Review

Expert Opinion

Delivery approaches for angiogenic growth factors in the treatment of ischemic conditions

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Introduction: Despite current medical treatments, cardiovascular disease resulting in local ischemia remains a significant clinical problem. Therapeutic angiogenesis, that is, the growth and remodeling of new blood vessels from pre-existing blood vessels to the ischemic area, is a promising solution to this problem.
Areas covered: Therapeutic angiogenesis can be generated *in vivo* through the local release of various proangiogenic factors. This review describes the various formulation approaches that have been devised for this purpose, highlighting the advantages and disadvantages of each.
Expert opinion: Formulations that release single proangiogenic growth factors have not yet been demonstrated to achieve functional therapeutic angiogenesis. Formulations capable of multiple growth factor delivery are needed; however, the complexity of the physiologic process requires the examination of appropriate growth factor doses, as well as release sequence, to guide effectively new formulation design. Furthermore, new formulation approaches need to be tested *in vivo* in appropriate animal models over extended time periods to assess clearly the potential of the delivery approach.

Keywords: angiogenesis, controlled release, growth factor, ischemia, neovascularization

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1. Introduction

Ischemia, or oxygen deprivation, of the tissue of the heart (myocardial ischemia) and limbs (peripheral ischemia) represents a significant health issue in the developed world [1]. Both are chronic conditions that result from occlusion of arteries, primarily as a result of atherosclerosis. As the condition progresses and the degree of blood restriction increases, blood flow to the downstream tissues is impeded. This reduction in blood flow produces ischemia in the tissues fed by the diseased artery. Left untreated, myocardial ischemia can lead to infarct, resulting in permanent muscle damage [2]. In severe cases of peripheral ischemia, healing of minor injuries is impaired, leading to ulceration, gangrene or tissue loss. This condition is referred to as critical limb ischemia [3].

There are several treatment options for myocardial ischemia, including drugs such as beta-blockers and nitrates, angioplasty with or without stenting and bypass grafting surgery. However, drugs only slow down the progression of the disease, and angioplasty and stenting is not an option for a significant population of patients in whom the pathological condition is widespread [4]. Critical limb ischemia can be treated by either a surgical distal bypass procedure or angioplasty. Nevertheless, in most circumstances these approaches are unsuccessful, owing to inadequate autologous veins to use in bypass procedures, occlusion of downstream arteries, or the presence of microvascular disease that hampers healing despite a successful bypass procedure. Thus, ~ 10 – 40% of patients with critical limb ischemia require amputation [5]. For perspective, this translates to ~ 50,000 patients annually in the US [6]. Limb amputation is associated with

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Article highlights.

- Although therapeutic angiogenesis through local administration of proangiogenic factors has large potential, so far no formulation has demonstrated efficacy in a large *in vivo* study.
- Delivery strategies that provide sequential delivery of proangiogenic factors may be necessary.
- The test for efficacy of the delivery strategies should include long-term studies wherein there are measurements of functional improvement in the afflicted tissue.
- A minimally invasive delivery vehicle is preferable; however, implantable delivery systems may be suitable when combined with artery bypass grafting for patients with ungraftable arteries.
- Effective formulations are likely to arise following further examination of the appropriate doses and timing of dosage of the various proangiogenic factors.

This box summarizes key points contained in the article.

significant morbidity, as perioperative mortality is 5 – 10% for below-the-knee amputation and 15 – 20% for above-the-knee amputation. For those patients who survive, nearly 30% will die within 2 years. Moreover, a second amputation will be required in one-third of the survivors, and full mobility will be achieved in less than half of them [7,8]. Thus, an alternative effective treatment for tissue ischemia is an important objective.

Therapeutic angiogenesis, that is, the growth of new capillaries from pre-existing capillaries to bypass the site of occlusion, is a promising solution. In this manner, blood flow to the afflicted region can be re-established. Before describing various drug delivery approaches that have been used to induce therapeutic angiogenesis, it is useful to overview the structure of capillaries and the process of angiogenesis. Capillaries consist of a layer of endothelial cells that are flattened and which possess tight gap junctions. They are typically between 5 and 20 μm in diameter. The endothelial cells are surrounded on the abluminal side by a basement membrane and are enveloped, although not completely covered, by pericytes. The extent of pericyte coverage varies depending on tissue type, species and developmental stage. This variation in coverage is believed to regulate the barrier properties of the vessel; the greater the pericyte number and density, the higher the barrier property of the vessel [9,10].

Normal blood vessel formation is a complex and incompletely understood process. Angiogenesis can be achieved by abluminal sprouting, bridging from existing vessels, or intussusception. During angiogenesis, endothelial cells in their stable, growth-arrested state become activated by locally produced stimuli, such as hypoxia or inflammation. These stimuli activate endothelial cells by initiating the autocrine or paracrine production and release of growth factors or cytokines (**Figure 1**). The blood vessels become hyperpermeable, which allows for extravasation of proteases and extracellular matrix components from the blood. Pericytes are removed from the vessel wall and the basement membrane and extracellular matrix are degraded by the secretion of proteases such as matrix metalloproteinases by the endothelial cells. The extracellular matrix is remodeled, and new matrix synthesized by pericytes or smooth muscle cells is produced. The endothelial cells divide, begin to migrate into the extracellular matrix in response to the presence of soluble and matrix-bound chemical signals and form a migrational column. Behind the migrational front, the endothelial cells stop proliferating and form a new luminal vessel. The endothelial cells then secrete growth factors that recruit pericytes from the surrounding tissue. The pericytes migrate towards and envelop the vessel wall. There they differentiate, deposit matrix and interact with the endothelial cells, stabilizing the newly formed vessel. The endothelial cells become quiescent, strengthen cell–cell contacts and secrete components to complete the extracellular matrix. In the absence of pericyte recruitment and nascent vessel envelopment, the newly formed vessels regress. Finally, the newly formed vessels fuse with existing vasculature and blood flow is initiated [10,11].

The sequence of events in angiogenesis is controlled by several growth factors that coordinate endothelial cell activation, proliferation and migration, and pericyte recruitment and differentiation at different stages of vascular development (**Figure 1**). There are some excellent reviews on the various proangiogenic growth factors involved and their roles [13-16], so the discussion is limited to those factors that have been incorporated into drug delivery devices for the purpose of therapeutic angiogenesis. The roles and physical properties of the main proangiogenic growth factors for this purpose are summarized in **Table 1**.

In situations where there is a major arterial occlusion, the formation of new capillaries alone cannot correct the problem because the primary cause of the ischemia is stenosis of a feeding artery. Thus, in addition to angiogenesis, pre-existing collateral vessels need to be remodeled such that they have an increased luminal diameter to accept increased blood flow. This process is called arteriogenesis, and it is initially triggered by fluid shear stresses caused by differences in pressure upstream and downstream of the occlusion, not by hypoxia [24,25]. Arteries consist of multiple layers: the intima composed of endothelial cells, pericytes and a basement membrane; the media, which is composed of smooth muscle cells and their extracellular matrix; and the adventitia, which is composed of fibroblasts and their extracellular matrix. The endothelial cells are activated by the fluid shear stresses and produce adhesion molecules and chemokines, such as monocyte chemoattractant protein-1 (MCP-1). Monocytes are attracted to, adhere and migrate into the vessel wall. Therein they express proteases that may produce protein degradation fragments that stimulate proliferation and migration of smooth muscle cells, and they release a variety of growth factors, such as FGF-2, HGF and PDGF, which mediate structural remodeling of the extracellular matrix [24].

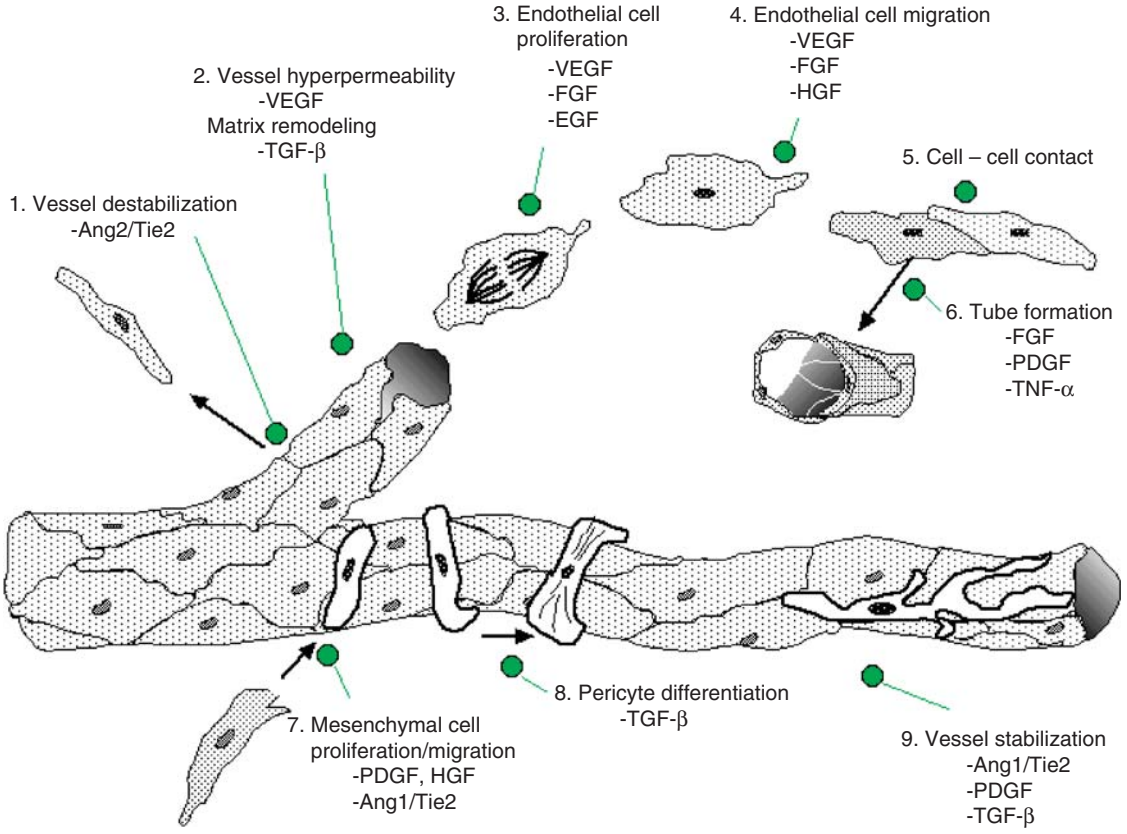

Figure 1. The process of angiogenesis and the soluble factors involved at each stage.

From Hughes [12] as adapted in Papetti and Herman [11], used with permission.

Ang: Angiopoietin; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; HGF: Hepatocyte growth factor; PDGF: Platelet-derived growth factor; TGF-β: Transforming growth factor-β; VEGF: Vascular endothelial growth factor.

Therapeutic angiogenesis and arteriogenesis can be accomplished through delivery of stem or progenitor cells to the ischemic site [26,27], or the local administration of various proangiogenic growth factors [15,28]. Whereas it was initially thought that injected stem cells would differentiate into endothelial cells, it has become clear that therapeutic responses following stem cell transplantation are due to the production of growth factors by the stem cells [5]. Cell source, number and expansion issues need to be solved, and this approach suffers from very poor cell survival on implantation into ischemic tissue, which has led to only very modest benefits in clinical trials involving therapeutic angiogenesis in ischemic regions in the heart [29]. Moreover, in a recent clinical trial involving implantation of bone marrow-derived stem cells, limb amputation was still required in three of four patients [30]. Consequently, growth factor delivery represents a promising alternative.

A continuous and local administration of growth factors at the ischemic site is necessary to induce effective neovascularization [31-34]. This is because most angiogenic factors also act as survival factors for endothelial cells, and exposure to a single growth factor needs to be sustained to prevent early apoptosis of the migrating endothelial cells. The presence of the growth factors at the ischemic site is required for periods of from 3 to 4 weeks [15,31]. However, the elimination half-life of these growth factors is typically short (**Table 1**). To attain sufficient concentrations of these growth factors by means of systemic injection requires multiple large doses, which may result in unwanted blood vessel growth and possibly atherosclerosis, restenosis and carcinogenesis [35]. To achieve effective local low-dose delivery, two alternative strategies have been examined: polymeric delivery and gene therapy. Gene therapy approaches have been investigated in clinical trials, albeit with disappointing results [28,33]. These results can be attributed to variability in individual responses as a result of differences in transfection efficiencies and low transfection rates producing variable intra-individual doses. The different gene therapy vectors that have been examined differ in their cell transduction efficiency, whether proliferating or non-proliferating cells are transduced, and in the duration and extent of transgene expression [34-36]. As a result, there is an inconsistent level of expression achieved with the same administered dose of gene vector in different individuals. A further issue is the need to achieve a balance between prolonged angiogenic growth factor expression and the need to avoid indefinite expression to prevent unknown long-term complications.

Table 1. Proangiogenic growth factors that have been examined in controlled release formulations for therapeutic angiogenesis and their properties.

Growth factor	Activities	Molecular mass (kDa)	pI	$t_{1/2}$ (min)
VEGF-A	Stimulates endothelial cell proliferation; inhibits endothelial cell apoptosis; enhances endothelial cell migration	46 [17]	8.5 [17]	50 [18]
FGF-2	Stimulates endothelial cell proliferation and migration; stimulates endothelial cell tube formation	17.3 [19]	9.6 [19]	3 [20]
HGF	Induces production of VEGF-A and PDGF-BB; induces endothelial cell activation; induces endothelial cell motility; chemoattractant for pericytes	82.9 [21]	9.5 [21]	5 [21]
PDGF-BB	Stimulates proliferation and motility of smooth muscle cells and pericytes; chemoattractant for smooth muscle cells and pericytes; induces VEGF expression in cardiac endothelial cells	26 – 32 [22]	9.8 [22]	2 [23]

FGF-2: Basic fibroblast growth factor (also known as bFGF); HGF: Hepatocyte growth factor; PDGF-BB: Platelet-derived growth factor-BB; VEGF: Vascular endothelial growth factor.

In comparison with gene therapy, polymeric delivery systems have several advantages, which include: precise knowledge of the delivered dose; the ability to achieve prolonged delivery; and the ability to combine several proteins into a single formulation. Polymer delivery strategies used for proangiogenic growth factor delivery have included degradable porous polymer matrices and microspheres, hydrogels, polyelectrolyte complexes and liquid-injectable hydrophobic polymers.

2. Growth factor delivery strategies

When comparing the various formulation approaches for achieving effective proangiogenic growth factor delivery, it is necessary to consider the desirable features of the delivery system. Growth factors are subject to deactivation by several chemical and physical processes [37], which must be considered when designing a formulation approach, and the means by which that formulation is prepared. Moreover, these growth factors are expensive, so efficiency in growth factory loading must be achieved. A large growth factor burst effect is to be avoided as it may produce the undesired effects noted with bolus systemic injections. An *in vivo* sustained delivery of 3 – 4 weeks is needed, within or at the boundary of the afflicted hypoxic tissue. The delivery rate, however, does not need to be constant to be effective. A study by Lee *et al.* [38] showed that VEGF released over 7 days in a diffusionally dependent (i.e., decreasing rate with time) manner from alginate gels at widely varying total doses from ~ 7.2 to 36 ng were effective at generating new blood vessels in 7-week-old SCID mice, and the number of effective blood vessels formed was not affected by the initial dose, but the thickness of tissue revascularized increased as the initial dose increased. Given the nature of the afflicted tissue, it would be beneficial for the formulation to be injected or implanted in a minimally invasive manner. Also, the polymers chosen should be biocompatible and biodegradable within a reasonable timeframe so as to provide effective multi-week growth factor delivery yet not persist at the implantation site long enough to cause chronic inflammation. Furthermore, the degradation of the polymer should not deactivate the protein within the delivery device before it is released.

2.1 Poly(lactide-*co*-glycolide) microspheres

Polymeric microspheres have been developed that are capable of delivering a prolonged and sustained amount of encapsulated protein [39-44]. These formulations generally consist of a biodegradable copolyester, poly(lactide-*co*-glycolide) (PLG), throughout which the protein is distributed as solid particles. The protein is released in three phases: an initial burst; diffusion-controlled release; and polymer erosion-controlled release. The initial burst is due to surface-resident protein particles, whereas the diffusion-controlled release is a result of dissolved protein diffusing through the water-filled pores and channels within the microspheres. To obtain sustained release rate from PLG microspheres, the diffusion phase must overlap with the erosion release phase such that new pores or channels are created.

PLG microspheres have the advantages of not just providing nearly constant release, but of being easily injected to the target site, providing a long release duration, consisting of proven biocompatible materials, having a reasonable shelf-life and degrading to completely bioresorbable compounds. However, owing to polymer erosion, a large problem with this delivery system is maintenance of protein stability [45]. When these polymers degrade, they generate acidic oligomers and monomers. The presence of these acidic compounds decreases the local pH at the surface of the polymer and in the pores and channels of the device [46,47], reaching to as low as 1.5 [48] to 1.8 [49]. These acidic degradation products have been implicated in the denaturation of incorporated protein therapeutics [50-58] and may cause tissue inflammation surrounding the implant [59].

Despite these potential problems, PLG microspheres and matrices have been investigated for VEGF delivery. Cleland *et al.* [31] prepared microspheres having an initial VEGF loading of 9% w/w. These same authors also compression-molded the VEGF PLG microspheres into disks that demonstrated a low initial *in vitro* release and a lag of 10 days followed by complete release over the next 28 days. They found that the released VEGF aggregated and hydrolyzed over time and lost heparin affinity (15% after 8 days), but not its affinity for its mitogenic receptor in an ELISA-based assay. Similarly, Kim and Burgess [60] examined the release of VEGF from PLG microspheres *in vitro* and *in vivo* following subcutaneous injections in rats. They found that the VEGF release rate followed that of the polymer degradation rate both *in vitro* and *in vivo* and that 25% of the activity of the VEGF was lost *in vivo*. They attributed this loss of activity to the acidic microenvironment within the PLG microspheres. In a more recent study by Rocha *et al.* in which VEGF was released from PLG microspheres made from PLG of 85 mol% lactide, it was claimed that there was little VEGF denaturation within the microspheres up to 14 days of release [61]. Nevertheless, the VEGF was released in a large initial burst of ~ 30% of the initially loaded VEGF within the first hour, reached ~ 45% released by 4 days, and was effectively complete at ~ 50% of the initially loaded VEGF by 14 days, with little significant release to 30 days. The VEGF released over the first week or so would not be exposed to significant concentrations of polymer degradation products, as PLG does not degrade that quickly. However, the remaining VEGF would be subject to acidic degradation, and this effect was not explored.

Attempts to overcome this pH issue have included the incorporation of basic salts [62] and blending PEG into the matrix [63-65]. The inclusion of a basic excipient does not prevent the internal pH of the microspheres from decreasing significantly over a 3-week period [66]. Blending PEG with PLG in a microsphere formulation maintained the internal pH at between 5 and 5.8 over 4 weeks in PBS [63]. Nevertheless, when this approach was attempted for VEGF release from PLG microspheres, a significant VEGF burst effect of ~ 60% was observed within the first time point (4 days) [67]. Another approach is to reduce the amount of PLG in the formulation in an attempt to reduce the amount of acidic degradation products. For example, Zhu *et al.* have prepared microspheres containing HGF from poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) blended with PLG in a 1:1 ratio [68]. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) degrades significantly more slowly than PLG [69]. However, the release obtained was biphasic, with a burst over the first 6 days reaching 33%, then little release until day 30, followed by a second phase of release reaching 100% by 60 days. This release profile is inappropriate for achieving therapeutic angiogenesis.

Advancement of this approach to a clinical setting would require that the integrity of the released growth factor be demonstrated, or at least that any changes in growth factor structure are not problematic, and the existing evidence strongly suggests that they are denatured, particularly at the latter stages of release where the PLG microsphere interior has an acidic microenvironment. A further issue of utilizing microspheres is the need for relatively complex manufacturing procedures necessary for maintenance of growth factor bioactivity while achieving efficient entrapment within the microspheres [70]. For example, VEGF encapsulation efficiency in PLG microspheres by the common double-emulsion method has been reported to be only ~ 29 ± 10% [61].

2.2 Osmotically-driven release

One approach to overcome protein degradation resulting from the production of acidic products is to achieve complete protein release before significant degradation of the hydrolyzable polymer occurs. This may be possible by using polymers prepared from monomers such as ε-caprolactone or trimethylene carbonate, which undergo hydrolysis more slowly than PLG [71] and/or by utilizing a different release mechanism. In an attempt to generate a nearly constant release that did not rely on polymer erosion kinetics, Gu *et al.* utilized osmotic pressure-driven release from biodegradable elastomers for the delivery of VEGF *in vitro* [72]. In this approach, the protein is lyophilized along with trehalose, which acts as the osmotic pressure-generating compound, and the solid particles obtained are initially distributed as discrete entities throughout the polymer matrix at a volumetric loading level of < 30%. On implantation of the device, water absorbs into and diffuses through the polymer until it reaches a polymer-encapsulated particle. The water dissolves the particle, generating a saturated solution. Water is then drawn into the capsule formed under the reduced water activity gradient. A pressure is generated in the capsule equal to the osmotic pressure of the solution. This pressure pushes against the surrounding polymer, which undergoes extension and eventually cracks. The cracks formed propagate throughout the matrix, forming an interconnected network of channels that ultimately reach the device surface. The polymer contracts under the reduction in pressure, forcing the dissolved drug solution through the channel network. The process repeats itself in a particle layer-by-layer fashion, moving towards the center of the device [73]. The elastomers were prepared through photocrosslinking a star-poly(ε-caprolactone-*co*-dl-lactide) (50:50 mol:mol dl-lactide and ε-caprolactone) prepolymer that contained terminal acrylate groups. Both cylindrical and slab forms were used. The photoencapsulation technique did not denature the incorporated VEGF [74] and a constant release and high bioactivity (at ~ 80% compared with as-received VEGF) were achieved for 2 weeks. Nevertheless, significant deactivation of the VEGF occurred rapidly after 2 weeks, corresponding to a decrease in internal pH to < 5 [75]. Reformulating the polymer such that it contained 25:25:50 mol ratio of dl-lactide:ε-caprolactone:trimethylene carbonate eliminated the acidic degradation issue [76]. Trimethylene carbonate was chosen for this purpose because, in contrast to PLG, poly(trimethylene carbonate) undergoes hydrolysis slowly [77-79] and produces propanol and carbon

dioxide as degradation products. Moreover, its homopolymer has a low glass transition temperature (ranging from -40 to -17°C, depending on molecular mass [80,81]) and it is biocompatible [78,79,82]. VEGF and HGF were released from cylindrical elastomers at nearly constant rates and were highly (> 80%) bioactive throughout the release period [83]. Drawbacks of this approach are the relatively slow degradation rate of the polymer and the fact that the solid polymer may block tissue from the forming blood vessels.

2.3 Porous matrices/scaffolds

In comparison with solid monolithic approaches such as the osmotically-driven systems described above, porous scaffolds have the advantages of providing a large surface area for host cellular infiltration, thus not blocking tissue from developing blood vessels, and reduced fibrous encapsulation, or fibrosis [84]. Fibrosis is deleterious as it may result in decreased growth factor release efficacy by providing a barrier to transport into the surrounding tissue. Different strategies for releasing growth factors and other therapeutic proteins from various porous scaffolds have recently been reviewed [85]. These approaches include adsorbing growth factors to the scaffold [86,87], coating a protein-containing polymeric emulsion on the internal surfaces of the scaffold [88], blending growth factor containing microspheres or nanospheres into the scaffold [89-91], or directly mixing growth factor containing protein powder into the scaffold during scaffold preparation [92-94]. Adsorbing growth factors onto the scaffold results in low loading efficiencies, potentially rapid release and/or a significant portion (25 – 30%) of the protein initially being released within the first few hours. Both approaches of loading growth factor into microspheres or a protein-containing polymeric emulsion coating utilize a water-in-organic solvent emulsion, which has been associated with a loss in protein bioactivity due to shear and protein conformation changes at the water–solvent interface [95]. Incorporating growth factor directly into the scaffold as solid particles can potentially avoid these shortcomings, as the incorporation of solid protein particles into polymer devices using a polymer-solvent solution, with solvents such as tetrahydrofuran, has been shown to have no effect on protein activity and structure [95]. However, this approach requires that the protein particles be of very small diameter to avoid a large burst effect and subsequent rapid release.

Sheridan *et al.* [96] prepared macroporous PLG scaffolds containing VEGF that produced a large burst of 20% of the initially loaded VEGF over the first 2 days, and reached a total of ~ 40% released after 44 days. They demonstrated that the bioactivity was > 90% over the first 7 days of release, where the total released fraction was ~ 25%, but did not measure the activity of the released VEGF beyond that period, nor did they determine whether the protein was intact or had aggregated. In a subsequent paper, the same group examined the ability of this scaffold to enhance angiogenesis and perfusion in a lower limb animal model of ischemia [97]. In that study, the *in vitro* initial burst reached 40% after the first 24 h and release reached a maximum of 80% after 30 days. No attempt was made to evaluate the bioactivity or integrity of the released VEGF. Nevertheless, on implantation the scaffold produced an improvement in lower limb extremity perfusion in comparison with the controls. Similarly, Ennett *et al.* prepared porous PLG scaffolds for VEG delivery, but pre-encapsulated VEGF within PLG microspheres that were then distributed throughout the PLG scaffold before pore formation through particulate leaching. This was done to improve on both the encapsulation efficiency and the release properties of the VEGF. The influence of PLG monomer composition of the microspheres was shown to influence the release rate of VEGF, with a 75:25 or 85:15 (lactide to glycolide) ratio providing the lowest burst effect and fastest release rates [98]. When implanted *in vivo*, the scaffolds released VEGF with an initial burst of ~ 26% within the first day, followed by a sustained release reaching ~ 72% by 15 days, and 80% by 35 days. The presence of greater numbers of blood vessels surrounding the implants was noted at 2 weeks; however, the overall percentage of the VEGF released that was bioactive was not assessed. Smith *et al.* [99] incorporated HGF in biodegradable PLG porous scaffolds by immersing PLG microspheres in an alginate/HGF solution, followed by lyophilization to achieve a powder, pressing this powder in the presence of NaCl to form disks, then subjecting the disks to 800 psi CO_2 for 24 h followed by rapid reduction of pressure to ambient. The NaCl was then leached from the disks by immersion in 0.1 M $CaCl_2$ for 16 h. Only 55% of the initial amount of HGF was successfully incorporated into the disks using this complicated procedure. Owing to the porous nature of the disks formed, there was an initial period of very rapid release wherein 35% of the total loaded HGF was released within 24 h, 55% was released by 4 days and 63% was released at 10 days. Afterwards, release proceeded very slowly, reaching a total of only 70% after 35 days. Moreover, the HGF retained only 60 – 80% of its original bioactivity over this time.

Polyurethanes with a degradable caprolactone soft segment [100] and poly(ester urethane) ureas composed of polycaprolactone extended with putrescine [94] have also been examined in this regard. These thermoplastic elastomers can be fabricated into protein containing porous scaffolds using solvent casting techniques. Guan *et al.* prepared FGF-2 particles of 0.5 ± 0.3 µm and distributed these particles within a solution of a poly(ester urethane) urea and formed a porous scaffold from this suspension using thermally induced phase separation [94]. FGF-2 release began with a high initial burst of 39% of the initially loaded protein within the first day. Subsequent release was much slower, reaching ~ 70% cumulative mass released after 28 days. The fabrication process involved temperatures as high as 80°C, which probably resulted in protein denaturation, and was lengthy, requiring ~ 10 days to complete. Bioactive FGF-2 was only measurable for up to 21 days, but the fraction of FGF-2 released that was bioactive was not measured. Furthermore, the elastomer degraded very slowly *in vivo*, reaching only ~ 13 – 15% mass loss after 35 weeks *in vivo* [94].

2.4 Hydrogels

Hydrogels are highly water-swollen, crosslinked polymer networks and have potential advantages as protein delivery vehicles. Their high water content makes their mechanical properties similar to tissue, so inflammation resulting from mechanical irritation of the tissue is avoided, they can be prepared from biodegradable and biocompatible materials, and proteins can be effectively incorporated and released from them, controlled by both the crosslink density of the gels and by electrostatic interactions with the polymer chains. Several different hydrogel delivery approaches have been explored, including those using hyaluronic acid, chondroitin sulfate, alginate, chitosan, collagen, fibrin, gelatin and heparin.

Hyaluronic acid is a linear copolymer of 2-acetamide-2-deoxy-D-glucose and D-glucuronic acid whereas chondroitin sulfate is a linear copolymer of *N*-acetylgalactosamine and D-glucuronic acid. Both are natural components of the extracellular matrix of many tissues and so are biodegradable and the devices prepared from them are likely to be biocompatible. Heparin-conjugated hyaluronic acid gels were prepared by Liu *et al.* by reacting aminated hyaluronic acid with aldehyde groups on oxidized heparin [101]. FGF-2 was incorporated into the gels by mixing following gel formation. The FGF-2 was released in a sustained manner over 10 days in Dulbecco's modified Eagle medium, and the released FGF-2 was highly bioactive as assessed by a fibroblast proliferation assay. This approach suffers from the need to load FGF-2 into the gels following their formation, and there was no measurement of the FGF-2 encapsulation efficiency, but it did demonstrate the potential of utilizing heparin-incorporated hyaluronic acid gels for effective FGF-2 delivery. *In situ* setting hydrogels of hyaluronic acid and chondroitin sulfate have been prepared by crosslinking thiolated derivatives of them with poly(ethylene glycol) diacrylate through Michael addition, and this *in situ* gelling approach examined for the controlled delivery of VEGF, FGF-2 and PDGF [102-104]. In each case, thiolated heparin was also included in the gel to provide control over the release of the growth factor. The heparin was included to provide control over the release of and help stabilize the growth factors. It is well established that both VEGF and FGF-2 bind strongly to heparin [105] and that this binding effectively reduces the denaturation of these proteins [106,107]. In the absence of heparin, FGF-2 was released *in vitro* from preformed gel disks with little burst and in a diffusionally dependent, two-stage manner for both chondroitin sulfate and hyaluronic acid gels. The first stage plateaued after 3 days with little release over the next 5 days, followed by a secondary release period lasting to 28 days [102]. This type of release implies a gel degradation mechanism. Incorporation of heparin into the gel matrix reduced the release rates of both stages of release for hyaluronic acid gels, as well as limiting the overall fraction of FGF-2 released; the release rate decreased as the amount of heparin included increased. Inclusion of hyaluronidase in the release medium increased both the release rate and the total fraction released in an enzyme concentration-dependent fashion. Recent studies have examined the effects of the *in vivo* release of VEGF and PDGF as well as FGF-2 from these gels on their ability to elicit angiogenesis in a mouse ear pinnae model; however, growth factor release rates and extent were not provided, making interpretation of the relevance of the data difficult.

Alginate is a block copolymer of D-mannuronic acid and L-guluronic acid residues. The primary advantage of alginate is that it is readily and mildly crosslinked through ionotropic interaction between negatively charged guluronic acid blocks and divalent cations such as Ca and Zn, so proteins can be readily incorporated with no deactivation owing to the crosslinking process. Highly purified alginate is biocompatible, and it can be rendered degradable through partial oxidation using sodium periodate [108]. Alginate has been examined extensively for controlling the release of VEGF [38,109-113]. Although a sustainable *in vitro* release of VEGF can be observed for 4 – 7 days following encapsulation, there is a low protein encapsulation yield, a significant initial burst and a low total fraction released. These problems are probably caused by an electrostatic attraction between the positively charged VEGF and the alginate carboxylate groups, and the large pore size of Ca alginate gels [114]. It has been demonstrated recently that the total fraction released, observed for Ca^{2+} crosslinked alginate gels, can be overcome by the use of Zn^{2+} as a crosslinking cation [113]. Despite this issue, it has recently been shown that the optimal VEGF release profile for generating blood vessels in an *in vitro* endothelial cell sprouting assay consisted of a high initial concentration followed by reduced concentrations of VEGF with time, the type of release profile expected from diffusion of proteins from gel implants [115]. These authors then demonstrated this release profile from oxidized alginate gelled *in situ* via co-injection with calcium sulfate slurry to deliver effectively VEGF *in vivo* in a mouse hindlimb ischemia model. The released VEGF promoted the formation of new blood vessels in the ischemic region, which remained stable up to 6 weeks following injection. Moreover, the new blood vessels improved blood flow into the ischemic region. Nevertheless, the *in vivo* release behavior was not assessed and the impact of a large initial release of VEGF on the generation of undesirable side effects, as has been noted with bolus injections, needs to be examined. A different injectable version of alginate has also been used to examine the potential of HGF to induce angiogenesis *in vivo*. Ruvinov *et al.* used sulfated alginate to form electrostatically complexed HGF bound within alginate matrices [116]. The alginate was made injectable and *in situ* gelling by inducing partial crosslinking *in vitro*, with complete crosslinking occurring *in vivo* through reaction with endogenous calcium. However, following *in vivo* injection into ischemic myocardial tissue, within 6 h 75% of the initially loaded HGF was lost from the injection site and subsequent release was slow; after 5 days, only ~ 85% of the HGF had been released.

Chitosan is a non-physiologic glycosaminoglycan that has been demonstrated to be biocompatible [117], to accelerate

wound healing [118] and to have antimicrobial properties [119]. Chitosan, however, is insoluble at neutral pH. To take advantage of its favorable properties while still achieving a hydrogel, a water-soluble derivative of chitosan would be required. Such a water-soluble chitosan has been prepared by grafting 4-azidobenzoic acid to available free amine groups of lactose-modified chitosan [120]. The azido groups provide the chitosan with photocrosslinking ability. Exposure to UV irradiation converts the azide group to nitrene groups, which are highly reactive with amino groups, inducing reaction with the free amines on the chitosan. A possible disadvantage of this approach is nonspecific interaction of these nitrenes with amino groups on the incorporated protein itself, resulting in protein denaturation and/or grafting of the protein to the chitosan. This is probably the reason for the observed incomplete *in vitro* VEGF and FGF-2 release from matrices in which the modified chitosan was photopolymerized in the presence of these proteins in solution [121,122]. For example, Yeo *et al.* reported that 80% of initially loaded VEGF was permanently retained within azide-grafted chitosan following crosslinking [122]. This approach has been used to deliver FGF-2 to ischemic regions in rabbit hearts, and the released FGF-2 induced angiogenesis in the ischemic myocardium [123]. Unfortunately, *in vivo* release rate and extent were not assessed.

Although designed for use as tissue engineering scaffolds that stimulate vascularization of the engineered tissue, angiogenic growth factor-releasing porous scaffolds have been developed from naturally occurring polymers such as collagen and fibrin that could also be used for therapeutic angiogenesis. For example, Nillesen *et al.* prepared chemically crosslinked collagen I scaffolds containing co-crosslinked heparin. These scaffolds were then soaked with either VEGF or FGF-2 solutions, followed by extensive washing. Although the authors demonstrated angiogenesis following implantation of these scaffolds into subcutaneous pockets in rats, there was no determination of the rate and extent of release of the growth factors [124]. HGF has been incorporated in acidic collagen microspheres by soaking premade collagen microspheres/slabs in solutions of HGF [125-127]. The positively charged HGF was electrostatically bound to the collagen and was released as the collagen matrix degraded *in vivo* by means of enzymatic action. Release proceeded at a decreasing rate with time over 30 days. The disadvantages of this approach are that the HGF bioactivity was significantly reduced by the electrostatic complexation [127], the rate of collagen degradation will vary from individual to individual, the electrostatic complex may be immunogenic and the preparation procedure resulted in low HGF loading efficiency.

As the main constituent of blood clots, which serve as the natural scaffold for cell ingrowth, fibrin supports each stage of blood vessel formation. Moreover, commercial fibrin glues are widely used in the clinic and can be applied *in situ*. Fibrin is gradually resorbed by the secretion of fibrinolytic enzymes, and as such represents a useful starting point for formulating a depot system for proangiogenic growth factor delivery. In an early clinical experiment, 100 μg of VEGF was injected within an *in situ* forming fibrin matrix into the right popliteal region of a 66-year-old man suffering from claudication. The treatment improved the patient's condition and generated substantial growth of new vessels in the infrapopliteal area 4 months later [128]. Nevertheless, the release of VEGF from these formulations proceeds with a large initial burst of 70 – 100% of the initially loaded growth factor within the first 24 h [129]. To overcome this issue, VEGF-121 was covalently linked to the fibrin matrix and released by cell-associated enzymatic activity [129,130]. The VEGF-121 was engineered so as to possess the fibrin-coupling factor XIIIa substrate site at its N terminus, which is a costly and complicated process, and which may result in immunogenicity. This formulation induced the formation of significantly more blood vessels than the bolus release of unconjugated VEGF-121 on subcutaneous implantation; however, the blood vessels did not remain stable [130], possibly owing to the non-hypoxic *in vivo* model used or because only a single growth factor was used (see below). In a different approach, heparin was immobilized to a fibrin matrix to bind FGF-2 electrostatically [131]. *In vitro* release of FGF-2 from heparin-conjugated fibrin plugs was sustained for 3 weeks, and in a mouse hindlimb ischemia model *in situ* formed depots generated a greater capillary density than administration of FGF-2 released from fibrin depots containing FGF-2 and free heparin after 4 weeks. However, this approach has similar drawbacks to those of collagen matrices discussed above: the rate of fibrin degradation will vary from individual to individual, the electrostatic complex may be immunogenic and the *in vivo* release rates are probably much higher than those observed *in vitro*, although this remains to be determined.

Gelatin is derived from collagen from animal sources, has a long history of clinical use and is enzymatically degraded. To make it suitable for controlled release applications, it is crosslinked using chemical crosslinkers, such as, for example, glutaraldehyde or carbodiimides. Crosslinking controls the degradation rate of the gelatin and the release of entrapped proteins; increasing the crosslink density reduces the biodegradation rate and protein release rate. It can be readily prepared into sheets, foams or microspheres by simple techniques. It can also be either positively or negatively charged, depending on how it is processed, and the charge can be utilized to complex electrostatically oppositely charged proteins, adding a further release rate controlling element [132]. Gelatin-based delivery systems have been used for the delivery of FGF-2 [133], HGF [134] and VEGF [135] both *in vivo* and *in vitro* and the released growth factor was bioactive and capable of increasing capillary density and enhancing blood flow into a hindlimb ischemic area in mice [134]. However, the growth factors were loaded into the gels through simple soaking techniques, which are slow, inefficient and make it difficult to sterilize the device terminally. Growth factor release rates from the gelatin appeared to be governed to some extent by the affinity of the growth factor to the gelatin, increasing in the

order: FGF-2 < HGF < VEGF. Furthermore, release proceeded with a significant initial burst effect of between 25 and 30% for FGF-2 and HGF [134], and 65 and 70% for VEGF [135].

To take advantage of the binding affinity of proangiogenic growth factors to sulfated polysaccharides, crosslinked heparin-only hydrogels have also been used to provide sustained release of VEGF and FGF-2. For example, Tae *et al.* functionalized heparin to possess pendant hydrazide groups, then crosslinked the heparin with bis(*N*-hydroxysuccinimidyl)-PEG [136]. The gel slabs formed were then soaked with a VEGF solution. Promisingly, *in vitro* release from the crosslinked slabs was sustained and prolonged (40% total release over the 20 days reported) and proceeded with no burst effect. Moreover, following subcutaneous implantation of the slabs in the dorsal area of mice, enhanced capillary density was observed surrounding the -VEGF-releasing implants at 2 weeks, relative to non-VEGF releasing controls. Nevertheless, the growth factor loading technique suffers from the same issues as described above for the gelatin hydrogel approaches. It would be difficult to overcome this issue with this strategy by preloading the conjugated heparin with growth factor, as the hydrazide would probably react with the free amines on the proteins. In a similar approach, Zieris *et al.* recently crosslinked heparin directly using conventional carbodiimide chemistry and a four-armed, amine-terminated star-PEG [137]. These researchers examined the release of both VEGF and FGF-2 from the gels formed, with growth factor loading achieved by soaking. In contrast to the results of Tae *et al.*, VEGF was released quickly, being complete within 4 days. This difference in release might be due to differences in the growth factor loading approach used, as Tae *et al.* dried the gels to ~ 50% of the initial water content before loading, whereas Zieris *et al.* soaked their gels in growth factor containing buffer for 24 h. FGF-2 was released faster than VEGF despite having a stronger binding affinity to heparin [105]. This result was explained in terms of the difference in hydrodynamic radii of the two molecules; FGF-2 has smaller radius owing to its lower molecular mass and thus would diffuse out faster.

2.4.1 *Cell-mediated growth factor release from hydrogels*

Endothelial cell migration into the extracellular matrix follows a growth factor concentration gradient, is controlled by extracellular matrix degradation through secretion of enzymes such as matrix metalloproteinases (MMP) and the cells receive cues to directional growth through integrin activation via recognition of ligands within the extracellular matrix. In an effort to reproduce the natural process, researchers have created semi-synthetic hydrogels whose design provides incorporated VEGF, enzyme degradable sites and arginine-glycine-aspartic acid (RGD) cell-adhesive ligands [138,139]. The VEGF is released as the migrating endothelial cells secrete enzymes, and migration of the endothelial cells into the network is facilitated by integrin binding to the tethered RGD groups. These matrices have been designed so far with PEG as the basic building block, as it provides a protein-resistant and cell non-adherent background. An MMP-degradable peptide sequence modified so as to undergo reaction with the functionalized PEG was used as a crosslinker, and an RGD containing peptide was also modified so as to react with the functionalized PEG and thereby be co-crosslinked into the network. Zisch *et al.* used star-PEG-vinyl sulfone to react with bis-thiol groups on a MMP-degradable polypeptide, a thiol-bearing RGD containing oligopeptide and a thiol containing VEGF derivative in a Michael-type addition approach [138], whereas Phelps *et al.* used PEG diacrylate photocrosslinked in the presence of MMP-degradable polypeptide functionalized with two PEG-acrylate groups, a mono-PEG-acrylate RGD containing peptide group and mono-PEG-acrylate VEGF [139]. The hydrogels can be readily formed *in situ*; the Michael-type addition reaction approach occurs rapidly under mildly alkaline conditions (pH 8) on mixing the vinyl-sulfone-modified PEG with thiol containing polypeptides, whereas the photocrosslinking approach requires initiation of a photosensitive, biocompatible initiator with long-wave UV irradiation. Recently, Phelps *et al.* have demonstrated the potential utility of this approach in a mouse hindlimb femoral artery ligation model wherein the gels were crosslinked in the muscle surrounding the femoral artery with low-intensity UV irradiation. After 7 days, the mice that received VEGF in gels containing RGD and MMP-degradable sites showed a 50% increase in perfusion to the legs and a 100% increase in perfusion to the feet as compared with untreated mice. This result is highly promising; however, the approach possesses some disadvantages. The implantation requires an invasive procedure to allow for UV crosslinking, although this might not be problematic with the Michael-type addition approach. The proof-of-concept experiment was undertaken over a relatively short term, and longer-term studies are required to demonstrate non-regression of the newly formed vessels. Also, there does not appear to be a simple means of providing sequential delivery of multiple growth factors, which may be necessary to induce a stable vasculature (see Section 2.7).

2.5 Nanoparticles

Nanoparticulate delivery systems have also been explored for growth factor delivery, as they are easily injected and perhaps better incorporated into tissue engineering scaffolds than microspheres. Recently, varying approaches have been used to achieve angiogenic growth factor release from nanoparticulate formulations, including incorporation into PLG [140] and PLG:poloxamer blends [141], electrostatically binding growth factor to PLG nanoparticle surfaces [142] and incorporating growth factor directly into electrostatically formed nanoparticles [143,144]. A common issue with all these approaches is that release proceeds with a large initial burst effect and release duration is generally short.

Incorporation of VEGF directly into PLG nanoparticles was achieved using a double-emulsion (water/oil/water) technique and using high shear homogenization [140]. Such high shear

conditions are responsible for protein denaturation in the preparation of microspheres [45], and per cent VEGF bioactivity on release was not measured. Release proceeded with a large initial burst of ~ 46% of the incorporated VEGF released within the first 24 h, and release was 90% complete by 4 days. Such a short release duration and large initial burst would be problematic in utilizing this approach for effective therapeutic angiogenesis. Building on a formulation strategy that improved protein stability within PLG microspheres, d'Angelo *et al.* entrapped FGF-2 and PDGF-BB in PLG:poloxamer blends [141]. Poloxamers are triblock copolymers composed of a central poly (propylene oxide) block flanked at each end with poly(ethylene oxide) blocks. A high shear double-emulsion process was also used in this approach. In optimal conditions, the encapsulation efficiency of PDGF-BB was 87% whereas that of FGF-2 was 63%, and the production yield was a respectable 73%. Moreover, the use of poloxamer maintained high growth factor bioactivity on release. Nevertheless, release of both growth factors was less than optimal for achieving angiogenesis; a large initial burst of 50 – 60% was observed within the first 24 h and release was essentially complete at 78 ± 7% by 7 days. To overcome denaturation resulting from entrapment in PLG in the formation of nanoparticles, Chung *et al.* first prepared PLG nanoparticles, then adsorbed poloxamer then heparin to the surface [142]. Following heparin adsorption, VEGF was electrostatically bound to the heparin surface by incubation in a VEGF solution overnight. The loading efficiency was not assessed, and this process is probably highly inefficient. *In vitro* release of VEGF from the nanoparticles alone began with an initial burst of ~ 40% and was complete by 5 days. To improve on this release profile, the nanoparticles were incorporated into a fibrin gel. *In vitro* release from the nanoparticle-embedded fibrin gel in disk geometry was markedly improved, showing a reduced burst effect of 18%, and VEGF release was sustained, reaching ~ 80% by 33 days. When these gels were implanted in a rabbit hindlimb ischemia model, newly formed blood vessels appeared to remain stable to 4 weeks. The fibrin gel had degraded and disappeared by 2 weeks, so it is likely that the *in vivo* release was much faster than found *in vitro*; unfortunately, the *in vivo* release rate was not assessed. One issue that needs to be explored further with PLG nanoparticles in this application is the ultimate fate of the nanoparticles. It is presumed that they ultimately have little to no negative interaction with the surrounding tissue, yet this has not been demonstrated in long-term studies.

Efficient encapsulation of VEGF was demonstrated through complex coacervation of dextran sulfate and chitosan to form polyelectrolyte complexes [143]. *In vitro* release of VEGF from these nanoparticles, which had an average diameter of 240 nm, showed a low burst of ~ 12%, but was complete by 9 days. There was no measurement of the bioactivity of the VEGF following release. Similarly, VEGF and PDFG-BB were separately encapsulated in hyaluronic acid/chitosan polyelectrolyte complexes [144]. VEGF was more efficiently entrapped, at 94%, than PDGF-BB, at 54%, probably owing to the differences in their hydrodynamic radii; PDGF-BB is the smaller protein and therefore more likely to be lost through diffusion during entrapment. Comparably sized nanoparticles (200 nm) were achieved in this work as in Huang *et al.* [143]; however, VEGF and PDGF-BB *in vitro* release was much faster, being complete within 4 h. An in vivo demonstration of efficacy with these formulations has not been reported; however, the release behavior from these polyelectrolyte nanoparticles is probably inappropriate for inducing effective angiogenesis.

2.6 Liquid-injectable, hydrophobic polymers

Viscous, amorphous, biodegradable liquid polymers have been developed and examined for delivery of a variety of drugs [145]. Use of these polymers for the delivery of angiogenic growth factors provides possible advantages, such as simple loading by mixing, injectability through standard gage needles and thus administration by means of minimally invasive means; and the liquid nature of the polymer may limit irritation when implanted in soft tissue [146]. So far, there have been few reports on the use of this delivery approach to achieve therapeutic angiogenesis.

A VEGF formulation based on semisolid poly(ortho esters) composed of 3,9-diethylidene-2,4,8,10-tetraoxaspiro [5.5]undecane, triethylene glycol and triethylene glycol diglycolide was first reported by van de Weert *et al.* [147]. These polymers have melting points within the range 25 – 40°C and hydrolyze *in vivo* into biocompatible compounds [148-150]. The VEGF was lyophilized with trehalose as a cryoprotectant and serum albumin as a bulking agent and mixed into the polymer with a mortar and pestle to a total solids loading of 4% w/w after warming the polymer to 30 – 40°C. *In vitro* VEGF release duration was 8 days, but the release was non-continuous, showing a burst of 20% followed by a lag in release of ~ 5 days, and then rapid release over the next 3 days. The inclusion of heparin in the lyophilized powder had no effect on the release kinetics. Furthermore, a low-molecular-mass polypeptide component was found at the later stages (times undisclosed) of release, attributed to the deamidation of the heparin-binding domain of the VEGF catalyzed by the acidic degradation products of the hydrolysis of the polymer.

In an attempt to overcome the deleterious influence of acidic degradation products on VEGF stability, low-molecular-mass (1600 Da) poly(trimethylene carbonate) has recently been investigated as an injectable delivery system for VEGF [151]. The VEGF was co-lyophilized with trehalose (95% w/w) and serum albumin (4.9% w/w), the particles ground and sieved to < 45 μm diameter, and mixed into the polymer to a final loading of 1% w/w. This low loading was used to take advantage of the osmotic pressure driving force generated by dissolution of the trehalose in the particle [152]. The osmotic release mechanism has not been completely elucidated, but is proposed to occur as follows. Dissolution of the particle on contact with water in the polymer forms a saturated solution composed primarily of trehalose. This solution generates a pressure, approximately equal to the osmotic

pressure of the trehalose, which pushes outwards on the surrounding polymer. In response to this pressure, the polymer flows and an aqueous channel is formed that ultimately reaches the surface, releasing the VEGF that has been dissolved into the solution. *In vitro*, VEGF was released in a sustained manner over 40 days, with a nearly constant rate of 15 – 20 ng/day, followed by a monotonically decreasing rate with time. An endothelial cell-based assay indicated that the released VEGF retained > 90% bioactivity throughout the release. Injection of VEGF-loaded poly(trimethylene carbonate) subcutaneously into the backs of rats elicited the formation of blood vessels; however, the blood vessels formed were resorbed with time, reaching the same density as those in the control implants by 3 weeks. The VEGF was released faster *in vivo*, owing to the polymer being distributed into the tissue as droplets as a result of muscle action. A drawback of this approach is that the polymer degraded very slowly *in vivo*, despite its low molecular mass, losing only ~ 55% of its initial mass after 40 weeks [146].

2.7 Combination growth factor delivery

The discussion to this point has been concerned with the release of individual growth factors from various formulations. This analysis has provided information on facets for each approach that are advantageous and aspects of each approach that require improvement. However, a consistent finding in clinical trials with VEGF and FGF-2 is that for newly generated blood vessels to become stabilized and thus provide therapeutic benefit, the administration of a single growth factor is not sufficient [31-34]. This is not surprising given the process of angiogenesis and the need to express and coordinate several molecules in a concentration and time-dependent fashion in order to induce endothelial cell activation, proliferation, migration and tube formation as well as to attract pericytes to stabilize the blood vessels.

This finding has led to the investigation of co-administration of different angiogenic factors. Formulation strategies for the combined delivery of multiple growth factors have recently been reviewed [153], so the focus here is on those used for therapeutic angiogenesis. The first to examine the co-release of different factors for this purpose were Richardson *et al.*, who demonstrated that the co-delivery of VEGF and PDGF-BB at rates of ~ 79 and 3 ng/day, respectively, in a mouse hindlimb ischemia, generated larger and more mature blood vessels than the delivery of either factor alone as measured 2 weeks following implantation [154]. The VEGF and PDGF-BB were released from a porous scaffold device, made by entrapping PDGF-BB in PLG microspheres through a standard double-emulsion process, then compressing these microspheres with particulate PLG and VEGF particles that had been co-lyophilized with alginate and NaCl. The compression process fused the PLG particles together. Subsequently, the NaCl was removed through soaking in water. VEGF and PDGF-BB release occurred simultaneously and was sustained *in vitro* for 34 days. It was reported that the growth factors remained bioactive over the first 3 weeks; however, the data were not shown and the level of bioactivity was not reported. Despite the success of this approach, the longer-term stability of the blood vessels was not measured. Moreover, this manufacturing approach is inefficient in terms of VEGF and PDGF-BB encapsulation efficiency, and potentially suffers from growth factor denaturation due to acidic degradation products from the PLG at the latter stages of release.

In a different approach, Cao *et al.* examined the co-release of FGF-2 and PDGF-BB as well as VEGF and PDGF-BB adsorbed onto heparinized Sepharose beads that were distributed within Matrigel [155]. These gels were injected into the ischemic regions of both rat and rabbit hindlimb models of peripheral limb ischemia. In contrast to the findings of Richardson *et al.*, the combined release of VEGF and PDGF-BB did not stimulate angiogenesis or induce vascular stability, whereas the combined release of FGF-2 and PDGF-BB did. These findings suggested that PDGF-BB cannot stabilize nascent blood vessels produced from VEGF. Unfortunately, the rate, dosage and extent of release of VEGF, FGF-2 and PDGF-BB were not reported. The variable results are probably due to differences in the amounts and duration of the released growth factors. In support of this conclusion, a recent study implies that VEGF may inhibit the response of pericytes to PDGF-BB [156].

Other approaches for combined and co-release have included co-incorporation of VEGF and other growth factors into *in situ* setting hyaluronic/heparin/gelatin/poly(ethylene glycol) hydrogels [103,104] and co-adsorption of VEGF and FGF-2 onto heparinized collagen scaffolds [124]. Both approaches demonstrated that co-administration of VEGF with another growth factor used to induce pericyte envelopment of the nascent blood vessels was more effective than the release of VEGF alone. However, the release rates and concentrations of the growth factors were not provided, and neither approach was tested in a tissue ischemia environment so the prolonged stability of the formed vessels could not be assessed.

Chapanian and Amsden recently demonstrated the possibility of utilizing osmotically-driven release from biodegradable elastomers composed of photocrosslinked, terminally acrylated star-poly(trimethylene carbonate-*co*-DL-lactide-*co*-ε-caprolactone) to achieve both co-release and sequential release of VEGF and HGF from cylinder geometries [83]. Co-release was achieved by co-lyophilizing both growth factors into the same particle (with albumin, trehalose and NaCl to generate sufficient osmotic pressure) and then distributing the particles throughout the prepolymer, pouring the suspension into a glass mold and photocrosslinking with UV light. To obtain sequential delivery, an inner core cylinder contained HGF particles, and this core was covered with an outer polymer layer containing VEGF particles. The HGF particles in the inner core contained a slightly greater amount of NaCl to drive its release. *In vitro*, this formulation showed nearly constant VEGF release from time 0 to 18 days and a lag in HGF release of ~ 4 days, followed by nearly constant HGF release for > 30 days. The released growth factors were highly bioactive, as assessed from cell-based assays. The

approach, however, required relatively large diameter cylinders (3 mm) that would be difficult to implant and was not tested *in vivo*.

3. Conclusions

Therapeutic angiogenesis through local angiogenic growth factor delivery from polymer devices has significant potential for the treatment of ischemic conditions, and these ischemic conditions represent a large and growing health problem. Many different formulation approaches, utilizing the full armament of controlled release devices, have been examined for this purpose, yet no single formulation possesses all the ideal features outlined above, nor has any single approach been demonstrated to be effective in a large *in vivo* study.

4. Expert opinion

Many of the issues with the formulation approaches examined are common to the design of any protein delivery system. The use of hydrolyzable polymers such as PLG for *in vivo* degradation is advantageous in that these polymers are generally well tolerated and their biodegradation rates are appropriate and predictable. However, it has been demonstrated clearly in the literature that the acidic degradation products of these polymers adversely affect protein structure and activity. Alternative hydrophobic and easily processed polymers should be explored as a possible substitute. For example, poly(ester amides) are potentially interesting as they are both enzymatically and hydrolytically degradable, and their hydrolytic degradation leads to a less drastic decrease in pH [157].

Several natural polymer-based delivery scaffolds have been examined, and these approaches have the significant advantage of being degraded *in vivo* through naturally produced enzymes while not producing acidic degradation products. Nevertheless, many of the formulations must be implanted through invasive means. These formulations would probably only be suitable for use in situations where the patient was already undergoing an operation. Such a situation would be in patients undergoing coronary bypass grafting. For patients with multivessel coronary artery disease, bypass grafting is recognized as the treatment of choice, and up to 300,000 Americans have this procedure each year [4]. However, in 25% of these patients, grafting may not be completely successful because of the existence of ungraftable arteries that supply a viable, but underperfused, tissue area [158]. As a result, they have continuing symptoms of angina after surgery and a decreased survival rate. For these patients, a potentially useful strategy might be to combine bypass grafting with localized growth factor delivery to generate new blood microvessels in the ischemic tissue that cannot be treated through grafting [159,160].

Many proof-of-principle experiments have been done with implantation into non-hypoxic regions. Although new blood vessel formation has been noted, which demonstrates some activity of the growth factors once released from the delivery vehicle under examination, the overall objective is to generate stable blood vessels. This can be assessed only by investigating release into a therapeutically relevant situation, that is, ischemic tissue. Moreover, the timeframe of many of the studies has been too short (4 weeks or less) really to determine any efficacy of the approach in terms of maintenance of blood vessel stability. Furthermore, measurement of only the presence of stable blood vessels may not be sufficient, and experiments should also include measurements of functional improvements in the afflicted tissue. For example, the generation of new blood vessels in infarcted regions of the heart has been demonstrated in porcine models, but this was not accompanied by functional improvement in the heart [161].

Given the complexity of angiogenesis and arteriogenesis, it is now accepted that combination growth factor delivery will be the most appropriate approach to generate stable blood vessels. Continued investigation into the mechanisms and roles of each growth factor in this process should prove beneficial in deciding on the appropriate growth factors to use. So far, there have been some demonstrations of efficacy in animal models of the combined co-release of VEGF or FGF-2 with other growth factors; however, the results are variable and long-term vessel stability has not been demonstrated. Given the recent finding that VEGF might inhibit pericyte recruitment by PDGF-BB, delivery strategies that are capable of sequential delivery of proangiogenic factors may be necessary. Such delivery kinetics may not be possible with natural matrices such as collagen and fibrin alone, or by using heparin binding, as VEGF and FGF-2, for example, have very different binding affinities for heparin [105]. Moreover, exploration of the release of other potential proangiogenic molecules such as stromal cell-derived factor-1α, platelet-derived factor, or angiogenic protein fragments is warranted. Stromal cell-derived factor has been shown to induce homing of stem cells [162-166], which have been postulated to coordinate angiogenesis. Platelet-derived factor induces endothelial cell growth, migration and survival and stimulates recruitment of pericytes and fibroblasts [167]. In a recent paper, Roy *et al.* demonstrated the angiogenic potential of an HGF fragment, termed 1K1, which may be less susceptible to degradation, and showed that its release from PLG nanoparticles embedded within Matrigel induced the formation of blood vessels after 12 days [168]. Although promising, longer-term experiments are needed to demonstrate efficacy and analysis of the structural stability of the released 1K1 is required. Coupled with the exploration of different angiogenic factors is the need to examine appropriate doses as well as timing in the delivery of multiple growth factors. These parameters need to be measured during *in vivo* release to assess clearly the potential of each delivery approach.

Declaration of interest

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