to the chain backbone of many biopolymers mentioned so far (e.g., proteins, polysaccharides, and GAGs). This was done to enable photo-cross-linking [116]. However, these modified polymers are not suitable for extrusion bioprinting due to their low viscosity and consequently poor printability [117], so photo-cross-linking becomes an obligate part of the total printing and production process with these hydrogels and is performed sometimes even before printing. A major disadvantage is the possible cytotoxic effect of photo-cross-linking to printed cells.

An alternative and much more promising quality that can be introduced in hydrogels chemically is their "self-healing property." The term "self-healing" refers to the ability of materials to restore automatically after damage [115]. As outlined earlier, hydrogels with weak mechanical properties are easily deformed by mechanical forces [118]; therefore, "self-healing" would be appropriate to realize form stability while avoiding photo-cross-linking procedures with their cytotoxic side effects. Self-healing hydrogels employ novel strategies of in situ cross-linking, e.g., via Schiff base formation between nitrogen from an amine or hydrazide and the carbonyl group of an aldehyde or ketone [119]. Such imine or hydrazone hydrogels exploit dynamic covalent bonds with significantly higher strengths than physical bonds. However, these bonds are reversible and depend on a constant equilibrium between the bound and unbound states [118]. Kirschning et al. developed a versatile and highly adaptable modular system for polysaccharide-based hydrogels, extensible for further chemical functionalization and for use in TE [120, 121]. These gel components offer the advantage that, contrary to the principle of alginate and calcium chloride, they allow spontaneous in situ gelation immediately after mixing the two components without the need for secondary cross-linking and feature tunable properties. Bacterial and plant-based polysaccharides are used in this process; alginate, HA, dextran, and pullulan have been derivatized and used to generate hydrazine hydrogels [122, 123]. These hydrogels were first successfully used as a matrix for myocardial tissue [122–124] but have not been evaluated for bioprinting so far.

GelMA [125] is a hydrogel used in microfabrication research because it possesses many advantages that lead to successful experiments [126]. The natural component of GelMA is the gelatin backbone of the hydrogel. Gelatin is a hydrolysis-evoked product of collagen. The complex structure of collagen is denatured during this reaction, which removes inconsistencies in the protein's structure. The leftover gelatin has a much more uniform structure, a crucial factor in GelMA's success as a hydrogel. The uniform structure of gelatin allows for the consistent addition of cross-linking substituents, increased biocompatibility, and decreased antigenicity compared to collagen. These characteristics make GelMA a more suitable hydrogel choice for TE than collagen. GelMA is formed when the gelatin backbone is reacted with methacryloyl (MA) substituent groups. These groups give GelMA its structural integrity and physical characteristics after cross-linking; thus, the quantity of MA substituents controls the mechanical characteristics of GelMA-generated print products. As photo-initiators for acrylation are cytotoxic and the process has cytotoxic side effects, removing not fully reacted MA from the reaction or the necessary water-soluble photo-initiators used for light-induced cross-linking remains challenging. Furthermore, in terms of genotoxicity or proliferating capacity, cells can be affected in the resulting 3D GelMA construct by ultraviolet light used for polymerization. Another problem is the resulting pore sizes in GelMA, which could affect oxygen and nutrient diffusion and inhibit cell migration and intercellular interactions. This review considered the latter point to be overcome by adjusted bioprinting resolution in the future.

Semisynthetic hydrogels could be a worthwhile alternative to purely synthetic hydrogels as vascular bioinks. It has to be postulated that the relatively rigid synthetic share could be held as low to make up with cell growth and migration and that non-cross-linking methods such as self-assembly by in situ gelation could be optimized. HA blends display excellent physicochemical characteristics associated with high survival rates of printed MSCs [127].

Physiological performance of bioinks

Testing of cell viability and adhesiveness

The biological performance of a hydrogel for use as a bioink in TE depends on its biocompatibility, adhesiveness, biodegradation, and functionality [128]. Cell viability can easily be qualitatively evaluated through live/dead staining or colorimetric tests. A test substance is quantitatively modified by metabolically active cells, producing a photometric signal proportional to the metabolic cell activity commonly referred to when defining cell viability.

Bioadhesiveness is a phenomenon where materials adhere to biological surfaces [129] and can be estimated by washing-off approaches [128] or vinculin staining to detect focal adhesion points [130]. Both objective measures are reasonable to assess possible cytotoxic or nonadvantageous effects of bioinks.

Confirmation of cell-specific biomarkers

Biofunctionality can be assessed by the detection of tissue-specific markers by immunostaining [128]. Potential target markers for ECs are the cytoskeleton (F-actin) or tight junction proteins such as zonula occludens-1 [131]. Further relevant markers in ECs are VE-cadherin, CD31, von Willebrand factor, or endothelial NO synthase 3. Suitable markers for VSMCs next to α-smooth muscle actin (α-SMA) are calponin 1 (CNN1) and transgelin (TAGLN) [131]. AFs can be judged by SMA or their secretory activity in producing collagen. Checking for cell character is crucial to exclude possible dedifferentiating effects of bioinks on printed cells.

Confirmation of vascular functions

The vasoreactivity of vascular cells must be evaluated after printing. The corresponding vasoconstricting or dilatating stimuli should be tested in the bioprinted construct to confirm the full functionality of the bioprinted vessel [9]. To check for contractility and the SMC phenotype, bioprinted vessels can be perfused with physiological concentrations of the vasoconstrictor phenylephrine to examine vasoconstriction responses as a functional test. Similarly, acetylcholine, a vasodilator, could counteract contractions of SMCs induced by phenylephrine. The effect can be assessed visually under a microscope [132]. The antithrombogenic phenotype of ECs can also be tested [133]. AFs could be tested for proliferation after agonist induction with platelet-derived growth factor (PDGF) [134].

Hemocompatibility, thrombosis, and immune reactions

In general, there are significant obstacles associated with the medical application of printed constructs, favoring increased foreign body reactions or rejection events to the implant. Therefore, interactions between vascular graft materials and the involved cells, including blood cells, and other blood components must be considered to avoid severe immunological reactions [6, 135]. These interactions and the immune response to TEVGs are a very complex subject that is beyond the scope of this review. Thus, this review referred to recently published reviews dealing with failure analysis of 3D printed TEVGs and immunomodulation strategies for TEVGs made of synthetic, hybrid, and biological materials [136]. Because it is an extremely important topic, this review briefly discussed some crucial keywords. The response to the surface or by-products of the foreign material changes over time after implantation of the vascular graft: (i) hemostasis (protein adsorption followed by adhesion of platelets and blood cells), (ii) innate immune system response and acute inflammatory reaction (influx of neutrophils and macrophages interacting with each other and T helper cells), and (iii) foreign body reaction and failure of the implant (recruitment of fibroblasts encapsulating the foreign material) [135–137]. This can lead to thrombosis, atherosclerosis, and severe inflammation, especially if the material has antigen-like properties, thereby triggering macrophage activation.

Because ECs line the inner vascular wall, these cells play a key role in the successful implantation and function of TEVGs. The complete endothelialization of bioprinted vessels prevents thrombosis and other reactions of the human body and favors the long-lasting patency of TEVGs [136, 138]. Furthermore, the application of autologous cells decreases rejection events. However, there are further effects caused by the chemical structure of the scaffold material [139, 140], potential degradation products, or incomplete endothelialization. These include humoral and cell-mediated immune responses. Some EC subtypes are considered semiprofessional antigen-presenting cells playing a key role in immune modulation that depends, among others, on the EC origin [141]. They express genes involved in antigen capture, processing, and presentation. For example, immunologically stimulated human renal vascular ECs have

long been shown to express major histocompatibility complex II (MHC-II) surface molecules, such as human leucocyte antigen DR (HLA-DR).

Therefore, systematic tests for hemocompatibility include the study of thrombosis, partial thromboplastin time, coagulation, aggregation, platelet adhesion, and hemolysis. Lastly, animal studies are required to evaluate the sustainable patency of TEVGs, immunological response in vivo, and the mechanical efficacy of the bioink and printed construct [6].

Printability and verification of printability

Printability and later biomechanical stability are crucial for the successful fabrication of precise tissue by 3D printing. The key factors affecting printability are bioink composition (physical properties and flow behavior), scaffold design, and printing process (cross-linking, printing method, and parameters). It is assessed by measuring rheological properties and shape fidelity and analyzing the quality and accuracy of the printed structure. Rheological measurements using a rheometer provide information on bioinks' viscosity, shear-thinning behavior, and elasticity. These parameters are important for determining the bioink flow behavior under different shear rates and temperatures and for approximating shear forces acting on the cells. In the ideal gelation state, uniform strand deposition is observed and products maintain their shape. Imaging techniques, such as scanning electron microscopy (SEM), confocal microscopy, and microcomputed tomography (micro-CT), can be used to evaluate the uniformity and microstructure of a printed construct. These data provide information on the shape fidelity compared to the target CAD and also on the interconnectivity, cell distribution, and porosity. Comparison to CAD models is often evaluated by extruding continuous filament of single lines or grids.

The uniformity of linear filaments is affected by process parameters, such as the printing pressure, nozzle speed and diameter, distance between the nozzle and the printing bed, and printed geometry. Due to their limited elastic properties, viscoelasticity, and tendency to deform structurally, printed filaments can flow and collapse by merging filaments or layers. Ouyang et al. proposed the printability value for quantifying strand merging through grids [142]. Because physiological structures do not exist in straight geometries, Gold et al. introduced fidelity ratio quantification to take bridging, line deposition, and infill density into consideration [131]. Therefore, they deposited bioink over gaps of defined sizes without underlying support materials and defined the ratio of the theoretical void to the actual void area of the print as fidelity ratio quantification. The fidelity ratio should ideally be equal to "1." With a fidelity ratio smaller than 1, actual voids are smaller than theoretical voids, which might come from overextrusion, swelling of the extrudate, or low print velocity. Fidelity ratios greater than 1 might arise from air bubbles, clogging, drag, or high print velocity. The extrusion force required to push the bioink through the printer nozzle can be measured using a force sensor [143]. This measurement provides information on the bioink's flow behavior and printability under different printing conditions. Shape retention and stability can be assessed through compression and tension testing, which evaluates the ability of the printed structure to maintain its shape and stability after printing [142, 144, 145].

Measuring print accuracy in 3D extrusion hydrogel printing involves evaluating the dimensional accuracy, uniformity, and microstructure of the printed objects. Different imaging techniques, such as SEM, confocal microscopy, and micro-CT, can assess these aspects, each with its strengths and limitations.

Table 4 gives an overview of different methods to quantify the printability of diverse bioinks.

Resistance against shear stress

Within circulation, blood pressure induces shear stress on the vessel wall. ECs react to the shear stress through various mechanosensing systems, such as ion channels, integrins, and cell junction molecules [42]. A unidirectional high shear stress induces antithrombogenic effects on the EC surface and prevents platelet aggregation and thrombus formation [146, 147]. In particular, endothelial colony-forming cells as a fraction of endothelial progenitor cells are a visionary cell source for TEVGs [133]. Because the bioink would serve as a printable vehicle for these cells to be decorated on tubular scaffolds, the ink must resist shear stress. Near-wall shear stress reaches 5 to 10 dyn/cm^2 in venous vessels and up to 25 dyn/cm^2 in middle-sized arterial vessels. Thus, a form-stable bioink must be resistive and elastic enough as a stand-alone structure (scaffold-free bioink) to tolerate maximal forces arising during the natural pulse wave of the physiological blood flow, or it could fulfill the role of a mediating material between stronger tubular scaffolds provided by 3D printing out of biodegradable polymers [148, 149] to enable the exact positioning of cells on top of these polymer-based resistant scaffolds.

A semisynthetic and tunable hydrogel (GelMA) with high flow resistance was already outlined above. The tunability of this hydrogel was exceptionally shown by Wang et al. who used photoactivatable HA methacrylate (HAMA) mixed with GelMA, followed by selectively enzymatic digestion of HAMA [150]. This process led to a tunability of mechanical properties. The biocompatibility in their work, assessed using rat fibroblasts, was sufficient for reliable cell proliferation, but there have been no attempts to transfer the results to primary vascular cells.

Table 4 Print assessment: current methods with strengths and limitations

Imaging technique	Strengths	Limitations
Scanning electron microscopy (SEM)	Submicron resolution: revealing fine details on a nanoscale level	Sample preparation, because samples need to be coated with a conductive layer, possibly introducing alterations/artifacts
	Surface analysis: providing insights into surface roughness, porosity, and overall surface quality	Limited depth information: providing only 2D images
		Vacuum environment: samples need to be placed in a vacuum chamber, possibly altering hydration and hydrogel appearance
Confocal microscopy	Optical sectioning: capturing images at different depths within the hydrogel, allowing for 3D reconstructions and accurate assessment of the internal structure	Limited resolution compared to SEM, less detailed for submicron structures
	Nondestructive: confocal imaging is noninvasive and does not require extensive sample preparation	Penetration depth into the hydrogel may be limited, depending on the hydrogel's optical properties and thickness
	Fluorescence capabilities: fluorescently labeled, confocal microscopy can provide additional information about the distribution of specific components	Time-consuming: capturing multiple optical sections for 3D reconstructions is time-intensive
μCT	3D imaging: able to generate volumetric data and 3D reconstructions of the printed hydrogel, allowing for comprehensive analysis of the internal structure	Limited resolution compared to SEM, images are less detailed for submicron structures
	Noninvasive, μCT does not require extensive sample preparation	X-ray attenuation: the hydrogel's X-ray attenuation might be weak, potentially leading to reduced contrast and limited visualization of subtle features

However, most hydrogels used as bioinks that derive from physiological sources do not fulfill the criteria of a scaffold-free resistant and form-stable material. They thus would have to be combined with more rigid scaffolds printed out of filamentous materials.

Creating a suitable microenvironment and enhanced consistency for bioinks

There are several methods to equip bioinks with specific features to overcome physiological challenges, such as biomechanical resistance, cell decay, thrombosis, immune reactivity, and others as outlined so far. First, one could use protein delivery systems based on nanoparticle integration. As an example [151], 3D printed macroporous scaffolds were used for EC repair. In detail, a polylactic acid, PEG, and pluronic F-127 formulation was prepared as a semisolid viscous bioink. The bioink was loaded with vasoactive drugs, such as either HIF-regulating dimethyloxalylglycine or red blood cells regulating the hormone erythropoietin. This technology represents a visionary approach for generating

composite scaffolds to set therapeutic effects in cardiovascular diseases.

Second, one could use genetic programming and induce mRNA and consequently protein expression of specific factors in printed cells responsible for cell differentiation and angiogenesis. The signal cascades of proangiogenic factors, such as VEGF, bFGF, and HIF-1, are interesting. An example was given by Malecki et al. [152], who evaluated overexpressing plasmid vectors carrying VEGF and FGF to induce angiogenesis and present new vessel formation. However, VEGF overexpression bears a high risk of carcinogenicity for the vascular implant recipient.

Nowadays, dosed approaches with only limited and calculable risks of side effects are based on EVs. EVs are used to transfer specific RNAs, distinct proteins, or lipids to induce signal cascades or create specific physiological microenvironments. Such nanovesicles carrying a mixture of interesting effector molecules can be bound to the matrix and integrated into the bioink design. Concepts to engineer such EVs could rely on modified electrospun scaffolds that imitate natural EV-ECM complexes. One example was given by Hao et al., who identified an integrin $\alpha_4\beta_1$ ligand binding to human placenta-derived mesenchymal stem cells (MSCs) [153]. Thus, they developed MSC-derived EVs from the placenta carrying integrin $\alpha_4\beta_1$ to improve EC migration and vascular sprouting in an ex vivo rat aortic ring assay. As another example, small vesicles released by cells with bioactive proteins and RNAs were used [154]. These "exosomes" were added to bioinks to promote proangiogenic tissue development or set immunomodulating effects. In an exemplary study, VEGF was incorporated as a spatiotemporally defined pattern in an implant using extrusion-based bioprinting [155]. The resulting implants presented a spatially distributed VEGF profile useful to equally promote vessel invasion, especially when compared to implants with a simple VEGF coating on top. Furthermore, proangiogenic microRNAs (miRNAs) and anti-miRNAs can be used to manipulate the 3'-untranslated region of targets, such as VEGF, FGF, and HIF, affecting Notch signaling or NO production [156].

3D printed constructs made up of bioink combinations have also been reported to suffer from biomaterial-related infections, resulting in structural decay and the need for replacement. Including antiinfective agents in bioinks, e.g., metal ions (copper, silver, gold, zinc oxide, and titanium dioxide) or small amounts of some immune reactive chitosans, could help prevent infections during and after the bioprinting process [157]. Their side effects must be explored.

However, it is important to note that the design and optimization of bioinks are complex processes that require careful consideration of the interactions between these components, printed cells, and the surrounding microenvironment [157].

Animal studies

In a further phase of vascular bioink development, evaluation heavily relies on animal experiments. However, no standard for in vivo models or study design has been defined, hampering interstudy comparisons and translational efficiency [158]. A meta-analysis by Koch et al. revealed that TEVG patency was the most warranted study endpoint [158]. Animal studies often did not pay attention to host factors for TEVGs, such as age, comorbidity, or immune status, especially concerning interspecies differences. This was reflected by the fact that only studies performed after 2010 reported such factors, following the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. After TEVG implantation, these factors are crucial to test for a possible impact on thrombogenicity.

Implant–host tolerance was also biomaterial dependent. Small animals are often used to evaluate TEVGs for in situ TE. Here, it was possible to use knockout and transgenic mice to research TEVG functionality in simulated pathologies or assess TEVGs seeded with nonautologous cells. So far, only one immunocompetent animal (mouse) model has been used to test GelMA biocompatibility [159]. A successful example of a pure bioink-consisting TEVG with two cell types was given by Gao et al. [111]. Their blood vessel graft comprised vascular tissue-derived ECM bioinks, processed with a triple-coaxial cell printing technique. The graft stayed patent with intact endothelium and remodeled the SMC layer after three weeks into a rat abdominal aorta. Although this is only a proof-of-concept study, this approach is encouraging and proves that semisynthetic bioinks can withstand in vivo conditions for quite a while.

Current multimaterial bioinks and printing strategies

Despite advances in hydrogel design, the use of hydrogels still has limitations. One-component hydrogels, including those mentioned above, are limited because properties that enhance cell viability and function often conflict with properties that enhance printing. For example, natural low-viscosity polymers with cell-binding domains facilitate cell spreading and show superior biocompatibility but little structural integrity to maintain the deposited shape. Elevating viscosity via increased polymer concentration allows tuning for better printability and mechanical properties but negatively influences cellular requirements [160]. Likewise, a single component will not provide cells with sufficient biochemical cues for proper tissue remodeling. This compromise between biocompatibility and printability properties to make acceptable bioinks is referred to as the biofabrication window [161]. Limitations in the properties of single-component

bioinks have led to the emergence of advanced multimaterial strategies. In multimaterial bioprinting, different materials are simultaneously or successively delivered and positioned on the printing bed from separate reservoirs or cartridges.

Depending on their print-head system and working mechanism, recent multimaterial bioprinting approaches for TEVGs can be divided into three main categories: (i) single-head bioprinting with manual application of the cross-linking solution, (ii) multihead multimaterial bioprinting, and (iii) multiaxial multimaterial bioprinting. Tubular macrochannels can be printed in a traditional layer-by-layer approach on a conventional planar printing bed, but hydrogels printed in this way suffer from deformation, tend to sag, and do not allow bridging due to insufficient support [148]. Therefore, recent strategies changed to a rotating rod as a cylindrical printing bed, allowing for more flexibility concerning length and porosity [148].

Table 5 summarizes bioink compositions for TEVGs published in recent years with various physiological outcomes. In recent years, bioink compositions have become increasingly intricate. A noticeable trend is the preference for natural materials, specifically protein-based gels, such as GelMA/gelatin, collagen, and fibrinogen, which have gained popularity. Conversely, the usage of alginate has decreased. Polysaccharides are added in minimal quantities, primarily to alter viscosity. Except for PEGDA as a cross-linker and to enhance printability, synthetic polymers are not widely used. Fibrillary polymers are used to provide biochemical and physical guidance to cells. One special multimaterial bioink, UNION bioink, combines gelatin, HA, recombinant elastin-like protein, and PEG [162].

Recent bioprinting techniques have resulted in TEVGs with only two layers instead of three. This may be attributed to the fact that creating a thicker wall necessitates more sophisticated techniques and the presence of vasa vasorum. Although the mechanical properties of these constructs are approaching those of native vessels, they have not yet achieved parity.

Conclusions and future perspectives

3D bioprinting has long been subjected to trade-offs between physicochemical and biological outcomes. However, bioinks that satisfy mechanical and biological needs are still lacking. Due to their extensive interactions with their ECM, vascular cells are more demanding for TE. Hydrogels must provide physiological environments that guide cellular behavior and facilitate cell attachment and cell–cell interactions by providing ligands for integrins and DDRs. Replicating the complex and diverse vascular ECM is essential for developing bioinks that recapitulate key features of native blood vessels, including lumenization, barrier performance, and expression of vascular-specific markers. Stiffness and elastic modulus are not primarily important as printing parameters for a dimensionally stable product but decisive parameters for guidance of cellular behaviors during the entire degradation and remodeling process. Despite the continued increase in various biocompatible synthetic materials available, there has been a shift toward using natural rather than synthetic bioinks for extrusion bioprinting, dominated by alginate and gelatin alone or in combination with other biomaterials. Current bioinks are complex multimaterial blends that combine the advantages of several materials and overcome the individual limitations of the single components.

The successful transformation into a functional TEVG is highly dependent on cellular behavior, which is connected to the biochemical and mechanobiological signaling properties of vascular ECM tissue. Therefore, establishing a native-like tissue organization is key for overcoming current bioink limitations, and natural or biomimetic polymers are most suitable. ECM degradation in response to specific angiogenic stimuli leads to the release of factors that further impact ECM maturation in terms of vascular remodeling [60]. This also bears the risk of degradation or uncontrollable partial modification of matrix molecules. However, to take advantage of this finely tuned natural process, natural or biomimetic polymers should incorporate as many native signals as possible into the hydrogel. It is not yet understood which signals are vital to make cells thrive and which are sufficient to stimulate cells. Therefore, the idea is that the bioink used must provide a micromilieu that allows as many native signals as possible. The key factors to consider must include the engagement of integrins for VEGF, FGF, EGF, or PDGF.

Substrate stiffness should be adjusted to about 30 kPa; thus, nanofibers within the hydrogel may facilitate motility and cell adhesion. Incorporating these cues into the hydrogel creates a microenvironment that regards the specific features of a vascular cell type in a manner that resembles native tissue. Thus, specific bioinks should be developed for bioprinting each specific vascular cell wall element: the tunica intima, media, and adventitia.

This review considered the scaffold-free construction of TEVGs out of bioinks as very challenging. To the authors' knowledge, no clinically established TEVG is produced solely consisting of a form-stable bioink. With a view on all bioink materials available so far, this review considered semisynthetic bioinks with self-assembly tendency and no need for cytotoxic agents for cross-linking to have the best features for that purpose.

Table 5 Currently used bioink compositions and printing strategies for vascular TE. Recent bioprinting approaches of different research groups to create macrochannels using diverse bioinks (derived from a natural source or semisynthetic hydrogels), procedures, and printing technologies

Method	Product	Bioink composition	Outcome	Reference
Single-head bioprinting on a rotating rod, thrombin cross-linking	Macrochannel bilayer	10 mg/mL fibrinogen 7.5% (0.075 g/mL) heat-treated gelatin	Increased elastic moduli and high burst pressure	[202]
Single-head bioprinting and photo-cross-linking with Irgacure 2959	Macrochannel bilayer; 4 mm diameter	ECs: 6% GelMA + 2% gelatin; 0.3% HA + 10% glycerol; SMCs: 4% GelMA + 4% gelatin; 0.3% HA + 10% glycerol	Suturable graft, barely tight junctions, but expression of cell-specific markers	[203]
Printed cylindrical vessel and photo-cross-linking with Irgacure 2959 and manual EC seeding	Macrochannel bilayer	5%–10% GelMA 0%–5% PEGDA 1%–5% 2D nanosilicates	Conserved SMC proliferating phenotype	[131]
Electrospun PCL scaffold on rotating rod, single-head bioprinting of SMCs, photo-cross-linking/seeding of ECs	1.78 mm macrochannel bilayer	5% GelMA; 3% gelatin; 0.35% HA; 10% glycerol	Suture retention, intact EC monolayer, partly spindle-shaped morphology of SMCs	[204]
Single-head droplet-based bioprinting of a sacrificial HUVEC-containing gelatin rod using fibrinogen/SMCs and thrombin as cross-linker followed by fibroblast-laden collagen–fibrinogen blend	Three-layered macrochannel with 1 mm wall thickness and 425 μm wall thickness	ECs: 5% gelatin; SMCs: 0.625%–2.5% fibrinogen; Cross-linker: 4 U/mL thrombin, 0.51 mg/mL $CaCl_2$; AFs: 0.18% (volume fraction) collagen / fibrinogen (25 mg/mL) and $CaCl_2$ (0.51 mg/mL)/antibiotics and transglutaminase and tranexamic acid (0.16%, volume fraction)	Network formation and highly stretched morphology after four days of cultivation	[68]
Triaxial concentric bioprinting with sacrificial core (pluronic + Ca^{2+}), HUVECs and SMC shell by shell	Macrochannel bilayer; 2–4 mm diameter	3% vascular dECM (0.5%), alginate; SMCs/AFs: alginate and $CaCl_2$; ECs: collagen	Intact EC monolayer and circumferentially orientated SMCs, in vivo anastomoses in a rat model	[106]
A coaxial 3D plotter platform for SMCs, manual seeding of ECs	Macrochannel bilayer; 1.6 mm	SMC-laden 10% GelMA; 1% PEGDA; 1% alginate; 0.01 mg/mL lyase	Histological analyses indicating angiogenesis	[195]
Coaxial (venous grafts), triaxial (arterial grafts) bioprinting with $CaCl_2$ cross-linker in the core	Macrochannel bilayer; 1–5 mm diameter	1% alginate, 15% gelatin, transglutaminase	Intact monolayer; vasoreactive SMC layer; Young's modulus of 63.4 kPa; 240.4% stretchability; high burst pressure	[127]

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Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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