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Hydrogel vehicles for sequential delivery of protein drugs to promote vascular regeneration

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ABSTRACT

In recent years, as the mechanisms of vasculogenesis and angiogenesis have been uncovered, the functions of various pro-angiogenic growth factors (GFs) and cytokines have been identified. Therefore, therapeutic angiogenesis, by delivery of GFs, has been sought as a treatment for many vascular diseases. However, direct injection of these protein drugs has proven to have limited clinical success due to their short half-lives and systemic off-target effects. To overcome this, hydrogel carriers have been developed to conjugate single or multiple GFs with controllable, sustained, and localized delivery. However, these attempts have failed to account for the temporal complexity of natural angiogenic pathways, resulting in limited therapeutic effects. Recently, the emerging ideas of optimal sequential delivery of multiple GFs have been suggested to better mimic the biological processes and to enhance therapeutic angiogenesis. Incorporating sequential release into drug delivery platforms will likely promote the formation of neovasculature and generate vast therapeutic potential.

Contents

1. Introduction

Human vasculature is responsible for delivering nutrients and oxygen to nearly all organs and tissues of the human body and for removing the waste generated by organs, both of which are crucial for tissue maintenance and regeneration [1–4]. Vascular dysfunction can range in severity from mild to moderate diseases, including Raynaud's disease and chronic wounds, to severe diseases, including peripheral vascular disorders, coronary artery disease, and cerebrovascular diseases [5–7]. All of these conditions affect the circulatory system and can potentially lead to significant reductions in quality of life, disabilities, and even death. For example, peripheral vascular disease, the most common arterial disease, narrows and blocks circulation to the brain, viscera, or

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limbs, leading to infarction, stroke, or amputation and causes enormous public health costs [8,9]. The standard clinical treatment for coronary and peripheral artery disease is to re-route flow via the bypass of damaged and blocked vessels. While bypass surgery can re-establish homeostatic flow to sufficiently supply blood to target organs and tissues, the damaged vessels and target organs are not repaired via bypass surgery. Furthermore, limitations to bypass surgery include difficulty in obtaining autologous vessels due to the patients' disease or advanced age, [10–12] and the susceptibility of synthetic grafts to both immunogenic and thrombogenic events that can lead to clinical complications and graft failure [13,14].

The goal of improving the blood flow of damaged vessels has been studied extensively in the field of vascular regenerative medicine, and new vessel formation may be achieved by means of cellular implantation or acellular delivery of therapeutic protein drugs using engineered materials [15–18]. Cell therapies may consist of a variety of cells types, including stem cells and progenitor cells, that can differentiate into vascular cells and re-form functional vasculature within the surrounding matrix [19–22]. However, these technologies are relatively new and are still being actively developed and optimized. Optimization of cell therapies should include thorough consideration and addition of relevant cell types. Most hydrogels developed towards cell-based vascular regeneration focus on the delivery of stem and progenitor cells, mature endothelial cells, smooth muscle cells, pericytes, and fibroblasts, all which can aid in supporting developing vasculature if selected appropriately. The optimal combinations and concentrations of delivered cell types are subject to current and future studies. Additionally, the design of delivery platforms or strategies (e.g. spheroid delivery versus single cell suspension delivery) to enhance survival following implantation or injection is a fascinating new arm of this field of research. In all cases, cell types should be assessed for ultimate long-term therapeutic efficacy, including a thorough understanding of the long-term consequences of the local immune response.

Alternatively, the therapeutic delivery of protein drugs, such as growth factors (GFs) and cytokines, has been studied as another treatment for many vascular disorders [15,23–25]. The processes of *in vivo* blood vessel formation are complex, and contain numerous molecular signals and transduction pathways that direct vessel progression and the expression of different cell phenotypes in a highly specific, time-dependent manner. These complex processes continue from embryonic development into adulthood, and can be activated to facilitate vascular regeneration and positive clinical outcomes in diseases such as chronic wound healing, artery diseases, and tissue ischemia [26,27]. In native tissues, an array of highly diverse signaling molecules is presented and retained in the extracellular matrix (ECM) [28–30]. During the process of angiogenesis, cells respond to these signals in the ECM to undergo morphogenetic changes leading to the formation of neovasculature. Constant feedback between the cells in the vasculature and the signaling molecules in their surrounding ECM leads to the formation of vascular sprouts, which extend and expand through proteolytic degradation to guide sprouting [31–33]. Degradation may also activate ECM-bound signaling molecules and reveal cryptic cues to further coax blood vessel formation.

Much of the work in this area has attempted to deliver these signaling factors to enhance vascularization and make new vessels more robust. However, clinical success and long-term treatment utilizing systemic injections have failed, due to the short half-lives of signaling molecules and their rapid diffusion or loss from targeted sites [34,35]. Without the protection of carrier matrices, most molecules are cleared from the body rapidly [35]. Moreover, off-target angiogenesis could develop if these angiogenic GFs accumulate at nontargeted sites such as the retina, leading to severely detrimental clinical outcomes [36]. Additionally, excess delivery of protein drugs may cause vascular leakage, hypotension, or potential tumor formation, while transient or insufficient delivery may result in new vessel regression [37,38]. Therefore, targeted, highly controlled and sustained release therapies are vital for the establishment of functional neovasculature [39,40].

To address these constraints and to achieve robust therapeutic effects, hydrogels, composed of three-dimensional (3D) polymeric networks, can function as ideal matrices for localizing and delivering angiogenic factors for regeneration of the vasculature, due to their ECM-like architecture, viscoelasticity, and diffusivity [41–48]. As material technologies have progressed, a large number of hydrogels, composed of either synthetic or natural polymers, have been developed as vehicles for delivering various signaling molecules [49,50]. By conjugating pro-angiogenic agents, pre-designed hydrogels can easily be directly implanted or injected into the target sites with controllable, local delivery [51–54]. To mimic the native ECM, synthetic hydrogel networks can be further modified to contain additional peptide sequences for cell adhesion and degradation for cell-mediated control of the release kinetics of the angiogenic cargo [55–58]. These functional hydrogels are capable of delivering GFs and therapeutics more precisely to the target regions, and are able to sustain their release in order to elicit an optimal healing response.

Due to the complexity of natural angiogenic pathways, multiple GFs have been combined and co-loaded into hydrogels in an attempt to attain maximum therapeutic effects [59–61]. However, these combined delivery trials have failed to incorporate sequential delivery mechanisms best suited for introducing different molecular signals for complete regeneration, resulting in limited success. Therefore, elucidating the mechanisms of the sequence of cellular and molecular processes leading to vascularization will help us to better understand and design delivery approaches that mimic natural biological processes. Recently, sequential drug release methods have been suggested to potentially address this concern and enhance therapeutic angiogenesis [62–67]. The studies aimed at achieving sequential drug delivery in vascular regeneration are of interest to a wide range of researchers, since knowledge of both biological events and engineered material science are required. Here, we provide an overview of the basic biology of vascular regeneration, the roles and functions of angiogenic factors, and the approaches to engineering hydrogels as delivery vehicles. Specifically, we focus on discussing the strategies of sequential drug delivery and their effects on neovascular formation and maturation into stable and functional vessels.

2. Natural vascularization mechanisms

In the most basic terms, development of the human vasculature during embryogenesis occurs through two steps: first, vascular precursors, angioblasts, give rise to endothelial cells (ECs), which form the primordial vascular plexus and initial blood vessels through vasculogenesis, by first clustering into blood islands, then rearranging into lumen and tube-like structures; and second, the vascular network continues to grow and remodel by sprouting and branching from the vascular plexus via angiogenesis [26,68]. Thus, the two distinct mechanisms for the development of vasculature can be described as vasculogenesis and angiogenesis.

Vasculogenesis is the process of *de novo* blood vessel formation, which was originally postulated to only occur during embryogenesis, but has since been recognized as an important mechanism for postnatal vasculogenesis [27,69,70]. The cells of the vasculature originate from the mesoderm, one of three germ layers, which forms from the process of gastrulation during embryonic growth. After mesoderm formation, angioblasts, which differentiate into endothelial cells (ECs), surround hematopoietic progenitor cells to form blood islands. ECs may also organize in isolation from hematopoietic progenitors. In both scenarios, EC vacuoles can coalesce to form the lumenal structures and generate the primary capillary plexus within the developmental ECM. The primary capillary plexus is a small initial network of vessels that can be used as the basis for further vessel assembly and expansion, through angiogenesis [68].

Vasculogenesis is also found to occur in adults through accumulation and network formation of bone marrow-derived circulating endothelial

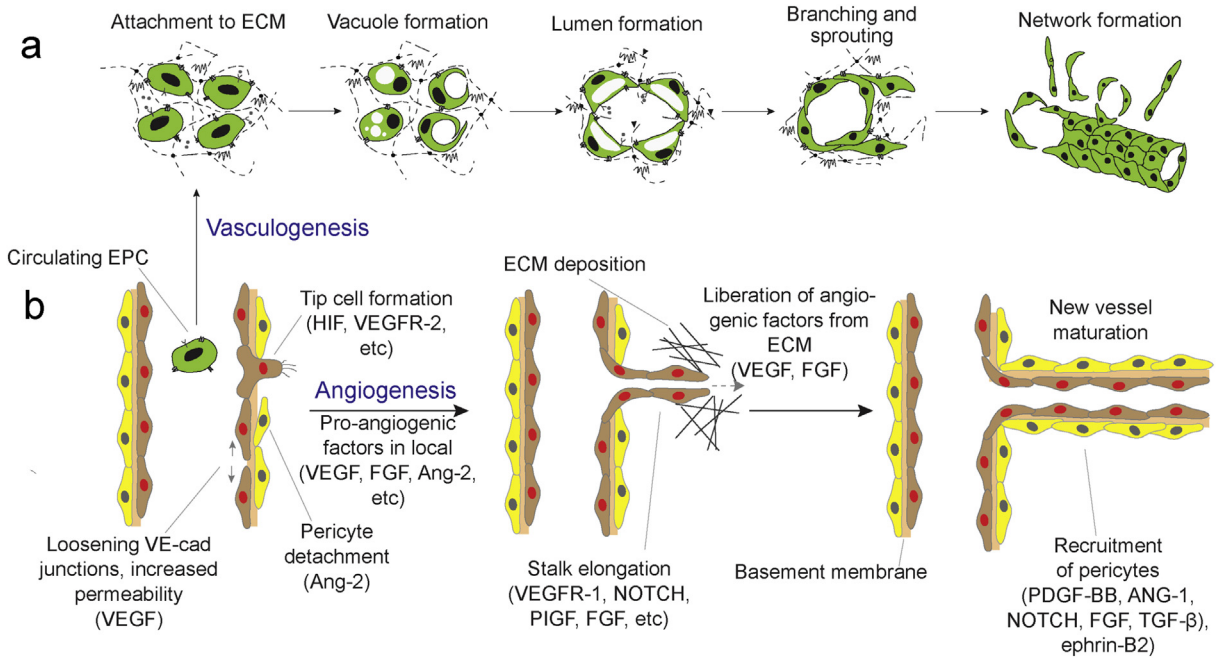

Fig. 1. Graphical illustration of vasculogenesis and angiogenesis. (a) Schematic of vasculogenesis starting with vacuole and lumen formation of circulating EPC (ECFC), followed by sprouting and branching to form vascular networks. (b) Angiogenesis occurs when a quiescent vessel is activated, leading to sprouting from the existing vessel, elongation of new branches, and lumen formation to create new vessels. Adapted from [15,18,22].

progenitor cells (EPCs), in postnatal or tumor vasculogenesis [15,71,72]. EPCs represent an ever-evolving class of cells that contribute to neovessel formation. The defining characteristics for these cells continue to be refined and can be broadly grouped into cells that contribute to angiogenesis primarily through paracrine effects (e.g. myeloid angiogenic cells (MACs)) or those cells that possess intrinsic capacity to form vascular structures (e.g. endothelial colony forming cells (ECFCs)) [193]. The direct contribution of ECFCs to adult vasculogenesis has been made clear through extensive *in vitro* and *in vivo* testing [193–196]. The circulating EPCs (specifically those cells that directly contribute to the formation of new blood vessels, ECFCs) can be recruited and regulated to differentiate into vascular lineages through the existing GF and physical or chemical cues in the ECMs Fig. 1a shows the specific vascular morphogenesis of EPCs: initially, vacuoles form within the ECFCs that will merge and coalesce with neighboring cells into a larger structure with open lumen. Then tubulogenesis occurs through sprouting and branching, and a continuation of the process proceeds toward comprehensive multicellular networks with patent lumenized structures. Finally, with the attachment of mural cells around the EC tube, the newly formed vasculature stabilizes and matures [73–76]. Studies suggest that the signals and cues for regulating EPC recruitment and mobilization are vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP-9), basic fibroblast growth factor (bFGF), hypoxia-inducible factor-1α (HIF-1α) and stromal derived factor-1 (SDF-1) [77], which are also summarized and reviewed by Rafii *et al.* [78].

Angiogenesis, on the other hand, is a much more common process. Here, new vessels sprout and expand from the existing vessels of mature ECs. This mechanism for neovessel formation has been studied extensively during wound healing, pregnancy, and tumor vascularization [76,79]. Angiogenesis occurs in three main stages; quiescence, activation, and branching [80]. As shown in Fig. 1b, in the quiescent state, ECs and supporting pericyte cells attach to a shared basement membrane composed of proteins and proteoglycans, including fibronectin, collagen IV and laminin [81]. The quiescent ECs are maintained and survive by molecular signals in the ECM, including VEGF, bFGF, Notch signaling (Notch) and angiopoietin-1(Ang-1) [82–86]. The supporting pericytes can also secrete additional Ang-1 and VEGF, which contribute to cell survival. In the activation state, the ECs and pericytes respond to numerous angiogenic signals including Ang-2, VEGF and bFGF which are released by inflammatory cells or cells that reside within a hypoxic microenvironment. Hypoxic conditions can be created either by injury wounds, tumor growth, or peripheral and coronary artery diseases [80]. These hypoxic conditions lead to differential levels of HIF-1α expression, and the level of HIF-1α present is highly spatiotemporally regulated and is cell type specific. Variations in signaling of HIF lead to upregulation of many signaling cues responsible for vascular regeneration. As such, hypoxia is a broad and potent angiogenic cue that can precisely tune spatiotemporal production of critical factors throughout the process of vascular regeneration. For further description and details of the importance of HIF-1α pathways in angiogenesis we point the reader to several reviews by Semenza et al. [212–215]. The vascular smooth muscle cells (VSMCs) or pericytes sensing Ang-2 will detach from the vessel wall and basement membrane by proteolytic degradation that is mediated by matrix metalloproteinases (MMPs) [84]. In the meantime, ECs loosen their cell-cell junctions, leading to an increase in the permeability of the EC layer. New ECM is then deposited adjacent to the permeable EC layer, and the activated ECs sprout in response to GFs, such as VEGF and bFGF. After activation, the sprouting EC, termed a tip cell, leads to the subsequent directed growth of new branches along GF gradients. In response to VEGF, Notch, placental growth factor (PlGF) and bFGF, neighboring ECs connected to the tip cell develop into stalk cells. These cells undergo proliferation, elongation, and form a lumen to shape the nascent vessel [87–89]. Recruited myeloid cells, (e.g. tumor associated macrophages (TAMs) and TIE-2- expressing monocytes (TEMs)) can act as “bridge cells” by directly interacting with the filopodia of the tip cells, or in a paracrine manner by both producing proangiogenic factors and proteolytically degrading the surrounding ECM to release additional GFs. As such, these cells play an important role in the continued growth and fusion of new vessel branches [189,190]. As the blood perfuses the vessel, the ECs return to the quiescent state and are stabilized by recruitment of VSMCs or pericytes. Pericytes stabilize capillaries, while VSMCs surround larger diameter vessels and control vessel conductance for the restoration of blood flow [17]. This recruitment is mainly stimulated by the release of platelet-derived growth factor (PDGF) [95–97] from the nearby ECs,

as well as by Ang-1, transforming growth factor-β (TGF-β), ephrin-B2, and Notch [90–92]. Basement membrane is also deposited to further stabilize the branched vessel; and subsequently, the junctions between ECs are restored.

Overall, the purpose of therapeutic angiogenesis is to enhance this native process of angiogenesis, via delivery of angiogenic drugs and to improve blood vessel density and tissue function to restore requisite delivery of nutrients and O_2 and removal of tissue and organ waste. Therefore, understanding the molecular pathways of vascular formation and maturation is critical to developing effective delivery strategies. Multiple GFs working simultaneously and sequentially are required for patterning functional vasculature and facilitating optimal blood vessel growth, from stimulation of sprouting to stabilization of new vessels. The key biochemical factors involved in angiogenesis are summarized in Fig. 1b. For much more detail on the role of angiogenic signals and molecular pathways, we point the readers to these reviews [17,29,80,93,94,98].

3. Single factor hydrogel carrier systems for therapeutic angiogenesis

The native physiological angiogenesis described above is not a sufficient cure for the range of clinical vascular diseases and disorders, including tissue ischemia and chronic wounds. The limitations of the body's natural vascular repair system highlight the need for additional intervention of therapeutic angiogenesis to treat debilitating vascular diseases and produce positive therapeutic effects. A variety of GFs and cytokines serve as vital stimulators and components in many of the steps of angiogenesis, as mentioned above. Most direct delivery attempts fail to show long-term improvements due to the inherent issues of rapid clearance and propensity for off-target systemic effects. Strategies using polymeric hydrogel materials as vehicles for delivering these factors, have been developed to solve these issues. The properties of these hydrogels can be tuned to meet a range of specific applications including injectability, stiffness, degradability, and pore structure [99–101].

Based on the source of the polymer backbones in the gel networks, the hydrogel carriers used for delivering angiogenic factors are divided into either natural-based or synthetic hydrogels. Macromolecules make up natural hydrogels and are derived from native ECMs which consist of proteoglycans, protein fibers, or glycoproteins [102–104]. Many of these macromolecules, such as collagen and fibrinogen, play a critical role in regulating angiogenesis [105–110]. These natural hydrogel carriers typically establish networks through physical interactions of non-covalent bonds between protein polymer chains, including hydrogen bonds, crystallization, hydrophobic interactions, electrostatic interactions, and π-π stacking [111]. Synthetic hydrogel networks most commonly consist of polysaccharide or polyethylene glycol (PEG) backbones, which have historically been crosslinked by stable covalent bonds or polymer-cation interactions, such as alginate-calcium hydrogels [112–116]. Overall, the use of both natural and synthetic hydrogel carriers for drug delivery has provided many unique solutions and has contributed to significant progress in promoting angiogenesis, which will be discussed in this section.

3.1. Natural-based hydrogel carriers

Many of the early attempts to create hydrogel systems that incorporate GFs for vascular regeneration used polymers derived from native ECMs, because of their bioactivity and biocompatibility. Fibrinogen is a large glycoprotein found in the blood plasma that works in hemostasis, fibrinolysis, and wound healing [117–119]. Fibrin hydrogels are usually polymerized by fibrinogen solution with thrombin or calcium ions through physical interactions, and have been widely used in tissue engineering due to their ECM-like nano fibrous structure within a macroporous hydrogel network [120,121]. Moreover, fibrin formed during blood coagulation is clinically permissible, and because it can be extracted from a patient's own blood, it provides a means of avoiding potential inflammatory and immune responses [122]. Over the last decade, fibrin gels have been used as local delivery matrices for biological agents such as VEGF, bFGF, and heparin. However, Shireman et al. reported that the release kinetics of bFGF and heparin from fibrin matrix lead to a burst effect, as 70% of the initially added agents were released into the surrounding media within only 24 hours [123].

In 2001, a more advanced delivery system was tested via the covalent incorporation of VEGF into a fibrin hydrogel for controlled and localized release [122]. VEGF is a secreted protein ligand including a disulfide-linked homodimer that plays a primary role in the initial stages of vessel formation, proliferation, migration, and survival [124,125]. The authors first developed an engineered VEGF protein sequence. This engineered VEGF was covalently conjugated to fibrin via transglutamination to obtain a covalent VEGF-modified fibrin gel. After injection *in vivo*, the surrounding cells invaded and degraded the matrix, causing the hydrogel carrier to release VEGF. Interestingly, a low but continuous level of VEGF content was achieved, as the release rate was directly related to the degradation of the fibrin hydrogel, that was dependent on localized invading cells. Moreover, dose-dependent responses were achieved by increasing the dosage of VEGF conjugated in the hydrogel. Additionally, free heparin sulfate-associated VEGF was encapsulated but unbound in the fibrin gel networks and was consequently capable of acting as an EC recruiter, leading to the formation of mature and functional vascular networks directly in the matrix [122]. Other complementary studies have focused on precisely controlling and slowing down the release rate of VEGF from fibrin carriers, by tuning their degradation rate. For example, fibrinolysis inhibitors have been introduced into the fibrin matrix to control and sustain delivery of VEGF for up to 3 months after injection *in vivo* [40].

Another vital component during angiogenesis is bFGF. Studies have shown the capacity for localized delivery and extended delivery time of therapeutic bFGF by using fibrin hydrogel carriers [126]. Similar to the previously described strategy, the release of conjugated bFGF from a fibrin hydrogel was mediated by fibrin degradation, which was driven by cell infiltration-mediated proteolysis. There are two known means of decreasing the release rate of the bFGF. The first is the preparation of fibrin hydrogels with a higher cross-linking density. By increasing the concentrations of thrombin and fibrinogen, it is possible to slow down the degradation rate of the hydrogel and, consequently, the diffusion rate of the released bFGF. The second method is the incorporation of heparin into fibrin structures. As bFGF can readily bind to heparin, incorporating heparin into fibrin hydrogel networks is a relatively simple method to further reduce the release rate. These approaches were all considered in the design of fibrin hydrogel carriers with controlled and sustained release properties.

3.2. Synthetic hydrogel carriers

In spite of extensive studies of natural-based hydrogels as carriers for angiogenesis, there are still numerous critical drawbacks that limit their clinical utility: 1) the narrow range of physical properties; 2) limitations of controlling matrix stiffness, pore size, as well as cell adhesive or proteolytic peptide densities independently; 3) inherent batch-to-batch variability and 4) inevitable inflammatory and immune issues [127]. To overcome these limitations of natural carriers, increasing efforts have been focused on developing synthetic hydrogels from biocompatible polysaccharides or synthetic polymers. Many of the synthetic hydrogel carriers utilized for controlled drug delivery can be tuned to control a myriad of parameters, such as matrix stiffness, pore size, proteolytic degradability and cell adhesion sites.

Poly(ethylene glycol) (PEG) is known as a representative synthetic polymer that can be cross-linked to form a 3D hydrogel carrier for releasing bioactive factors [128]. PEG-based hydrogels have advantages of biocompatibility, high hydrophilicity, and highly tunable physical

properties. However, because of the lack of bioactivity of PEG chains, PEG-based hydrogels are usually decorated with cell adhesive peptides, such as RGD or other integrin binding sequences, [129] and proteolytically degradable sites with MMP-sensitive peptide sequences, or directly mixed with biopolymers such as collagen, gelatin, or fibrinogen, to serve as bioactive carriers for controlled release applications [130,131]. One example of such a hydrogel is a VEGF-conjugated PEG-based hydrogel, synthesized by Michael addition of the vinyl sulfone-functionalized 4-arm PEG with RGD and MMP-sensitive peptide sites to mimic the cell-ECM interactions during revascularization [36]. In this study, an engineered VEGF variant was prepared with two unpaired cysteine residues, which could also be covalently bonded to PEG peptide hydrogel networks via Michael-type conjugation. The invading cells attached to RGD sites and remodeled the matrix by cell associated proteolysis of the MMP-sensitive peptide cross-linkers. The bound VEGF was then released locally, allowing for vascular network formation. A similar delivery system was developed by incorporation of VEGF into a diacrylate modified PEG (PEGDA) network cross-linked by cell adhesive and proteolytic peptides [132]. As described above, the manner of cell-mediated release could achieve controlled, sustained, and localized delivery of VEGF during remodeling of the hydrogel, thereby enhancing angiogenesis and eliminating the negative effects of a directly systemic release approach. Covalently bound growth factors allow for long-term control of growth factor availability and ensure that matrix-degradation, not diffusion, is the main mechanism of release from the hydrogel [200–202]. However, this chemical bonding of growth factor and hydrogel may ultimately impact final protein conformation and binding site activity. Consequently, approaches for shielding growth factors from potentially harmful covalent bonds have been developed. These approaches include the use of proteoglycans, glycosaminoglycans, [203,204] and heparin sulfates [205] to shield growth factors from unexpected reactions with the hydrogel.

In addition to VEGF, West's group developed a PEG-based hydrogel immobilized with PDGF-BB, to increase angiogenesis *in vivo* [133]. Unlike VEGF and bFGF, PDGF-BB is not involved in the initial steps of angiogenesis, but is an essential recruiter of pericytes for stabilizing newly formed vessels and can increase the production of collagenases utilized by ECs to facilitate migration [134]. Owing to the highly hydrophilic PEG chains, the stability and solubility of conjugating PDGF-BB increased while the immune response decreased, showing promising potential for regenerative medicine. The immobilization of SDF-1 in a star PEG-heparin hydrogel was also reported [135]. The sustained and controlled release of SDF-1 from the PEG-based hydrogel aided in the recruitment of endothelial progenitor cells allowing for angiogenic healing and improving the overall therapeutic effect.

Hyaluronic acid (HA), is another popular natural polymer backbone used for the synthesis and fabrication of hydrogel carriers for delivery of bioactive factors *in vivo* [136,137]. HA itself is an important ECM component, with angiogenic functions including regulation and stimulation of cytokines for EC proliferation [138]. HA hydrogels can also be cross-linked by MMP-sensitive peptides with cell adhesive RGD conjugated in the networks, allowing adhesion and degradation of ECs for morphogenesis into functional vasculature [20–22]. Prestwich et al. combined VEGF into the HA hydrogels, which leads to significantly more vessel formation than VEGF only or HA only groups, indicating a synergistic effect between the polymer backbone and loaded factors [139]. Moreover, the delivery of VEGF from HA hydrogels can be controlled by parameters of both degradation rate and initial pore size [140]. Another work, aimed to sustain and control the delivery of VEGF, involved utilization of sodium alginate (Alg) hydrogels [39]. Alg is another common polysaccharide with water solubility that can form stable ionic cross-links within seconds in the presence of divalent cations such as Ca^{2+} [141]. Owing to good biocompatibility and a facile gelation process, Ca^{2+} cross-linked Alg-based hydrogels have been used in a number of biomedical applications [142]. Early uses of these hydrogels by Mooney's group showed vasculogenic potential in providing a sustained local delivery of VEGF in hind limb ischemia models [39] as well as providing structural support when implanted as microporous beads [142] However, traditional alginate gels had poorly controlled degradability *in vivo*. Recent work has combatted this via techniques such as click chemistry. Click crosslinked Alg-based Ca^{2+} gels can crosslink post *in vivo* injection, provoke minimal inflammatory response, and resist fragmentation and cellular infiltration for up to two months [206]. More recently, Alg-based Ca^{2+} hydrogels have been used to create bio-printed perfusable vasculature conduits [207,208]. Shu's group has developed a novel three-stage crosslinking procedure of alginate and calcium allowing for gradual crosslinking before, during, and after printing, which results in finer control of mechanical properties [209]. By optimally controlling the release of VEGF, the physical properties of Alg-based hydrogels, such as pore size and degradation, can be easily adjusted to obtain desired parameters by partial oxidation of the polymer chains or molecular weight distribution [39].

3.3. Micro/nanosphere-hydrogel hybrid carriers

Although hydrogel systems alone can achieve drug release by cell-mediated degradation, they still lack effective control of sustained delivery. Owing to recent progress in materials science, more complex systems have been explored to enhance therapeutic delivery of GFs. For example, incorporating drugs into microspheres first, instead of directly loading drugs into hydrogels, results in a more controllable and persistent delivery [143,144]. In turn, microspheres capable of carrying protein drugs are usually encapsulated in hydrogels to minimize the drugs' diffusion at the target site. This reduces the initial drug burst and decreases release rates to achieve long term delivery [145,146].

For example, Lee et al. developed a microsphere-hydrogel combination delivery system, consisting of PLGA microspheres and Alg-based hydrogels formed via ionic cross-linking, as an injectable tool for localized and sustained delivery of VEGF [147,148]. This combination delivery system not only provided protection for the microspheres, but also maintained a prolonged release of VEGF, which promoted the formation of new active blood vessels near the ischemic site, after injecting in a hindlimb ischemia mouse model. Another similar hydrogel system reported making use of microspheres, containing VEGF, contained within hydrogels via the use of cross-linked pyrrole molecules. The prolonged drug release in this system was attributed to the strong hydrophobic association of cross-linked pyrrole groups with the VEGF molecules, and was sustained over 25 days [149].

In addition to microspheres, nanoparticles can be used to incorporate protein drugs into hydrogels, allowing an increased protection against drug degradation and a controlled delivery approach. VEGF has been encapsulated in nanoparticle polyelectrolyte complexes by first binding to dextran sulfate and then by coacervation with chitosan. The resultant VEGF nanoparticles were then suspended and incorporated into Matrigel [150]. The dextran sulfate combined with the heparin binding site of the VEGF, protecting it from degradation, while the chitosan in the nanoparticles stabilized the complex and contributed to sustained release of the drugs. Implantation of this composite system stimulated EC colonization and resulted in more efficient blood vessel formation than Matrigel loaded with free VEGF, regardless of the drug dose, implant time, and implant approach. Another study for optimizing the release rate of bFGF used heparin-conjugated PLGA nanoparticles, which were encapsulated in natural fibrin hydrogels. By optimizing these nanoparticles and fibrinogen concentrations before gelation, the release time of bFGF could be controlled for up to one month, which bolstered the angiogenic effects of these injectable hydrogels [151]. The localized, controlled, and sustained delivery of protein drugs achieved by the hydrogel carrier systems discussed above have resulted in enhancing the angiogenic response to form mature and functional vasculature.

4. Strategies for sequential delivery to promote angiogenesis

Despite the positive therapeutic effects seen with many of the hydrogel delivery systems mentioned above with *in vitro* or *in vivo* disease models, other aspects and steps in natural angiogenesis, aimed to promote more mature and robust vascular networks with long-term stability and effectiveness, should also be considered. With the need to understand the natural mechanisms of vasculature formation, the use of designer hydrogel carriers capable of combined delivery of multiple protein drugs has emerged and received much more attention in recent years [152–154]. Combinations of GFs incorporated in one hydrogel system have been developed for co-release and their synergistic effects have been evaluated, and have proven to enhance vascular regeneration [155–157]. For example, Seliktar's group recently used polyethylene glycol-fibrinogen hydrogels for sustained dual delivery of VEGF and ANG-1 to enhance myocardial repair and function [197].

However, in many of these combined delivery technologies and hydrogels, each protein drug is simultaneous or nearly simultaneously released, similar to the approaches discussed in Section 3. Natural angiogenesis *in vivo* is a complex, time dependent, multi-step process, requiring the precise temporal release and uptake of GFs. Therefore, developing hydrogel carrier systems that control both the delivery and timing of multiple protein signals may facilitate closer mimicry of natural angiogenesis, which can be denoted as temporal or sequential release [66,158–160]. In accordance with biological angiogenic pathways, most hydrogel carriers designed for sequential controlled release, first release the angiogenic initiators, such as VEGF or bFGF. These hydrogel carriers then deliver other signals that participate in the later stages of angiogenesis, such as PDGF, sphingosine-1-phosphate (S1P), or keratinocyte growth factor (KGF), to stabilize newly formed blood vessels. In this section, we will focus on discussing the novelty and efficacy of current sequential delivery systems and describe methods for optimizing sequential delivery of multiple biological factors for desired signaling pathways to better accommodate for the native complexity of angiogenic processes. These developments further accelerate the therapeutic potential of sequential delivery as a treatment modality for vascular diseases.

4.1. Affinity-based delivery approach

Natural interactions between protein factors and the ECM can directly modulate the partitioning of factors from the ECM to the environment and thus control their local concentration, diffusion and signaling [161,162]. Most GFs or cytokines are able to bind the proteoglycan components of the ECM [163–166]. However, researchers recently found that ECM proteins without highly negatively charged sugar chains, such as fibronectin, vitronectin, and fibrinogen can also bind with several GFs [167–169]. For example, unlike the synthetic covalent links between GFs and fibrin discussed in Section 3, GFs can be bound to specific heparin-binding domains on fibrinogen through natural self-assembly interactions [170]. The release of many GFs was tested in a fibrin hydrogel matrix, and showed a range of affinities dependent on the type of GFs. GFs with lower affinity for the fibrin hydrogel were released faster, while those with higher affinity had a sustained release over a longer period. For instance, more than 80% of the VEGF and PDGF-BB were released from the fibrin hydrogel after 1 day, but bFGF and placental growth factor-2 (PlGF-2) showed stronger retention, with only 32.5% released after 7 days [170]. Additionally, the different binding affinities of ECM components can sequester specific GFs and then deliver them when specific stimulators are activated. Fragments of the heparin-binding domain on fibrin could be modified into a synthetic PEG hydrogel to mimic natural characteristics, offering many more options for designing novel hydrogel carriers with functional temporal delivery. Hubbell's group presented such a fibrin-mimetic hydrogel, formed by eight-arm PEG-peptide conjugate, to greatly accentuate the effects of bFGF and PlGF-2 for chronic skin wound healing [170]. Although these studies have not clearly demonstrated the concept of sequential delivery, exploration of the binding affinities between a range of GFs and a range of ECM proteins may make it possible to achieve temporal control of protein drug delivery to better mimic the *in vivo* mechanisms for vascular regeneration.

Aside from studying the natural affinity between GFs and the ECM, other improvements for achieving temporal release have been explored, by modulating polymer/GFs affinities via the utilization of specific polymers. In other words, making use of the different binding affinities of each protein factor to various polymers provides a different solution for sequential drug release. In 2007, an injectable Alg-based hydrogel used for local sequential delivery of VEGF and PDGF-BB for treatment of myocardial infarction [171]. The Alg-based hydrogels were prepared by combining high and low molecular weight Alg cross-linked by cations. The PDGF-BB released from this Alg-based hydrogel exhibited a slower release than the VEGF, which was likely related to the two GFs' different affinities to alginate. The sequential delivery of VEGF and PDGF-BB showed an angiogenic effect and improved cardiac function. Furthermore, Cohen et al. employed hydrogel scaffolds consisting of alginate-sulfate/alginate to allow for controllable sequential delivery of three common GFs, including VEGF, PDGF-BB and TGF-β1 [66]. These three GFs specifically bind to alginate-sulfate by electrostatic interactions in a manner similar to their interaction via heparin binding. The release kinetics were found to correlate with the respective equilibrium-binding constant of each factor. The results showed that the sequential delivery of VEGF, PDGF-BB and TGF-β1 from the hydrogel system matched the signaling in natural angiogenesis, which is initiated by VEGF, followed by vessel stabilization by PDGF-BB-mediated recruitment of mural cells and finally, vessel remodeling of the ECM induced by TGF-β1, thus leading to the formation of mature and stable vasculature within the scaffold after *in vivo* implantation.

4.2. Microsphere-hydrogel hybrid delivery systems

The development of a hybrid hydrogel system containing both GF-microspheres and free GFs is another popular approach to achieving sequential drug delivery. In 2001, Mooney's group first reported a new polymeric system, formed from biodegradable PLGA, that allowed for delivery of multiple angiogenic factors with controlled dose and distinct release kinetics [172]. VEGF and PDGF-BB were incorporated into the structural polymer scaffolds using two different approaches. As shown in Fig. 2a, VEGF was directly encapsulated into the polymer scaffolds by mixing with PLG particles before fabricating into porous scaffolds, while the PDGF-BB was first pre-encapsulated into PLG microspheres. By mixing the PDGF-BB contained PLG microspheres with PLG polymers of free VEGF, a continuous and homogeneous PLG matrix was formed, leading to the delivery of two GFs with distinct release rates. The delivery of free VEGF from the scaffold depended on the porosity, allowing a rapid release rate. In contrast, the delivery of PDGF-BB, encapsulated in the PLG microspheres, was regulated by the degradation of the microspheres. The utility of this sequential delivery system resulted in a rapid formation of mature vascular networks. Although this PLG microsphere system is not hydrogel, the study can serve as a conceptual framework to guide the future development of microsphere/hydrogel hybrid sequential delivery systems.

Electrostatic attraction between protein factors and heparin is a well-known interaction [173–175]. Werner's group developed a modular star PEG-heparin composite hydrogel system to deliver the two heparin binding GFs, VEGF and bFGF, in parallel [176]. As large quantities of heparin were added to the gels, the delivery of both bFGF and VEGF occurred independently from each other, resulting in pro-angiogenic effects both *in vitro* and *in vivo*. This combined delivery method happened simultaneously. To achieve a sequential release with heparin-binding benefits, Wang and his co-workers prepared heparin-

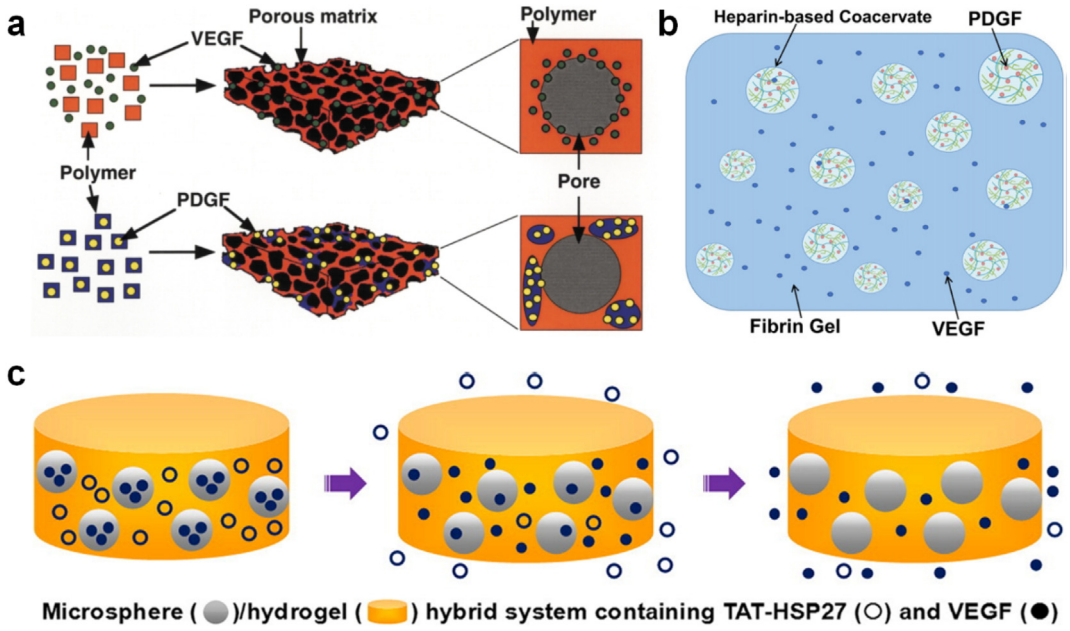

Fig. 2. (a) Schematic of scaffold preparation of porous PLG matrix consisting of free VEGF and PDGF microsphere. Reproduced from [172]. (b) The sequential delivery system comprised of a fibrin gel embedding free VEGF and PDGF-loaded coacervate microsphere. Reproduced from [177]. (c) The fabrication process of sequential delivery system containing free TAT-HSP27 and VEGF microsphere in Alg-based hydrogels. Reproduced from [178].

based spherical droplets, formed by mixing the oppositely charged polyelectrolytes of heparin and PDGF [177]. The complex coacervated spheres were then embedded into fibrin gels containing free VEGF, through polymerization of fibrinogen solution and thrombin (Fig. 2b). The resultant VEGF and heparin-PDGF spheres were homogeneously distributed in the same fibrin gel. This dual controlled delivery system provided a quick release of VEGF followed by a slow and sustained release of PDGF due to its affinity to heparin. This approach stimulated novel vessel formation and stabilization, improved cardiac functions *in vitro* and in a rat model of myocardial infarction.

Another sequential and combined delivery trial using sphere/hydrogel hybrid delivery vehicles was developed to improve the treatment for ischemic vascular disease [178]. Porous PLGA microspheres, embedded with VEGF, were incorporated into an Alg-based hydrogel containing free TAT-HSP27 (Heat shock protein 27 fused with transcriptional activator) (Fig. 2c). The free TAT-HSP27, acted as an anti-apoptotic agent, departed the hydrogel carriers within 7 days, while the VEGF, acting as an angiogenic agent, sustained released over 28 days. The release rate of VEGF could also be adjusted by varying the porous structure of the PLGA microspheres. Sequential delivery of VEGF and TAT-HSP27 was achieved in this hybrid system, protecting against muscle degeneration and fibrosis and promoting new vessel formation in the ischemic site.

4.3. Layered electrospinning delivery strategies

Electrospinning is a conventional technique used to prepare fibrous membranes as scaffolds with controllable and prolonged angiogenic factor release [179–181]. In the electrospinning process, a high voltage is applied to a droplet of polymer solution to eliminate surface tension and to enable the formation of fibers with diameters ranging from nanometers to micrometers [182–184]. Due to their structural similarities to the native ECM, electrospun fiber membranes can be used as vascular grafts or for encapsulation and delivery of GFs, particularly by preparation as a core/shell fiber structure. A coaxial electrospinning system was set up by Toh's group, [185] with the bFGF solution held in the inner channel of the set-up and the PLGA polymer solution held in the outer channel, providing improved extended release (Fig. 3a).

Inspired by the coaxial electrospinning system, Yuan and his colleagues created a double-layered coaxial-electrospun membrane for dually controlled sequential release of VEGF and PDGF for blood vessel regeneration, and investigated the adhesion, proliferation and vascular regeneration of ECs and VSMCs [186]. VEGF was loaded into the inner layer of chitosan hydrogel/poly (ethylene glycol)-b-poly(L-lactide-co-caprolactone) (PELCL), and PDGF was embedded in the outer layer of an emulsion/PELCL coaxial electrospun membrane. The PEG segment of PELCL macromolecules enhanced the hydrophilicity of the chitosan hydrogel, which accelerated the infiltration of surrounding PBS medium, and allowed for subsequent diffusion of VEGF from the electrospun fibers. In this dual-GF sequential release system, early release of VEGF initialized the angiogenic response while the PDGF showed a sustained release behavior for supporting and stabilizing new vessels. After seeding with cells, the EC proliferation was rapid in the first 6 days due to the higher cumulative release of VEGF during this period. The VSMCs then developed on the outer layer, stabilizing the vessels without thrombosis or bursting, owing to the sustained PDGF release facilitating revascularization. Additionally, the release profiles in this system could be modulated for variable requirements during vascularization via the tuning of compositions of chitosan hydrogels in the electrospun fibers. While the layered electrospun delivery systems discussed above are not hydrogels, many hydrogels can be prepared by via electrospinning [191,192,210,211]. Therefore, inspired by the idea of layered electrospun coating approaches, there is great potential to develop novel hydrogel systems for sequential GF delivery in the future.

4.4. Core/shell microcapsules and hollow fiber delivery systems

In addition to the strategies discussed above, other novel and creative material design delivering approaches have been attempted to enhance the efficacy of sequential delivery towards mimicking native angiogenesis. Little's group created a sequential delivery model based on a porous hollow fiber structure, permitting external control of the loading and release of angiogenic factors [160,187]. GFs loaded in the inner lumen of the cellulose hollow fibers were released into surrounding matrix by crossing the thick fiber wall, which was controlled and tuned to ensure the passage of large protein drugs. This hollow fiber

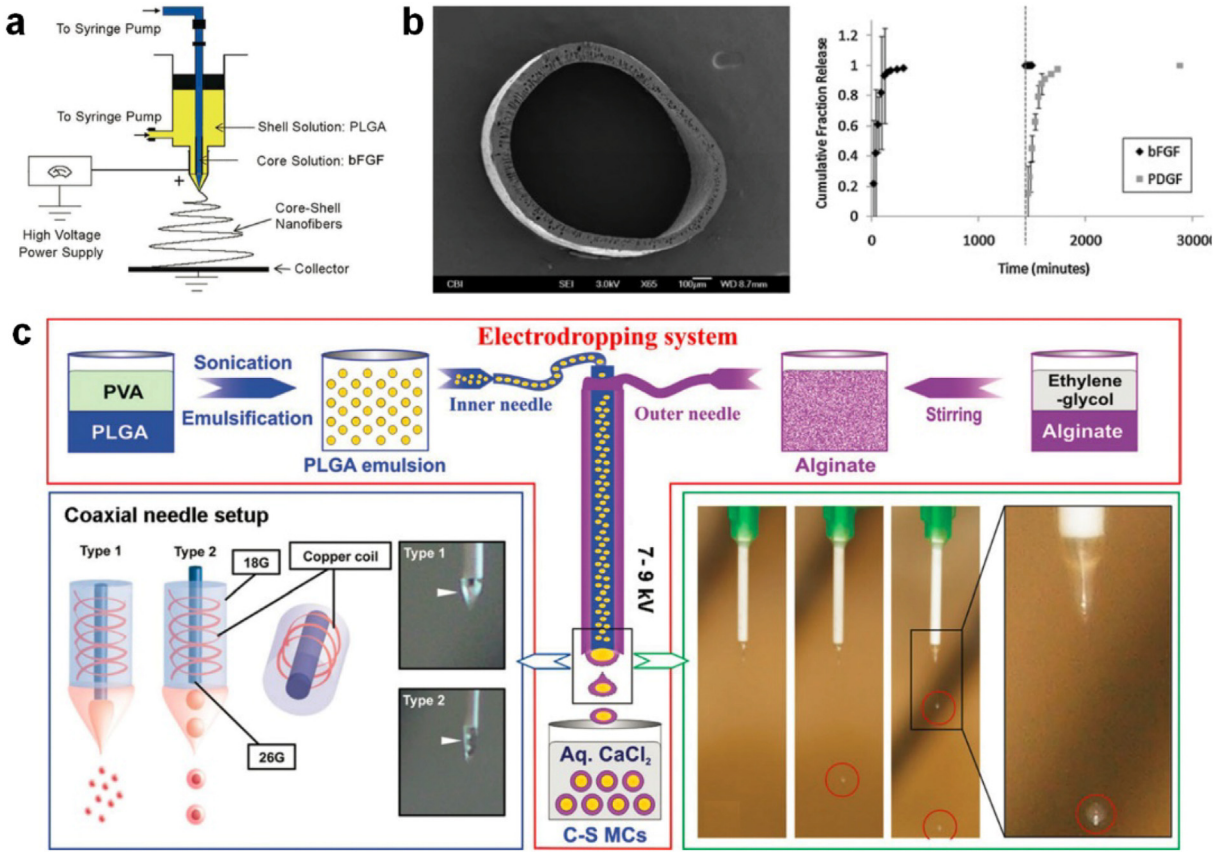

Fig. 3. (a) Schematic of coaxial electrospinning set up to generate layered core-shell structured nanofibers, using two different solutions passing through two coaxial needles. Reproduced from [185]. (b) The schematic of the combination of PLGA emulsion and Alg solution in the coaxial needles and extrusion to produce core-shell microcapsules by using an electrodropping system. (middle); The scheme of coaxial needle setup (left) and photographic images (right) of the extruded microcapsules' formation, including inner PLGA and outer Alg regions. Reproduced from [160]. (c) Scanning electron micrographs of a cellulose hollow fiber delivery carrier and the sequential release profiles of bFGF and PDGF. Reproduced from [188].

system was used to sequentially release VEGF and S1P via manual injection of the GFs at the desired time points. For example, the porous fibers were first loaded with VEGF for an initial period of vessel formation, then subsequently loaded with PDGF for vessel stabilization after the VEGF was delivered. With flexible control of the loading time points, a higher maturation index (high percent of capillary-like vessels co-localized with SMA+ cells) of the vessels was achieved by delivering VEGF for 3 days, followed by releasing S1P for the next 4 days. This same result was also observed when loading bFGF and PDGF in the same hollow fiber delivery system (Fig. 3b) [160].

In another instance, using the common polymers of PLGA and Alg, Park and his co-workers developed an optimized electrodropping system used to generate homogeneous core-shell microcapsules, for sequential release of VEGF and PDGF [188]. As shown in Fig. 3c, the system utilized coaxial needles, where the PLGA emulsion containing PDGF and Alg solution containing VEGF were loaded separately in each syringe. The two syringes had different diameters and the GFs were slowly extruded through inner and outer nozzles. The two immiscible polymers then merged and transformed into a core/shell droplet when they came into contact at the tip of the coaxial nozzle. Finally,

Table 1
The features of the various hydrogels mentioned throughout the review.

Polymer backbone	Hydrogel formation	Cell adhesion ability	Biosafety	Applications	Immune response	References
Collagen	Cross-linked by hydrogen bonds and crystallization between collagen fibers	Natural adhesion motifs	Safe	Growth factor delivery ECM mimic scaffold	Immunogenic	75
Fibrinogen	Cross-linked by thrombin or calcium ions	Natural adhesion motifs	Safe	Growth factor delivery ECM mimic scaffold Hind limb ischemia model	Immunogenic	109, 110, 120-121, 126, 151, 170
Alginate (Alg)	Ionic cross-linked by Ca^{2+} or cross-linked by "click" chemistry	Synthetic RGD peptide motifs	Safe	Growth factor release ECM mimic scaffold	Non-immunogenic	39, 52, 66,112, 114 116, 141, 142, 149, 171
Hyaluronic Acid (HA)	UV cross-linking or cross-linked by "click" chemistry	Synthetic RGD peptide motifs	Safe	Growth factor and cytokine delivery	Non-immunogenic	138-140
Polyethylene Glycol (PEG)	Cross-linked by "click" chemistry	Fibrinogen or collagen composites; Synthetic RGD peptide motifs	Safe	ECM mimic scaffold Growth factor delivery	Non-immunogenic	100-101, 109-110, 115, 130-131, 170, 176

the droplets fell into a $CaCl_2$ solution for gelation of Alg to form the microcapsules. The VEGF embedded in the outer Alg hydrogel diffused out much earlier and faster than PDGF in the core of PLGA. Moreover, the release profile of this sequential delivery system was tunable, by using different molecular weights of PLGA or varying the thickness of layer-by-layer assembly.

5. Conclusion and future perspectives

In recent years, various therapies have been proposed to localize and control the release of pro-angiogenic protein drugs to promote the formation of more mature and stable vessels for treatment of vascular related diseases and disorders. Researchers have developed hydrogel systems (Table 1) for single or combination delivery. Although multiple groups have designed hydrogel carriers capable of maintaining sustained release rates and local delivery, these attempts have failed to account for the complexity of natural angiogenic pathways, resulting in limited therapeutic effects. Obtaining an understanding of the basic biological mechanisms of the complex molecular and cellular interactions within native angiogenic microenvironments is essential for improving engineered approaches to regulating blood vessel formation, maturation, and stabilization. As a result, an optimal sequential delivery system with multiple GFs was considered to hold great potential to enhance therapeutic angiogenesis. Although limited quantities of the GFs are involved and angiogenic pathways are only partially stimulated concurrently, attractive synergistic effects were achieved by sequential release of the GFs both i*n vitro* and in animal models. However, there are still several limitations and difficulties in current sequential delivery systems for vascular regeneration, as well as ample room for further progress.

One major challenge is the need to expand on the types of factors released from the hydrogel carriers. Multi-step sequential release should be utilized when designing more complex and smarter materials, for better mimicry of natural angiogenesis, instead of the delivery of only two or three factors. Moreover, the release rate of each drug from the developed hydrogels should be easily temporally modulated to meet the biomimetic requirements of varying doses of a factor. Hydrogel carriers with different responsive segments, activated by specific cell types or other environmental cues, and complex activated structures may provide a solution for these temporal and kinetic issues.

Another limitation of the current sequential delivery systems is that the release of GF is a passive mechanism, which relies on diffusion and/or cell invasion. In particular, the delivery cannot be actively controlled after the scaffold is implanted *in vivo*. Recently, there are several studies on actuatable release systems that can release drugs "on demand" in response to an external stimulus. For instance, Fabiilli et al, developed acoustically-responsive scaffolds (ARSs) which are fibrin scaffolds doped with two kinds of sonosensitive payload emulsions containing fluorescent payloads of Alexa Fluor 488-labeled dextran and Alexa Fluor 594-labeled dextran, respectively. *In vivo,* the sequential release of the two fluorescent payloads was controlled non-invasively by using consecutive ultrasound exposures at different acoustic pressures [198]. Moreover, DeForest's group reported stimuli-sensitive hydrogel systems with precise degradative responsiveness by multiple environmental cues including enzyme, reduction and light, for sequentially stimulated release of multiple cell lines [199]. The potential of these strategies and platforms should be considered for designs of new smart hydrogels in the field of GF sequential release for blood vessel engineering in the future.

The third principal challenge of the sequential delivery system is the lack of testing in large animal models or clinical trials. Combining these pre-clinical therapeutic ideas with *in vivo* experiments would provide practical experience for material design and, in turn, suggest potential for widespread clinical usages. Overall, progress in any of these areas would greatly expand the potential of the sequential delivery of protein factors and the creation of next generation of hydrogel vehicles.

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