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Hydrogels to Model 3D *in vitro* Microenvironment of Tumor Vascularization

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Abstract

A growing number of failing clinical trials for cancer therapy is substantiating the need to upgrade the current practice in culturing tumor cells and modeling tumor angiogenesis *in vitro*. Many attempts have been made to engineer vasculature *in vitro* by utilizing hydrogels, but the application of these tools in simulating *in vivo* tumor angiogenesis is still very new. In this review, we explore current use of hydrogels and their design parameters to engineer vasculogenesis and angiogenesis and to evaluate the angiogenic capability of cancerous cells and tissues. When coupled with other technologies such as lithography and three-dimensional printing, one can even create an advanced microvessel model as microfluidic channels to more accurately capture the native angiogenesis process.

Keywords

Angiogenesis; Hydrogel; Tumor modeling; Three-dimensional cell culture; ECM remodeling

1. Introduction

Despite the overall decreasing trend of the cancer mortality rate, over 1.6 million people in the U.S are expected to suffer from cancer in 2013 with 580,000 estimated deaths [1]. In an effort to supersede the conventional treatments involving chemotherapy and radiation, various attempts have been made to discover new drugs with antitumor activity. However, clinical trials are very costly and often slowed down by high failure rates, commonly due to misguided preclinical models. Therefore, a more extensive analysis at the preclinical stage is required to more accurately predict the outcomes of clinical trials [2]. A growing number of researchers are now focusing on targeting biomarkers to accelerate the drug development process, minimize the cost, and maximize the benefit from early clinical trials [2, 3].

Particularly, angiogenesis has been an attractive target for anti-cancer drugs [4]. As the unregulated tumor growth continues, exacerbated oxygen and nutrient deprivation turns tumors into the angiogenic phenotype, triggering the release of angiogenic growth factors and cytokines, such as vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8), to the microenvironment [5, 6]. This dysregulated signaling pathway activates the nearby endothelial cells (EC) and perivascular cells, which ultimately results in the recruitment of new blood vessels to the area to support further tumor growth [6]. Eventually, these vessels would provide means for metastasis [7]. Inhibiting this angiogenic process has been one of the main foci of modern cancer research, but many of the recent clinical studies have reported various side effects of antiangiogenic therapies that utilizes small molecule inhibitors (such as bevacizumab, sunitinib, and sorafenib), including hypertension, impaired wound healing, coagulation, and, in some cases, increased tumor activity and metastatic acceleration [8–12]. More importantly, currently observed benefits from this strategy are transient since tumors are capable of overcoming the anti-angiogenic condition by employing different pathways (for example, vasculogenesis, vascular mimicry and vessel co-option) to remodel their neighboring blood vessels [6, 13–15].

More comprehensive investigation of tumor angiogenesis and identification of robust tumor angiogenic biomarkers are thus vital to developing viable cancer treatments. However, a lack of competent preclinical models often hinders successful subsequent clinical trials. Animal *in vivo* xenograft models are commonly used, but often cannot represent the disease sufficiently due to physical differences from humans. For example, tumors in a murine xenograft model grow relatively faster than human tumors, which results in immature blood vessels that cannot compare with tumorigenic vessels that have been established for a longer period of time [16, 17]. In addition, key parameters that affect tumor progression, including oxygen tension, nutrient gradients, and mechanical forces, cannot be easily controlled and manipulated in these models [9]. Imaging tumor vasculature *in vivo* has been particularly challenging as well, making it difficult to evaluate the benefits from anti-angiogenic therapies [15, 18]. To address these issues, investigators have been developing various alternative *in vitro* models for cancer cell growth and vascularization [19–24]. For this approach, the validity of a model would depend on how closely it can mimic the *in vivo* conditions. Up to this date, the majority of *in vitro* cancer studies have used two-dimensional (2D) monolayer cultures, where cells are usually grown on a plastic plane [25]. However, cell-cell and cell-extracellular matrix (ECM) interactions that are essential for tumor growth and angiogenesis cannot be recapitulated in 2D models, so these models may produce misleading results and provide wrong guidance for future clinical trials.

In fact, growing numbers of cancer studies are now utilizing three-dimensional (3D) culture models, and, not surprisingly, many have observed significantly distinct responses compared to the traditional 2D models. By encouraging cell-cell and cell-ECM interactions, 3D models support increased release of vascular growth factors, increased aggressiveness and metastatic potential, slower proliferation, increased resistance to anti-cancer drugs and radiation therapy, and physiological gene-expression profiles, all of which are characteristics of tumor cells *in vivo* [24–32]. In addition, integrin-mediated cell attachment to the 3D matrix and remodeling of ECM via matrix metalloproteinase (MMPs) is critical for

proliferation and survival for both tumor cells and ECs [27, 33]. Specifically for tumor angiogenesis, the remodeled ECM and immobilized molecular cues from tumor cells support EC recruitment and morphogenesis that leads to vascularization around the tissue [6, 33]. It has also been shown that ECs respond to different topography, geometry, and the mechanical stiffness of their 3D microenvironment. In their physiological environment, vessels exist as multi-cellular tubes with hollow lumens of circular cross-section, where ECs are polarized to interact with the ECM surrounding the vessels and respond to the shear stress from the fluid flow inside the lumens [33, 34]. Together with shear stress, 3D geometrical cues have shown to contribute to the alignment and the elongation of the ECs inside the vessels, which directly relate to cell function and survival *in vivo* and cannot be observed in a static 2D culture [35–38]. In addition, we have recently demonstrated *in vitro* that the 3D curvature on which the ECs are grown results in circumferential ECM deposition and organization [39]. These observations demonstrate the advantages of utilizing 3D architectural designs *in vitro* to model the physiological microenvironments of various tissues *in vitro*. These models are prevalent in the field of tissue engineering, which has allowed researchers to design systems that mimic the physiological cell-cell and cell-ECM interactions of a variety of tissue types [21, 40–42]. Since tumor vascularization occurs within a 3D physiological environment just like other tissues, similar engineering principles and techniques can be applied to the model in order to study cancer biology.

Hydrogels are hydrophilic polymeric networks that are commonly used for creating 3D *in vitro* models of tissues. Hydrogels provide means of tuning the mechanical strength and chemical structures of the cellular microenvironment. Studies have shown that different stiffness of gels created by varying crosslinking densities can effect the proliferation, survival, and migration of the embedded cells and can also cue differentiation of stem cells to specific lineages [43–45]. In addition, hydrogels can be chemically modified to present cell-attaching sites (such as RGD amino acid sequence) and MMP-degradable sites which is crucial for tumor progression, endothelial migration, and, ultimately, tumor angiogenesis [6, 28, 45, 46]. Recently, hydrogels have been incorporated with other technologies such as lithography, microfabrication, and microfluidics to develop complex blood vessels, which show promise for more advanced and clinically relevant tumor angiogenesis models [47–49].

The importance of 3D *in vitro* models is becoming evident as more and more studies benefit from the tunable platform by hydrogels that gives us more control over the microenvironment of a tissue. Here, we first review the mechanisms of tumor vascularization, and explore natural and synthetic hydrogels and design parameters commonly employed to form tumors and create vasculatures *in vitro*. We then examine hydrogel-based angiogenesis assays that are currently being used in cancer studies and move on to explore recent advanced *in vitro* models that recapitulate tumor angiogenesis from microvascular networks.

2. Tumor vascularization mechanisms

Angiogenesis is an intricate process that involves cell-ECM interaction and cell-cell interaction not only between ECs, but also between ECs and other cell types such as mural

cells (pericytes and smooth muscle cells), fibroblasts, and inflammatory cells. It has been one of the key topics for cancer biology for decades due to its close association with tumor development, maintenance, and survival. The dysregulated nature of cancer growth provides unique features to tumor-associated blood vessels that may be critical for cancer therapies and should be sufficiently replicated in *in vitro* models to obtain better guidance for clinical trials. In this section, we briefly describe biomolecular and cellular mechanisms of tumor vascularization.

Initially, a tumor can grow with passive diffusion of oxygen and nutrients from the surrounding stroma without any support from blood vessels. However, as the tumor lesion grows to 1–2 mm^3, the cells at its core start to experience hypoxia and nutrient deprivation and accumulate hypoxia inducible factors (HIFs) such as HIF–1α, which triggers a phenotypic transition known as the angiogenic switch [50, 51]. Activation of the pathway leads to overexpression of cytokines, growth factors, and other soluble factors that breaks the balance between pro- and antiangiogenic factors. This dysregulated cascade ultimately recruits new blood vessels to the tumor site. The generalized overview of tumor angiogenesis is illustrated in Figure 1.

The most well-understood tumor angiogenic signaling pathways involve VEGF, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and angiopoietin (Ang), which are intricately coordinated and overlapped. Tumor angiogenesis begins with activation of pericytes by tumor-secreted VEGF and Ang-2, which leads to the detachment of the cells from the vessel and acquiring more proliferative phenotype [8, 52]. The ECs at these sites thus are exposed to the cytokines and growth factors secreted by tumor cells and activated pericytes as well as to the interstitial collagen-rich ECM as the basement membrane is degraded [8, 33, 52]. In physiological conditions, the hyperpermeability of the vessel allows the plasma proteins to leak into the surrounding matrix, setting up a provisional matrix (composed of fibrin, vitronectin, and fibronectin from plasma mixed within the preexisting collagens) into which new vessels can sprout [33].

Vessel sprouting occurs through a close-knit combination of biomolecular signaling, ECM remodeling, and cellular migration. PDGFβ and VEGF secreted from tumor cells recruits pericytes to the tumor which secretes membrane MMP and paves the way for EC vessel sprout migration [53]. The ECs at the tips of the sprouts also express MMP on their membranes (membrane type-1 MMP) to help facilitate the expansion of the new vascular structure [54]. In the stalk cells of the new sprout, endothelium-specific vascular endothelial-phosphotyprosine phosphatase (VE-PTP) has shown to play an important role in EC polarization and lumen formation by inactivating VEGF receptor-2 in EC junctions [55]. Around certain types of tumors, hypoxia induces overexpression of lysyl oxidase (LOX) which stiffens ECM by cross-linking the collagen fibers in the tumor stroma [54]. Different types of tumors can overexpress other ECM remodeling enzymes and different types of MMPs that give tumor-associated ECMs features that differentiate them from normal ECM in terms of composition, orientation, density, and mechanical properties [56]. In addition, tumor-associated fibroblasts are known to deposit provisional or tumor-specific ECM in the tumor stroma as well [6]. These different enzymes remodel and cleave ECM into active

fragments such as endostatin, tumstatin, and canstatin, that can collaborate with other soluble factors to guide tumor vascularization [51, 54].

Recently, studies have suggested that tumor vascularization involves not only angiogenesis, but also vasculogenesis, where endothelial progenitor cells (EPCs) from the bone marrow are recruited to the vasculature site [8]. In fact, Kerbel and his colleagues observed marked suppression of tumor growth when antiangiogenic therapy was coupled with a low-dose of metronomic cyclophosphamide, a drug that inhibits EPC mobilization [57]. The mechanism for EPC recruitment and mobilization is not well established and remains to be explained, but several studies suggest it is regulated by factors such as VEGF, MMP-9, HIF-1α, FGF, and IL-8 [58–60]. This mechanism is covered in more detail in Rafii and his colleagues' review [59]. In addition, cancer stem cells can also differentiate into endothelial cells and pericytes to support tumor vasculature, which is a topic thoroughly explored in Liu and Ouyang's review [61].

In developmental angiogenesis, the last stage consists of new basement membrane formation and mural cell attachment to the newly formed vessels that leads to vascular stabilization and maturation [33]. In contrast, tumor-driven angiogenesis lacks this stage since tumors continue secreting hypoxic and angiogenic factors as they require more and more oxygen and nutrients to support their unlimited, dysregulated growth. For example, although pericytes are recruited to the tumor site, they do not properly attach to the newly formed vessels and facilitate basement membrane production [52]. These impaired vessels are therefore porous and leaky, causing irregular blood flow and providing a platform for tumor metastasis [6].

3. Engineering microenvironments for cancer cell growth and angiogenesis

Current approaches for the development of *in vitro* tumor models aspire to recapitulate the native microenvironments *in vivo* using 3D scaffolds in order to create better preclinical cancer models. Since the tumor microenvironments play critical roles in tumorigenesis [5, 62], many researchers have focused on reconstructing the complex and myriad microenvironment conditions, in which the native ECMs, using bioinspired materials. Specifically, hydrogels have been widely utilized as 3D cellular microenvironments due to their ECM-like biophysical properties [63–66]. Hydrogel materials can provide dynamic microenvironments to regulate cell fate through either cell-cell or cell-matrix interactions. These hydrogel materials have shown to be adequate 3D cellular microenvironments for supporting cell adhesion and growth of various cell types as well as cancer cells [65–67]. To create engineered microenvironments for supporting cancer cell growth and angiogenesis, a variety of natural and synthetic hydrogel materials have been used as 3D artificial tumor microenvironments that can provide mechanical support while regulating tumor behaviors within the matrix. Figure 2 illustrates the representative engineered tumor microenvironments created by using natural and synthetic polymers. These hydrogel materials are fabricated through numerous physical and/or chemical crosslinking reactions [68, 69]. In addition, by controlling physicochemical and biological properties of the hydrogel materials, such as cell adhesion ligands, proteolytic degradable sites, matrix stiffness, and 3D topography, more improved *in vitro* tumor models are engineered to better

understand cancer biology. In this section, we discuss the hydrogel materials from natural and synthetic hydrogels to provide 3D artificial tumor microenvironment for supporting tumor growth and angiogenesis.

3.1. Natural hydrogel materials

The ECMs, consisting of proteoglycans, non-proteoglycans, protein fibers, and glycoproteins, play a critical role in regulating cellular behaviors [70–73]. Thus, materials derived from natural ECM have been widely used as 3D microenvironments for supporting tumor growth and angiogenesis.

Matrigel, a basement membrane-derived hydrogel with laminin as the primary component, is extracted from Engelbrecth-Holm-Swarm (EHS) mouse tumor cells [74]. Matrigel has been widely utilized as 3D microenvironment to support tumor growth and angiogenesis owing to its inherent cytocompatibility, cell adhesion sites, and capacity to vary its physical properties [75]. Bissell and colleagues have successfully recapitulated *in vivo* characteristics of breast cancer invasion and their phenotypes through 3D culture of human breast cancer cells using Matrigel [76]. They demonstrated that each of the cells showed four distinct morphologies including round, mass, grape-like, stellate shapes, which were associated with tumor cell invasiveness and with cell lines originating from metastases. In addition, they also demonstrated that reliable differences in gene encoding signal transduction proteins emerge when tumor cells were cultured in a 3D microenvironment. In addition, Matrigel has shown to be an effective angiogenic assay tool alone or with various vascular cells, which can be used to identify pro- and anti-angiogenic molecules including those involved in genetic and signaling pathways of angiogenesis [77, 78]. It has been used with vascular cells as well, such as EPCs, human umbilical vein cells (HUVECs), endothelial cells derived from SV mice (strain A) (SVEC4-10), and human dermal microvascular endothelial cell-1 (HDMEC-1) [79–81]. For example, Watt and colleagues have utilized growth factor-reduced Matrigel along with umbilical cord blood-derived endothelial colony-forming cells (ECFCs) in a recent study [81]. They tested the inhibition of angiogenesis using two angiogenic inhibitors, suramin and SU6668, to find the best quantification method for evaluating the efficacy of angiogenic stimulants or inhibitors compared to two quantification methods, such as Angiosys and Wimasis. In addition, angiogenic assays using Matrigel are now combined with other emerging technologies to create more advanced tools to evaluate angiogenesis. Kleinman and colleagues have established a protocol that can provide a rapid, quantitative and reliable *in vitro* high-throughput angiogenesis assay [77].

Collagen, the most abundant proteins in the native ECMs, has been reported to support vascular morphogenesis and network formation of various kinds of vascular cells. Collagen hydrogels have been extensively used as cellular microenvironments for a broad range of biomedical applications due to its biocompatibility and biodegradability [82, 83]. Collagen hydrogels can be fabricated through multiple cross-linking methods that result in fibrous architectures similar to collagen found in native ECM [84, 85]. These hydrogels can provide bioactive microenvironments for supporting cellular behaviors due to their cellular activities, such as cell adhesion ligands and proteolytic-degradable sites that are crucial for ECM remodeling. Marissa and colleagues have established a 3D engineered tumor models as a

platform for better understanding *in vitro* solid tumor biology [30]. They demonstrated that MDA-MB-231 breast cancer cells cultured within collagen type I hydrogel formed 3D cancer spheroids (diameter 150–200 μm), inducing necrosis and hypoxia in the core. They also demonstrated that the bioengineered tumor showed promising angiogenic potential through upregulation of HIF-1α and VEGF-A compared to the 2D monolayer culture of MDA-MB-231 cells. More recently, they have investigated tumor angiogenesis induced by a tumor and endothelial cell co-culture system using collagen type I hydrogels [86]. They successfully utilized an *in vitro* tumor angiogenesis model driven solely by paracrine effect between MDA-MB-231 cells and telomerase-immortalized human microvascular endothelial (TIME) cells. The engineered tumor model consists of three hydrogel layers; 1) bottom layer, MDA-MB-231cells within collagen hydrogels; 2) middle layer, acellular collagen hydrogel; 3) top layer, TIME cells within collagen hydrogels. Using the models, they found that TIME cells co-cultured with the MDA-MB-231 cells showed a significant increase in cell proliferation and promote vascular morphogenesis of TIME cells as well as invasively sprout into acellualr collagen matrix, and the angiogenic effect dependent on VEGF secretion, matrix concentration, and culture periods.

Fibrinogen is a large glycoprotein found in plasma that plays a critical role in blood clotting, fibrinolysis, cellular and matrix interactions, inflammatory response, wound healing, and neoplasia [87]. Fibrin hydrogels are formed via polymerization of fibrinogen with thrombin and calcium ions. Fibrin hydrogels have been widely used as an artificial microenvironment because they have a nano/macro fibrous architecture that mimics the native ECMs. Huang and his colleagues have found that hydrogel stiffness plays critical roles in stem-cell-like cancer cell characteristics of murine B16-F1 melanoma cells [88]. They encapsulated the cells within fibrin hydrogels with different mechanical properties ranging from 90 to 1050 Pa and evaluated their morphology and stem-cell-like properties. When the cells cultured in 90 Pa fibrin gel, they formed larger cancer spheroids and expressed higher levels of stem cell markers, resulting in aggressive tumor metastasis into lung tissue even injecting 10~100 cells into mice subcutaneous tissues.

Despite the extensive uses of the natural hydrogel materials as 3D microenvironments for tumor growth and angiogenesis, they still have critical drawbacks to be utilized as well-defined tumor microenvironments due to relatively narrow range of physical properties (*i.e.* stiffness), limited ability to control the matrix rigidity and cell adhesion peptide density independently and inherent batch-to-batch variability [89].

3.2. Synthetic hydrogel materials

To overcome the limitations of natural hydrogels, increasing effort has been focused on developing synthetic hydrogels from natural and synthetic polymers. Many synthetic hydrogels have been utilized as engineered microenvironment to support the growth of cancer cells and tumor angiogenesis by controlling myriad parameters, such as proteolytic degradability, cell adhesion site, and matrix stiffness.

Hyaluronic acid (HA) has shown to be a critical ECM molecule for tumor progression and invasiveness [90, 91]. Farach-Carson and colleagues have developed a 3D culture system for poorly adherent bone metastatic prostate cancer cells (C4-2B) as an *in vitro* platform for

anti-cancer drug screening [92]. To generate a biomimetic *in vitro* tumor model, they synthesized HA derivatives including HA-aldehyde and HA-hydrazide that can be crosslinked *via* imine formation with dehydration. Cultured cells within a 3D HA matrix exhibited distinct clustered structures, reminiscent of real tumors, and also showed higher drug resistance compared to 2D monolayer cultures.

The spatiotemporal complexity of soluble factor signaling has been implicated as a crucial factor in tumor progression [89]. Recently, they successfully utilized an engineered tumor microenvironment in a bilayer using HA hydrogels and epidermal growth factor-loaded heparin hydrogel particles (EGF-HGP) [93]. To mimic the tumor and stromal interaction, EGF-HGPs were incorporated within the top gel layer while LNCaP prostate cancer cells were encapsulated within the bottom layer. Sustained release of EGF allowed tumor growth enlarging tumoroids with an average diameter of 85 μm after 7 days in culture. They demonstrated that the engineered tumor spheroids significantly increased the expression of VEGF165 and IL-8, suggesting that the HA tumor models provided a useful platform for the study of tumor cell responses to growth factors.

In addition, HA hydrogels have been utilized as 3D microenvironments for creating *de novo* engineered vasculature. Toward this, HA hydrogels should be tailored with cell adhesion ligands (*i.e.* RGD peptide) and MMP-sensitive cleavable sites to be utilized as cellar microenvironments. Recently, we have developed *in situ* crosslinkable HA hydrogels decorated with RGD and MMP-sensitive peptide to support vascular morphogenesis of ECFCs [21, 94]. We synthesized acrylated HA (AHA) polymer as a backbone and mixed it with RGD peptide and MMP cleavable crosslinker to form hydrogels though Michael-type addition reaction. We demonstrated that the synthetic AHA hydrogels support the process of vascular morphogenesis, including vacuole and lumen formation, vascular branching and sprouting, through integrin-mediated cell adhesion and MMP-mediated matrix degradation. We also have created *de novo* vasculature using the HA hydrogels with ECFCs [94] and early vascular cells (EVCs), derived from human pluripotent stem cells (hPSCs) [95]. AHA hydrogels provided 3D cellular microenvironments for supporting self-organized microvascular networks from the cells to yield multicellular networks that survived implantation *in vivo* and integrated with the host circulatory system. Moreover, we evaluated the effect of matrix remodeling of HA hydrogels on vascular morphogenesis and sprout of ECFCs through creating spatial pattering *via* secondary radical polymerization [21]. The results demonstrated that through controlling matrix degradation cues, the AHA hydrogels could either support or inhibit *in vitro* vasculogenesis and angiogenesis of ECFCs as well as angiogenesis from *ex ovo* choriallantoic membranes.

Poly(ethylene glycol) (PEG)-based hydrogel materials are known as the representative synthetic biomaterials. These PEG-based hydrogel materials have been shown to be used as 3D microenvironments to support cell fate due to biocompatibility, high water content, and multi-tunable properties. Due to the lack of bioactivities of PEG molecules, PEG hydrogels should be decorated with bioactive molecules (*i.e.* cell adhesion peptide, RGD; proteolytic degradable site, MMP-sensitive peptide sequence), or incorporated with biopolymers (*i.e.* collagen, gelatin, or fibrinogen) to serve as cellular microenvironments. These engineered matrices have been investigated for supporting tumor growth and angiogenesis. Recently,

West and colleagues have investigated the effect of PEG hydrogels with independently tunable biochemistry and mechanical properties on epithelial morphogenesis of a metastatic cell line (344SQ) and EMT in a lung adenocarcinoma model [67]. They demonstrated that 344sQ cells cultured within PEG decorated with RGD and MMP-degradable sites showed lumenized epithelial spheres comparable to that seen with 3D cultures in Matrigel. Moreover, they found that altering the matrix stiffness and RGD concentrations significantly affected epithelial morphogenesis. These results demonstrated that the engineered microenvironment could be utilized to define matrix cues that can affect tumor morphogenesis, suggesting their potential for cancer biology. Similar studies were conducted by Jabbari and colleagues. They investigated the effect of matrix stiffness of PEG hydrogels on the maintenance of cancer stem cells (CSCs) and their spheroid formation [96]. The PEG-based hydrogels were prepared with different mechanical properties ranging from 2.5 kPa to 47.1 kPa. Interestingly, 4T1 mouse breast cancer cells encapsulated within the hydrogels with 5.3 kPa modulus exhibited the largest tumorspheres and highest density of tumorspheres as well as highest expression of breast CSC markers CD44 and ABCG2. These results suggest that the PEG-based hydrogel can be used as a 3D engineered matrix to study the role of individual parameters in the tumor microenvironment on tumorigenesis and maintenance of CSCs.

4. Modeling tumor angiogenesis *in vitro*

One of the earliest preclinical methods for evaluating tumor angiogenesis was the use of *in vivo* murine models and histologically measuring the microvessel density using endothelial markers [97, 98]. Although this method is still a useful prognostic indicator, microvessel density is usually not predictive of antiangiogenic therapies, and its use has decreased over the years because of its inability to accurately predict clinical outcomes [18]. Recent efforts have focused on developing functional and targeted imaging techniques to better visualize and model tumor angiogenesis *in vivo* [99–101] but these approaches require specialized software and equipment to analyze, and *in vivo* samples are expensive and lack results that are consistent and highly comparable [102]. In addition, high-throughput drug screening through *in vivo* models is impractical.

Many 2D *in vitro* models' incompetence in recapitulating physiological tumor microenvironment and producing predictive results has halted many researchers to use them for imperative preclinical studies. Recent progress in developing matrices with comparable *in vivo* conditions using hydrogels has slowly helped *in vitro* studies to gain more reliability. In this section, we review different strategies that are currently being used to evaluate angiogenesis using hydrogels and explore advanced biomimetic models that can potentially be used for studying tumor angiogenesis and screening of antiangiogenic drugs.

4.1. Three-dimensional tumor angiogenesis assays

As mentioned in the previous section, various matrices including nature-derived and synthetic materials have proven to be able to manipulate the behavior of ECs to form vascular structures inside the hydrogel. Many studies have been harnessing ECs' ability to sprout in these environments to study the effect of different pro- and antiangiogenic factors on angiogenesis [102–104]. Generally, these models involve a thin hydrogel layer with a

monolayer of ECs seeded on the top of the gel (Fig. 3a). The degree of angiogenesis is evaluated and quantified by calculating the invasion density and measuring the distance the EC sprouts have invaded into the gel. Similar measurements can be obtained by coating microcarrier beads with ECs and embedding them in a matrix [104]. These approaches are currently used to study angiogenesis from a variety of perspectives, such as screening antiangiogenic reagents and varying the stiffness of the hydrogel matrix [21, 105]. Endothelial cells can also be co-cultured with tumor cells embedded inside the matrix to study angiogenesis induced by these cells in absence of exogenous growth factors [106]. In addition, one can manipulate different design parameters of the gel matrix to study the effect of ECM composition and mechanical properties on angiogenesis.

The EC invasion assay has allowed investigators to conduct basic experiments involving angiogenesis, but it lacks other cell types that play an imperative role during angiogenesis, such as pericytes. As discussed in the previous section, the recruitment of mural cells is a critical part of tumor vascularization, so modeling the interaction between tumor cells and other multiple cell types could provide a better understanding of tumor angiogenesis [107–109]. *Ex vivo* assays can provide a more physiologically relevant *in vitro* angiogenesis model compared to the invasion assay by allowing the regulation of neovascularization that involves several cell types [110]. These assays generally involve embedding vascular explants from animals or humans inside a hydrogel matrix (Fig. 3b). A rat aortic ring assay was first developed in 1990, but now the explants are collected from pigs as well [104, 110]. Recently, Bussolino and colleagues have developed a new *ex vivo* assay that uses arterial explants from human umbilical cords as it is more clinically relevant [34]. Their model addresses disadvantages of other animal *ex vivo* models such as age- and strain-dependent variability, autonomous angiogenic capability, and species-specific angiogenic markers that results in misleading data for the translation to clinical trials [34]. They showed that this approach can be used to model human tumor angiogenesis by co-culturing the explants with cancer cell aggregates embedded in the basal membrane extract (BME) gel without additional growth factors. Maximal angiogenic outgrowth was observed at days 30 to 35 with more branched structures compared to outgrowth in the presence of medium alone [34]. With the use of live-imaging technology, the authors note their model's potential for dynamic analysis of tumor angiogenesis within a controlled microenvironment [34]. Another *ex vivo* model was also established not long ago by Radisic and colleagues which creates directed microvessel growth from vein and artery explants. The vein and artery explants from human umbilical cords were placed on each side of a PDMS surface coated with chitosan-collagen hydrogel, 0.5–1mm apart from each other [111]. The gap between the explants was micropatterned with 50μm wide grooves created by soft lithography, which served as topographical cues to guide the outgrowth of vessels from the explants. The authors stimulated and accelerated vessel growth by an angiogenic factor Tβ4 encapsulated inside the gel, but the model has a potential for studying tumor angiogenesis similar to Bussolino's model.

4.2 Advanced biomimetic models for angiogenesis

Although the above assays may provide a general and preliminary guidance for studying the angiogenic capability of a given tissue model, these assays are limited in their ability to

predict the angiogenic activities *in vivo* due to their little anatomical resemblance to the native process, including continuous fluid flow that influences the EC gene expression profile [104]. In order to address these issues and incorporate additional complexity, recent efforts have combined hydrogel matrices with microfluidic channels to create native microvessels from which new vessels can sprout. Unlike the sprouting assay described above that studies invasion of individual ECs in one plane, these models allow true 3D observation of angiogenesis from native microvessels with fluid flow that closely resembles the *in vivo* conditions.

The robust method to create a microfluidic channel within a hydrogel matrix is to place a cylindrical mold (such as a needle) through the gel as it crosslinks. After the crosslinking of the hydrogel matrix, the needle is gently withdrawn, and the matrix is then left with a cylindrical microfluidic channel that can be used for perfusion studies. For example, Tien and colleagues used a 120μm diameter, 15mm long stainless steel needle coated with 1% bovine serum albumin to create a microfluidic tube inside their collagen hydrogel [112]. They then introduced ECs (HUVECs or HDMECs) into the tubes in a suspension to create a confluent cell layer attached along the tube. This created a microvessel with a viable barrier function and quick response to inflammatory stimuli [112]. Khademhosseini and his colleagues also used this method to test the capacity of their methacrylated gelatin hydrogel for creating microvessels. A gauge needle with 300μm diameter was used to mold the channel, and the gelatin hydrogel was embedded with fibroblasts [113]. Seeding HUVECs through the fabricated tube created functional perfusable microvessels that also demonstrated the potential for co-culture models for angiogenesis [113].

Strook and his colleagues have recently established *in vitro* microvascular networks supported by fluid flow by embedding microfluidic channels inside a collagen hydrogel [49, 114]. The 100μm wide grooves at the bottom of the top hydrogel layer are created by a lithographic process, which outlines the microvascular networks. When sealed in a plexiglass chamber with a bottom hydrogel layer, the grooved areas become microfluidic channels through which media can flow. Seeding HUVECs through the channel created a microvascular structure with a permeability barrier comparable to the physiological structure [49]. The ECs remodeled the surrounding collagen hydrogel during spreading and proliferation. Proangiogenic factors can be flowed through the channels and/or different types of cells can be embedded within the collagen hydrogel to induce angiogenesis in this model, which can be evaluated by the degree of EC sprouting from the established microvascular networks. In their study, Strook and his colleagues observed endothelial sprouting from the networks when human brain vascular pericytes, which are known to secrete both VEGF and FGF, were embedded into the interstitial collagen matrix [49]. Their model can also be used to investigate thrombosis by perfusing blood with physiological composition instead of media. The main purpose of the technique is to create vascularized tissue *in vitro*, but Strook highlights the model's high versatility that can also be applied to tumor systems to study the angiogenic capabilities and inflammatory profiles of tumors *in vitro* [6, 8]. A detailed method for fabricating the device can be found in their published protocol [114].

Advancement of 3D printing technology has recently inspired bioengineers to develop methods to —print organs *in vitro*. Termed —bioprinting, this technique utilizes layer-by-layer addition of —bioink in either droplets or strands that contain living cells [115]. Hydrogels have been used widely as the bioink that encapsulates the cells due to their high biocompatibility and tunable viscosity that allows the maintenance of the printed tissue's structural integrity [116]. Over time, these individual layers self-assemble into 3D organizations through spatial and environmental cues, similar to the process observed during the embryonic development [117]. By incorporating different cells types and hydrogels, bioprinting research aims to one day create complete functional organs with physiological complexity and sufficient vascularization. Using these principles, several attempts have been made to fabricate vessel-like constructs (Fig. 3c) [118, 119]. However, these efforts revealed several challenges, such as low scalability of the fabrication methods and prolonged time necessary for tissue self-assembly [120]. In addition, the resolution of the bioprinter presents a limitation in the size of the vascular structure that can be fabricated. Most importantly, engineering perfusable vascular lumen structures is also a difficult challenge [120].

Recently, Chen and his colleagues demonstrated a method to fabricate *in vitro* perfusable vascular networks using 3D-printed sacrificial molds (Fig. 3d). Unlike the traditional bioprinting method described above, their method can fabricate vessel molds with diameters as small as 150μm by changing the traveling velocity of the printing nozzle [121]. The 3D schematic of the vascular networks are first printed with carbohydrate glass, and this lattice is then embedded inside a monolithic ECM. The chemical stability and mechanical strength of the sacrificial lattice allow it to maintain its structural integrity during a wide range of ECM cross-linking processes, including chain entanglements (agarose), ionic interactions (alginate), enzymatic activity (fibrin), and protein precipitation (Matrigel) [121]. In addition, its optical transparency allows the matrix, such as a PEG-based hydrogel, to crosslink via photopolymerization without leaving shadowing artifacts [121]. Once the lattice is embedded, it is dissolved and flushed out of the network with water inflow, leaving hollow microfluidic channels from which vasculature can be constructed. To demonstrate their model's angiogenic capability, they co-cultured HUVECs with 10T1/2 mouse fibroblasts embedded in the interstitial ECM which resulted in single and multicellular sprouts extending from the established vessels into the gel [121]. Like Strook's model described above, a wide range of cells, including tumorigenic cells, can be embedded in the gel to investigate their angiogenic capabilities.

Lewis and colleagues took a step further and developed a new 3D bioprinting technique that combines Chen's sacrificial molding method with the traditional layer-by-layer bioprinting method. By co-printing sacrificial molds with two bioinks encapsulating different cell types, they were able to fabricate heterogeneous tissue constructs with perfusable microvascular channels [122]. For the molds for their vascular channels, they used Fluronic F127 triblock copolymer that transitions reversibly between liquid and gel state with a temperature change [123]. They were able to vary the diameter of these molds from 45μm to 500μm by changing the printing pressure and nozzle height [122]. To create a vascularized tissue construct, the fugitive Pluronic F127 ink is first printed directly onto a surface with methacrylated denatured collagen (GelMA) inks that encapsulate different cell types. The printed structure

is then embedded in pure GelMA ink to fully submerge the construct, and the final GelMA matrix is terminally crosslinked with a UV light source. After, the fugitive Pluronic F127 ink is liquefied and aspirated under low temperature, which creates hollow microfluidic channels that can be lined with ECs. Using this technique, Lewis and colleagues created a vascularized tissue construct with human neonatal dermal fibroblasts and 10T1/2 mouse fibroblasts that showed high viability after 7 days [122]. Like Chen's model, this technique allows the control of microvascular channel geometry, but in addition to that, type of cells as well as the location of these cell subunits can also be controlled by the co-printing method. Printing a complete organ is still far away, but with simple modifications, this model can enable a tighter control on engineering 3D cellular microenvironments and provide a pivotal *in vitro* platform for studying wound healing, angiogenesis, and tumor vascularization, all of which involve multiple cell types in physiological conditions [122].

5. Considerations and future directions for drug screening and nanomaterials applications

All the models above allow observation of angiogenesis from native-like vessels and exhibit features that can easily be adapted for cancer biology and oncology research. Possibilities extend from optimizing hydrogel parameters for tumor angiogenesis modeling to screening anti-angiogenic drugs for potential clinical studies in the near future. In addition, applying these models for studying cancer metastasis through blood vessels would require only a few adjustments to the designs. Changing the type of ECs may also be considered to model the lymphatic vessels as cancer cells can also metastasize through this system [124, 125]. Also, chemical composition of the flowing medium and the interstitial matrix as well as the cellular microenvironment of the system can be simultaneously controlled, which opens up an even wider variety of phenomena to be investigated. One can also utilize different cell-types of endothelial and tumor cells. For example, EPCs can be added to the flowing media to study their mobilization and recruitment to the tumor stroma *in vitro* to study tumor vasculogenesis in addition to angiogenesis.

Most synthetic hydrogels provide a uniform microenvironment with isotropic mechanical properties, but cells *in vivo* are exposed to a fibrous network of ECM that they interact with (such as collagen and fibronectin). Hydrogels that are formed by the self-assembly of amphiphilic peptides have gained much popularity for their ability to mimic the anisotropic fibrous nanotopography of natural ECM at a dimension that cannot be mimicked by electrospun fibers [126–129]. Recent studies suggest that these hydrogels can be tuned to create a favorable environment for angiogenesis and tumor growth [130, 131]. Recently, there has also been an effort to develop materials that combine the architectural features of the fibrous natural matrices with the tunability of synthetic hydrogels [132]. For example, Yu and his colleagues have developed a PEG-based hydrogel with collagen mimetic peptides that are incorporated to form crosslinks that mimic the hierarchic self-assembly of collagen fibers [133]. Incorporating advanced composite materials like this into the *in vitro* biomimetic models discussed in the above section could certainly be considered to make the models more physiologically relevant and specific for the tumor types under investigation.

In recent years, many studies have been exploring the use of nanomaterials for cancer therapeutics and tissue engineering [134]. Although an enormous amount of nanomaterials has been designed as tools for cancer therapeutics and diagnosis (defined as theragnosis), development of these therapies are still mostly done in traditional 2D or *in vivo* animal models. However, as discussed in the introduction, the 2D culture systems do not recapitulate the native 3D microenvironments, and *in vivo* models still have limitations. Therefore, many 3D engineered tumor models, which have the potential to bridge the gap between 2D culture systems and *in vivo* xenograft models, have been utilized as a platform to evaluate the therapeutic effects of nanomaterials. For example, Jia and colleagues have developed hydrogel-derived prostate cancer models to evaluate the therapeutic effect of doxorubicin (DOX)-loaded nanoparticles [135]. They prepared HA based hydrogels encapsulated with NCaP prostate cancer cells as a 3D tissue-engineered tumor model. They also generated DOX loaded-nanoparticles composed of amphiphilic polymer chains (hydrophilic PEG chains and hydrophobic polyester-based cyclic pendants) that can self-assemble in aqueous solutions. They observed that the cells cultured in HA hydrogels expressed significantly higher levels of multidrug-resistance (MDR) proteins compared to the cells cultured in 2D monolayer, resulting in higher resistance to the DOX-loaded nanoparticles. Zaman and colleagues have also utilized a 3D tumor model created by the encapsulation of multicellular tumor spheroids (MTS) into hydrogels to study tumor behavior and to evaluate the response of pharmacological activity of anti-cancer drugs in nanoparticles. Their MTSs were prepared with MDA-MB-231 cells and pediatric osteosarcoma (U2OS) cells and were encapsulated in collagen hydrogels [136]. In addition, they generated paclitaxel-loaded expansile nanoparticles (Pax-eNP) by oil-in-water mini-emulsion. Their Pax-eNP effectively prevented the growth of cancer spheroids within the 3D microenvironments compared to drug-free controls. These studies demonstrate that 3D engineered hydrogels can provide an *in vitro* tumor model with physiologically relevant drug resistance to evaluate the efficacy of nanomaterials based cancer therapeutics at the preclinical level. As the transport across the vessels into the tumor sites provides an additional drug delivery barrier, the *in vitro* biomimetic vascular models presented in the above section could recapitulate this challenge in combination with the drug-resistance that is emulated by the 3D engineered tumor model.

As mentioned previously, tumor-associated vessels have unique features such as higher permeability and tortuous vasculature organization. Recently, these unique features of tumor-associated vessels, termed enhanced permeability and retention (EPR), have shown to potentially be exploited for targeted drug delivery [137, 138]. However, the degree of the EPR effect can vary significantly depending on the type, the location, and the stage of tumor [139]. In addition, there can be significant heterogeneity of the vessel leakiness even within a single tumor type depending on the vessel structure, which is difficult to control in *in vivo* models [139]. Designing robust, biomimetic 3D *in vitro* models will enable researchers to test nanoparticles in a controlled environment. One way to generate a more physiologically-relevant models is by using with tumor-associated ECs, as these cells exhibit unique properties such as increased permeability, drug resistance, adhesion to tumor cells, angiogenic activities, and motility [6, 140, 141]. Another consideration could be controlling the leakiness of vessels in the *in vitro* model by utilizing nanoparticles that has been shown

to increase the vessel permeability, such as iron oxide or titanium dioxide, to develop an enhanced drug delivery strategy. Recent studies have shown that these nanoparticles induce EC leakiness via reactive oxygen species production or direct disruption of cell-cell junctions [142–144]. The degree at which these nanoparticles can extend the limitation of EPR effect would be an interesting topic to investigate, which would evidently benefit from using advanced *in vitro* vascular models based on the merits discussed in this review.

6. Conclusion

Three-dimensional culture models provide cell-cell and cell-matrix interactions that influences the morphology, gene expression profile, drug resistance, and other behaviors for multiple cancer cell types that cannot be recapitulated in 2D culture systems. Hydrogels have provided a powerful platform for fine-tuning and recapitulating the cancerous and angiogenic microenvironment, and many studies using natural or synthetic polymer hydrogels have successfully directed vasculogenesis from ECs. Also providing a favorable microenvironment for tumor growth, hydrogels are commonly used in ECs invasion assay and aortic ring assay to assess the angiogenic capability of tumor tissues. When coupled with other technologies such as lithography and 3D printing, it can create an advanced biomimetic model with microfluidic channels that serves as a schematic for microvascular structure. Further understanding of the differences between normal and cancerous environments is needed to enable the accurate mimicking of the vasculo- and angiogenesis in pathological conditions. Many parameters must be first defined before fully adapting these models to tumor angiogenesis, but hopes for creating efficient preclinical models that obviate the need for costly *in vivo* models remain high.

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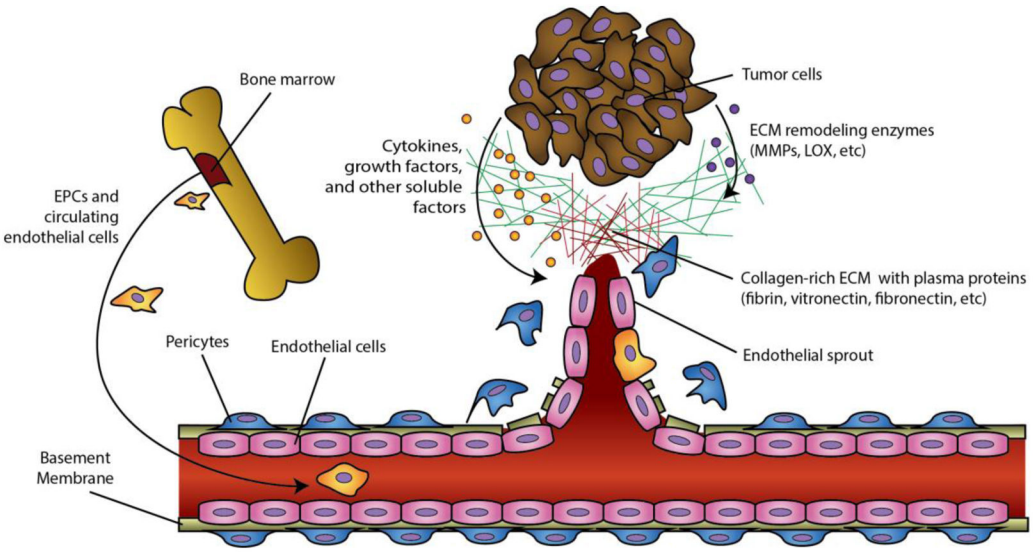

Fig. 1.

Graphical illustration of tumor vascularization. Cytokines and growth factors secreted from tumor cells induce recruitment of detached pericytes and activation of ECs that lead to vessel expansion and branching. Extracellular matrix in tumor stroma is remodeled by enzymes secreted by tumor cells, and its collagen-rich matrix is combined with plasma proteins leaking out the growing sprout. Drawing not to scale.

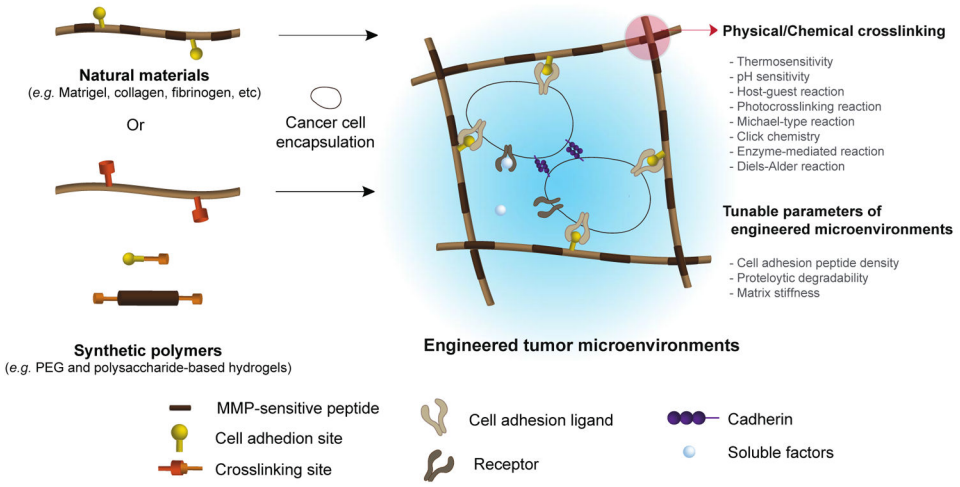

Fig. 2.

A schematic representation of engineered tumor microenvironments using natural/synthetic hydrogel materials for *in vitro* tumor models. Hydrogel matrices are fabricated through a number of physical/chemical reactions under physiological conditions. These hydrophilic networks provide 3D microenvironments to support tumor growth (*e.g.* tumor morphogenesis, proliferation, migration as well as tumor angiogenesis) by controlling myriad cues, such as cell adhesion site, proteolytic degradability, and matrix stiffness. Drawing not to scale.

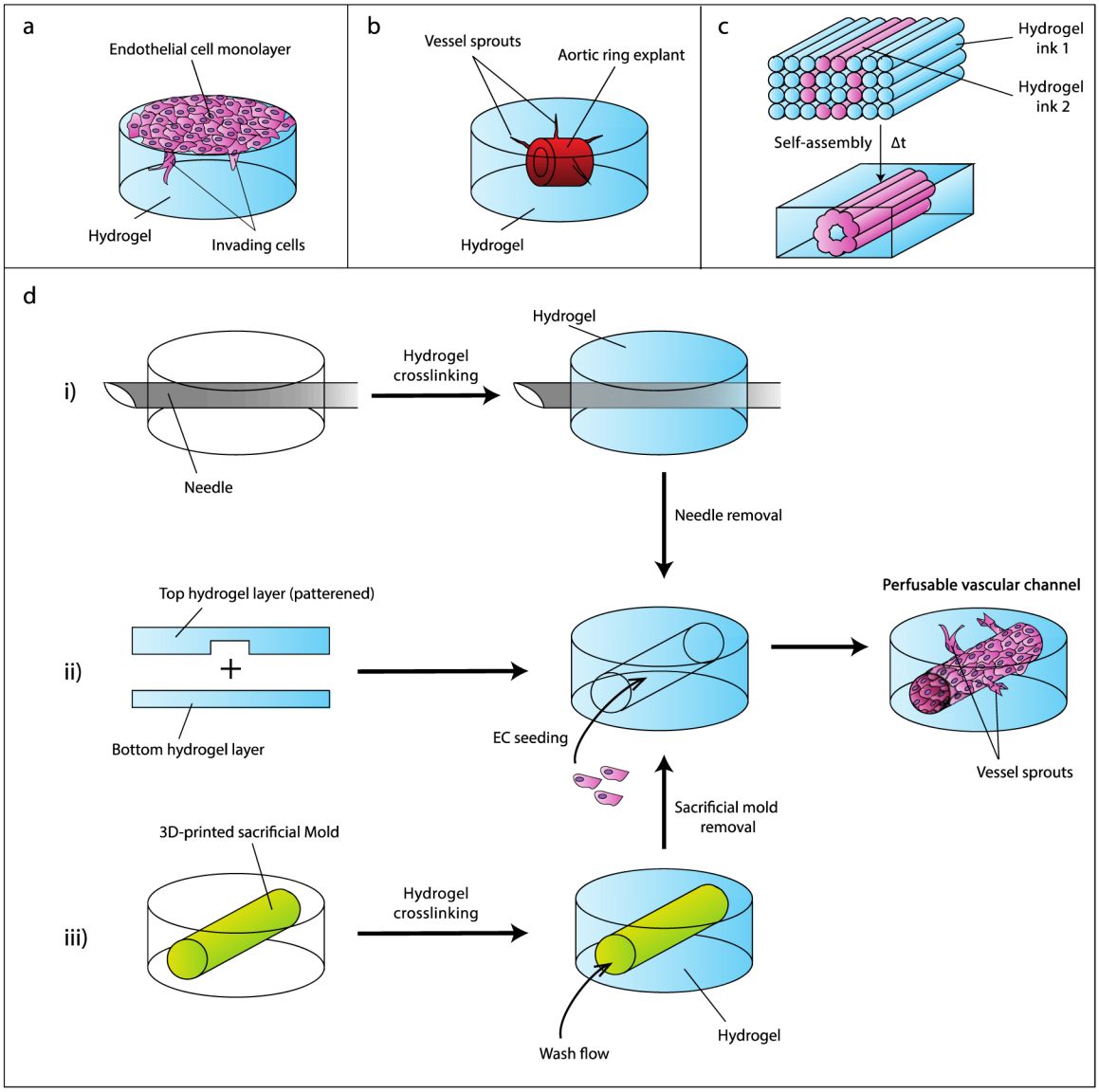

Fig. 3.

A schematic representation of *in vitro* angiogenesis models including a. monolayer EC invasion model, b. aortic ring model, c. bioprinted model, and d. advanced perfusable biomimetic model created by i) needle molding [113], ii) lithographic microfabrication [49], and iii) 3D-printed sacrificial molding [121, 122]. These models, when coupled with cancer cells embedded in the interstitial hydrogel matrix, can be applied to simulate tumor angiogenesis. Drawing not to scale.