

Bioengineering strategies for the treatment of peripheral arterial disease

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ABSTRACT

Peripheral arterial disease (PAD) is a progressive atherosclerotic disorder characterized by narrowing and occlusion of arteries supplying the lower extremities. Approximately 200 million people worldwide are affected by PAD. The current standard of operative care is open or endovascular revascularization in which blood flow restoration is the goal. However, many patients are not appropriate candidates for these treatments and are subject to continuous ischemia of their lower limbs. Current research in the therapy of PAD involves developing modalities that induce angiogenesis, but the results of simple cell transplantation or growth factor delivery have been found to be relatively poor mainly due to difficulties in stem cell retention and survival and rapid diffusion and enzymolysis of growth factors following injection of these agents in the affected tissues. Biomaterials, including hydrogels, have the capability to protect stem cells during injection and to support cell survival. Hydrogels can also provide a sustained release of growth factors at the injection site. This review will focus on biomaterial systems currently being investigated as carriers for cell and growth factor delivery, and will also discuss biomaterials as a potential stand-alone method for the treatment of PAD. Finally, the challenges of development and use of biomaterials systems for PAD treatment will be reviewed.

1. Introduction

Peripheral arterial disease (PAD) is a progressive atherosclerotic disorder characterized by narrowing or occlusion of the arteries that supply the lower extremities. PAD usually presents as claudication (leg pain and severe walking limitation), but some patients progress to critical limb ischemia (CLI; ischemic rest pain and tissue loss) and may lose their leg to amputation [1]. It is estimated that over 200 million people have PAD worldwide with a higher proportion of them being elderly [2]. Prevalence of PAD rises with age (up to 20% in over 65-year-old individuals) due to the increased rates of obesity, type 2 diabetes, as well as sedentary lifestyle [1].

The current standard of operative care is either open or endovascular revascularization. However, approximately 40% of patients are ineligible for these treatments due to high operative risk or inadequate vascular anatomy [3]. Medications are often prescribed to attenuate the underlying atherosclerosis but have modest or no effect

on the function and amputation rates among PAD patients especially in CLI [1]. Hypoxia in normal tissue typically triggers a revascularization compensatory response that includes vasculogenesis, angiogenesis, and arteriogenesis, but this response is defective in patients with CLI [4,5]. The induction of revascularization with the objective of improving circulation and perfusion has always been a key objective of the research on ischemic disease. Recent studies involving growth factor delivery and cell transplantation, combined with the use of biomaterials, highlight that the use of biomaterials has the potential to become an alternative treatment for the care of patients with ischemic diseases. The purpose of this review is to provide an overview of bioengineering approaches for the treatment of PAD.

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2. Pathophysiology of PAD and brief overview of current clinical trials that use advanced therapy medicinal products for PAD

2.1. PAD pathophysiology

Progressive atherosclerosis within the lower limb arteries is the initiating process in PAD [4]. When the blood flow to the lower limbs gets significantly compromised, the body responds with a series of molecular, cellular, and extracellular responses that remodel the ischemic tissue. In particular, vascular homeostasis, including angiogenesis (development of new capillary networks), vasculogenesis (tube formation by endothelial cells from their progenitors), and arteriogenesis (enlargement of pre-existing collateral arteries), act in concert to enhance the blood flow to the affected limb [4,5]. All of these are triggered by hypoxia-related and immunoinflammatory pathways (angiogenesis), circulating or local vascular progenitor cells (vasculogenesis), as well as changes in downstream luminal pressure and shear stress associated with redistribution of blood flow (arteriogenesis) [4,5]. However, in patients with PAD, these compensatory responses are inefficient, and the affected limbs suffer from insufficient tissue perfusion, endothelial dysfunction, chronic inflammation, and high levels of oxidative stress [6,7]. All these changes lead to mitochondrial injury, free radical generation, muscle fiber degeneration, fibrosis, and ultimately tissue loss and gangrene [6,8,9].

2.2. Brief overview of current clinical trials that use advanced therapy medicinal products for PAD

Currently, there are two major types of clinical trials for PAD that use advanced therapy medicinal products. The vast majority of these works focus on patients with CLI (because of the limb threatening nature of the problem) and use gene/growth factor therapy and autologous cell therapy.

2.2.1. Gene/growth factor therapy

Trials evaluating gene therapy, have mainly focused on genes encoding for angiogenesis-related growth factors, including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), hypoxia-inducible factor 1- α (HIF-1 α), and stromal cell-derived factor-1 (SDF-1). Gene therapy (usually delivered by injection in the muscles of the affected legs) has been shown to be safe, but its efficacy remains unknown. When comparing patients treated with gene therapy to controls, no clear differences are found in amputation-free survival, major amputation, and all-cause mortality [10]. Some evidence suggests that gene therapy may lead to improvements in complete ulcer healing, but this outcome needs to be further explored with objectively-defined measures and more detailed description of the type and size of ulcer and the rate of healing [10–12]. Trials investigating growth factor therapy for PAD patients have focused in the use of VEGF, HGF, and FGF. Some evidence suggests that these factors may improve hemodynamic measures and decrease the rate of limb amputations (especially minor amputations) [13–15], however, these trials have not shown clear improvement of ulceration and rest pain [16] while the factors need repeated injections due to their rapid diffusion and enzymolysis *in vivo* [17,18].

2.2.2. Cell-based therapy

Trials investigating the use of autologous cell-based therapies have focused on the use of mobilized peripheral blood stem cells, bone marrow mononuclear cells, bone marrow mesenchymal stem cells, perinatal mesenchymal stem cells, and CD34⁺ cells [19]. The clinical data about these cells have demonstrated they are safe and well-tolerated in patients. In terms of cell efficacy, current trials are very dissimilar, and this makes comparison of their results difficult, because these autologous cells have been derived from various sources, prepared using distinctive protocols, administered at different doses, and

delivered via diverse routes [20]. In particular, the efficiency of cell therapy on clinical end points is not as great as it was in preclinical trials in the randomized controlled trials [21,22]. Furthermore, the injected/transplanted cells experience many adversities, including the shearing force during injection and the lack of endogenous supporting cues, hypoxia, and oxidative stress of the recipient tissues. All of these issues lead to a diminished quantity of viable cells and only less than 10% of injected cells survive past the first week [23,24]. Using a larger number of therapeutic cells increases the costs for cell processing and the risks of side effects. Efficacy of autologous cell-based therapy in PAD patients would likely benefit from delivery strategies to enhance the specificity, efficacy, and reproducibility of cell therapy with minimized cell dosage and side effects [23].

3. Bioengineering approaches for the treatment of PAD

3.1. Biomaterials-mediated exogenous cell transplantation for the treatment of PAD

Current research has highlighted that biomaterials, especially hydrogels, can encapsulate cells and protect them against shearing force during injection [23,25]. Hydrogel is a three-dimensional (3D) network based on hydrophilic polymers, which are crosslinked through covalent bonds, hydrogen bonds, ionic bonds, or intermolecular hydrophobic association. Hydrogels can provide biophysical and biochemical cues to injected cells which influence their proliferation, migration, and secretory profile. Hydrogels have been applied to deliver various types of cells to treat PAD, including endothelial cells [26,27], macrophages [26], and stem cells [28]. For example, the group of Lee et al. have demonstrated that a biocompatible peptide amphiphile (PA) nanomatrix hydrogel substantially improved long-term survival of human pluripotent stem cell (hPSC)-derived ECs in an ischemic hindlimb environment (> 10 months). The hPSC-derived ECs, when encapsulated into PA hydrogel, showed better perfusion recovery and higher and more prolonged angiogenic and vascular incorporation capabilities than the bare hPSC-derived ECs [29,30].

Adipose-derived stem cells (ASCs) are also a potential resource for cell therapy in PAD. ASCs are much easier to obtain than bone marrow-derived stem cells. With low expression of surface histocompatibility antigens, ASCs could possibly escape host immune system without inducing allospecific T-cell proliferative responses [23,28,31]. Recently Li et al. have developed and used injectable 3D microscale cellular niches (microniches) based on gelatin. The primed hydrogel microniches protected hASCs from mechanical insults during injection, dramatically improved cell retention and survival following intramuscular injection. Most importantly, these microniches with cells have shown superior therapeutic efficiency with a cell dosage of 1×10^5 cells, which is 10 times less than the lowest dosage of 1×10^6 cells used in all previously reported therapy in treating CLI in a mouse model (Fig. 1) [24]. The primary action of stem cells for PAD/CLI treatment is paracrine secretion [1,3,28]. With the use of hydrogels, many research groups seek ways to support stem cell survival and increase their secretory profile. We have summarized current research related to hydrogels with exogenous cell transplantation for PAD treatment in Table 1.

When developing biomaterials as delivery vehicles for exogenous cells, biocompatibility is the first and major concern. Natural materials, like collagen and fibrin, provide excellent compatibility with cells and can be customized to deliver and protect different cell types but are associated with immunogenicity and inflammation [32]. On the other hand, synthetic material is less likely to cause an immune response, but some of the chemical reactions involved in crosslinking interfere with the biological processes of cells, which may disrupt cell survival [25]. Bioadhesive ligands are needed to conjugate to synthetic materials to enhance their biocompatibility. Furthermore, mechanical properties of hydrogels need to be carefully considered. This becomes especially important in PAD because most of the time the biomaterials are injected

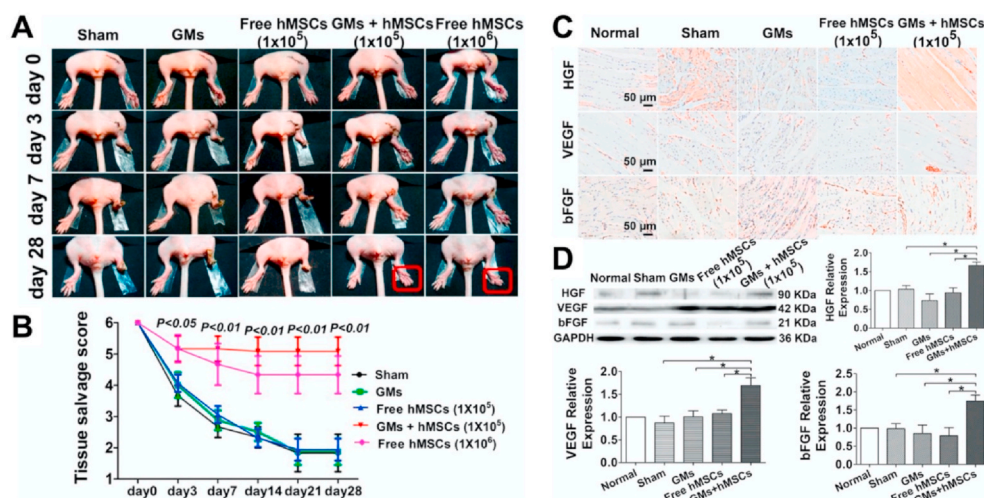


Fig. 1. Improved salvage and enhanced angiogenesis with increased expression of angiogenic factors in ischemic hindlimbs based on the 3D injectable microcylinders. (A) Representative photographs of sham ($n = 4$), blank gelatin microcylinders (GMs) ($n = 4$), free hMSCs (10^5) ($n = 8$), hMSCs (10^5) within GMs (GMs + hMSCs) ($n = 8$), and free hMSCs (10^6) ($n = 4$) at 0, 3, 7, and 28 d after treatment. (B) Tissue salvage score of ischemic hindlimbs in different treatment groups. (C) Immunohistochemical staining of ischemic muscle tissue sections 28 d after cell therapy for HGF, VEGF, and bFGF. (D) Images and semiquantitative analysis of Western blot for expressions of HGF, VEGF, and bFGF of ischemic muscle tissue 28 d after cell therapy. Relative expression of each protein was normalized to GAPDH, and results of each group were normalized

to the result of the normal group. $*P < 0.05$ ($n = 3$). The Figure is reproduced with minor adaptations from Ref. [24] with permission.

in the dynamic contracting muscle environment. Most natural substances lack mechanical resilience and are prone to early degradation, such as decellularized extracellular matrix (ECM). Synthetic materials can be modified to have tunable mechanical properties [23], but the properties that promote desirable outcomes still need to be identified. Finally, hydrogel microstructures need to be considered as they have dramatic effects on cell behaviors and also can affect tissue regeneration [33,34]. Finally, injectable hydrogel is considered superior to three-dimensional (3D) hydrogel scaffolds that require incisions for their placement in the recipient leg [24].

3.2. Biomaterials-mediated extracellular vesicle delivery for the treatment of PAD

Extracellular vesicles (EVs) are cell-derived vesicles comprising exosomes and microvesicles. They play a major role in cell to cell communication, in the exchange of proteins, lipids, and genetic materials, thus making them effective regulators of tissue repair [35]. Functional properties of EVs depend on their molecular composition, which has been shown to be similar to the cell of origin. Contrasting physiological or pathological conditions can alter the molecular composition of EVs. Changes in the microenvironment can induce a change of the EV contents. For example, exposure to an ischemic microenvironment elevates the expression of angiogenic proteins not only in cells but also in their EVs. These EVs contain many robust angiogenic factors and are capable of stimulating angiogenesis [36].

Various EVs derived from different cell sources, including MSCs [36], cardiac progenitor cells [37], glioblastoma cells [38], and CD34⁺ stem cells [39], have been explored for the purposes of therapeutic angiogenesis in PAD. Like growth factors, EVs diffuse rapidly and require large doses, thus treating with EVs alone is insufficient. Hydrogels have been shown to prolong the release of EVs (Table 2). For example, Zhang et al. have evaluated if chitosan hydrogel could boost the retention, stability and therapeutic effects of exosomes derived from human placenta MSCs. Chitosan hydrogel notably increased the stability of proteins and microRNAs in exosomes, and increased the retention of exosomes *in vivo* (Fig. 2) [40]. In similar work, the incorporation of EVs and miRNA antagonists, including anti-miR and antago-miR, in porcine-derived decellularized ECM hydrogels lead to a prolonged release as compared to the use of these biologic agents alone [37].

3.3. Engineering delivery methods for growth factors for the treatment of PAD

Therapeutic angiogenesis with direct delivery of growth factors holds great promise for treating patients with leg ischemia, but current methods have not found success in clinical trials. There are several major hurdles, including short half-lives of growth factors. In addition, long-term disease states and co-morbidities, such as diabetes and obesity, make patients resistant to angiogenic stimuli. As we have summarized in Table 3, various types of biomaterials have been used to deliver growth factors for PAD treatment. Incorporation of these signaling molecules into biomaterials, as opposed to a bolus injection, protects factors from rapid enzymatic degradation, resulting in sustained release over a longer therapeutic window [41]. Here we highlight several unique approaches that aim to address the major hurdles of growth factor delivery.

3.3.1. Harnessing activity of endogenous pro-healing factors

Several research groups have worked to harness the activity of endogenous pro-healing factors generated at the injury site [42,43]. For example, the Cool group has reported a heparan sulphate (termed HS7) tuned to avidly bind VEGF₁₆₅. HS7 stabilized VEGF₁₆₅ against thermal and enzyme degradation *in vitro*, and isolated VEGF₁₆₅ from serum via affinity-chromatography. Intramuscular injection of HS7 into vastus lateralis, vastus medialis, and gastrocnemius improved blood reperfusion in the footpad of C57BL/6 N mice with hindlimb ischemia with a recovered hindlimb blood volume two-to four-fold faster compared to the saline group. These results highlight the potential of an affinity-isolated heparan sulphate for limb ischemia treatment [44,45]. However, PAD and critical limb ischemia are more prevalent in aged and unhealthy populations, such as patients with diabetes. These patients have reduced expression of growth factors and their receptors, adding to the challenges of exploiting endogenous factors. For these PAD patients with diabetes, the combined delivery of exogenous growth factors and heparan sulphate is required. Heparan sulphate can stabilize the exogenous growth factors, reduce the amounts of growth factors required, and decrease side effects accompanying the high dose of growth factors in revascularization therapy.

3.3.2. Delivering co-receptors with exogenous growth factors

PAD patients who are in the greatest need of angiogenic therapies often have long-term disease states, such as diabetes and obesity, which make them resistant to angiogenic stimuli. Growth factor signaling is controlled at the cell surface level via binding to heparan sulphate

Table 1
Modalities of exogenous cell transplantation with hydrogels.

Biomaterial	Growth Factor	Cell Type	Comment	Animal	Model	Delivery Method	Ref.
SHIELD hydrogel: (1) Seven CC43 WW domains (2) An 8-arm PEG with proline rich peptides (3) PNIPAM	None	Human iPSC-ECs	Shear thinning, self-healing, injectable Enhanced iPSC-EC retention Improved neovascularization of the ischemic limb by arteriogenesis No significant improvement of capillary density or blood perfusion	NOD/SCID male mice; 12 weeks old	Femoral artery ligated and excised	Gastrocnemius; Delivery volume: 20 μ L; 1 \times 10 ⁶ cells/mouse	[100]
	bFGF	Rat MSCs	Thermosensitive and injectable Promoted MSC survival Engrafted MSCs differentiated into skeletal muscle and endothelial cells Blood perfusion completely recovered after 2 weeks The average muscle fiber diameter was similar to that of the normal limb after 4 weeks	Male wild type C57BL/6 mice; 8–10 weeks old	Femoral artery and vein ligation	Gracilis; Delivery volume: 200 μ L; 50 μ L/injection; Cell number not mentioned	[101]
	None	Human ASCs	Non-swollen and resilient Cells retained in the scaffold over 28 days Increased intramuscular vascular density by continued secretion of paracrine factor	NOD/SCID mice	Right femoral and saphenous artery and vein ligated and excised	Adductors; Delivery volume: 20 μ L; Single injection; 4 \times 10 ⁵ cells/mouse Hindlimb muscles; 2 \times 10 ⁵ cells/mouse	[23]
Peptide amphiphile nanomatrix gel	None	Human iPSC-ECs	Injectable Longer cell survival and retention (> 10 months) Better perfusion recovery	Athymic male nude mice	Ligation of the femoral artery and cauterization of large branches		[29,30]
Gelatin microcryogels	None	Human MSCs	Engrafted iPSC-ECs into the host vessels Injectable Improved cell survival and retention Increased angiogenesis, blood perfusion, and ischemic limb salvage	Female BALB/c nude mice	Femoral artery and its branches ligated and excised	Gracilis; Delivery volume: 150 μ L; 1 \times 10 ⁵ cells/mouse	[24]
Type-I collagen-based microgel with 4S-StarPEG	None	Human MSCs	Injectable microgels Reduced radial diffusion Complete limb salvage along with a reduced inflammation response	Nude mice	Femoral artery ligated at the proximal and distal region above the profunda femoris branch	Surgery site; Delivery amount 30 \pm 5 microgels/mouse; 1600 cells/microgel	[102,103]
Human platelet lysate-based hydrogel	None	Human MSCs	Injectable Improved perfusion with complete restoration of perfusion by day 8	Male NOD/SCID mice; 12 weeks old	Ligation and removal of femoral artery	Gastrocnemius; Delivery volume: 200 μ L; 1 \times 10 ⁶ cells/mouse	[104]
Chitosan-HA hydrogel	C domain peptide of IGF (IGF-1C)	Mouse ASCs	Injectable Improved cell survival and retention during injection Suppressed collagen deposition Enhanced angiogenesis	Male BALB/c nude mice; 8–10 weeks old	Left femoral artery and vein ligated and cut from just above the deep femoral artery to the popliteal artery and vein	Ischemic limbs; Delivery volume: 100 μ L; 34 μ L/injection site; 1 \times 10 ⁶ cells/mouse	[105]
Chitosan and β -glycerophosphate (β -GP) hydrogel	IGF-1C	Human placenta-derived MSCs	Accelerated ischemic limb function Injectable and thermosensitive Improved cell survival Provided a conducive niche for hP-MSCs to exert pro-mitogenic, anti-apoptotic, and pro-angiogenic effects, and inhibit fibrosis Enhanced neovascularization and limb salvage	BALB/c nude mice; 8–12 weeks old; Transgenic Vegfr2-luc mice	Femoral artery ligated and excised between the proximal location near the groin and the distal location close to the knee	Quadriceps and gastrocnemius; Delivery volume: 30 μ L; 10 μ L/injection site; 1 \times 10 ⁶ cells/mouse	[40,106]
Nap-GFFYK-thiol hydrogel	None	Human placenta-derived MSCs	Injectable Promoted survival and paracrine activity of hP-MSCs Improved the retention of hP-MSC Improved blood perfusion leading to superior limb salvage	BALB/c nude mice; 8–12 weeks old; Transgenic Vegfr2-luc mice	Femoral artery ligated and excised between the proximal location near the groin and the distal location close to the knee	Adductor muscle adjacent to and within 1 mm proximal or distal to the ligation site; Delivery volume: 100 μ L; 34 μ L/injection site; 3 \times 10 ⁷ cells/mouse	[107]

Table 2
Delivering extracellular vesicles for PAD treatments.

Biomaterial	Cell Type	Factor	Comment	Animal	Model	Delivery Method	Ref.
Glycosylated chitosan hydrogel	MSCs derived from human placenta	VEGF and miR-126	Augmented the angiogenic effects of HUVECs <i>in vitro</i> Superior angiogenic effects and ameliorated limb function	BALB/c nude mice; 8–12 weeks old; Transgenic Vegfr2-luc mice	The femoral artery ligated and excised between the proximal location and the distal location close to the knee	Adductor muscle; 100 µg exosomes in 100 µL PBS; 3 injection sites	[36]
Porcine-derived decellularized ECM hydrogel	Human cardiac progenitor cells	None	An advantageous platform for the delivery of miRNAs and EVs	None	None	None	[37]
Alginate beads	Glioblastoma cells (A-172)	miR-221, glypican-1, syndecan-4	Enhanced endothelial cell proliferation tubule formation <i>in vitro</i> Promoted revascularization in comparison to alginate gels and FGF-2 alone	C57BL/6 mice	The femoral artery ligated	Ischemic hindlimb; Delivery volume: 200 µL; ~13 billion exosomes/mouse;	[38]
None	Human CD34 ⁺ stem cells	miR-126-3p	Induced therapeutic angiogenesis mediated by exosomal miR-126-3p Internalized by endothelial cells in the ischemic tissue to induce cell cycle induction, angiogenesis and proliferation	Balb C mice; 8–10 weeks old	A ligation was made around the femoral artery and all arterial branches were removed	1.5 µg/mouse Ischemic hindlimb; Delivery volume: 20 µL; 4 injection sites; 5 × 10 ⁶ cells/kg	[39]

proteoglycans, such as syndecans. Syndecan-2 can bind VEGF and is essential for VEGF-mediated angiogenesis [46,47]. Recently the group of Baker et al. using a mouse model of diabetes and obesity, has demonstrated that these common disease states cause a profound shift in signaling pathways of growth factors, including FGF-2, VEGF-A, and platelet-derived growth factor (PDGF). In skeletal muscle, a high-fat diet in wild-type mice (C57BL/6J) increased levels of growth factor receptors and co-receptors including syndecan-1, syndecan-4, and PDGFR- α , but these increases did not occur in Ob/Ob mice (B6.Cg-Lep^{ob}/J) [48]. They have demonstrated that syndecan-4 with FGF-2 could improve the effectiveness of FGF-2 therapy for ischemia in the diabetic disease state [49,50]. Syndecan-4 therapy also induced a marked immunomodulation in the tissues, such as M2 phenotype polarization of macrophages [51]. In addition, they also created a novel therapeutic enhancer for growth factor activity consisting of glypican-1 delivered in a nanoliposomal carrier. This carrier enhanced FGF-2 trafficking by increasing both uptake and endosomal processing. Co-delivery of glypisomes with FGF-2 markedly increased the recovery of perfusion and vessel formation in ischemic hindlimbs of wild type and diabetic mice in comparison to mice treated with FGF-2 alone [49].

3.3.3. Delivering growth factor-mimicking peptides

Other researchers have been exploring growth factor-mimicking peptides instead of growth factors since these mimicking peptides have longer half-lives. For example, the angiogenic factor, secretoneurin, has induced angiogenesis, arteriogenesis, and vasculogenesis in the mouse hind limb ischemia model [52,53]. Albrecht-Schgoer et al. have identified the biologically active part of secretoneurin and modified it by induction of a cysteine residue. Afterwards, they packed it into S-protected thiolated chitosan nanoparticles, which gave secretoneurin higher stability against enzymatic degradation. They demonstrated that secretoneurin nanoparticles after intramuscular injection restored blood flow in a mouse hindlimb ischemia model within one week, whereas control particles did not [54,55].

3.3.4. Targeted delivery of growth factors to ischemic tissue

Cell membrane-functionalized nanoparticles have been employed to enhance natural targeting for therapeutic applications in PAD [56,57]. Bose et al. designed bioengineered stem cell membrane-functionalized poly(lactic-co-glycolic acid) (PLGA) nanoparticles. The group engineered hASCs to overexpress CXCR4 to coat VEGF-loaded PLGA nanoparticles. The CXCR4 receptors allow targeting ligands for SDF-1, thereby enhancing targeted delivery of VEGF to the ischemic site [58]. The functionalization enhanced the nanoparticle penetration across the endothelial cell barrier and significantly decreased the nanoparticle uptake in macrophages. Systemic retro-orbital injection of nanoparticles loaded with VEGF into mice with hindlimb ischemia resulted in accumulation of a large part of the particles in the ischemic limb, substantial enhancement of blood reperfusion, muscle repair, and limb salvage (Fig. 3) [59].

3.3.5. Facilitating the beneficial effects of growth factors with infused stem cells

It is worth noting that very few studies have been performed in clinically relevant animals that have characteristics that are closer to human PAD such as size, comorbidities, metabolic syndrome, and increased age [60,61]. Recently Anderson et al. have applied alginate hydrogels to provide sustained release of VEGF and insulin-like growth factor (IGF) into ischemic hind limbs in middle-aged and old mice and in young rabbits [62]. As mice aged, spontaneous perfusion recovery after ischemia decreased. But the combination of VEGF and IGF delivery from hydrogels significantly rescued perfusion in middle-aged (13 months) and old (20 months) mice. In rabbits, the delivery of VEGF alone or in combination with IGF from alginate hydrogels, enhanced perfusion recovery when given immediately after surgery, or as a treatment for chronic ischemia. Moreover, dosages were 2 orders of

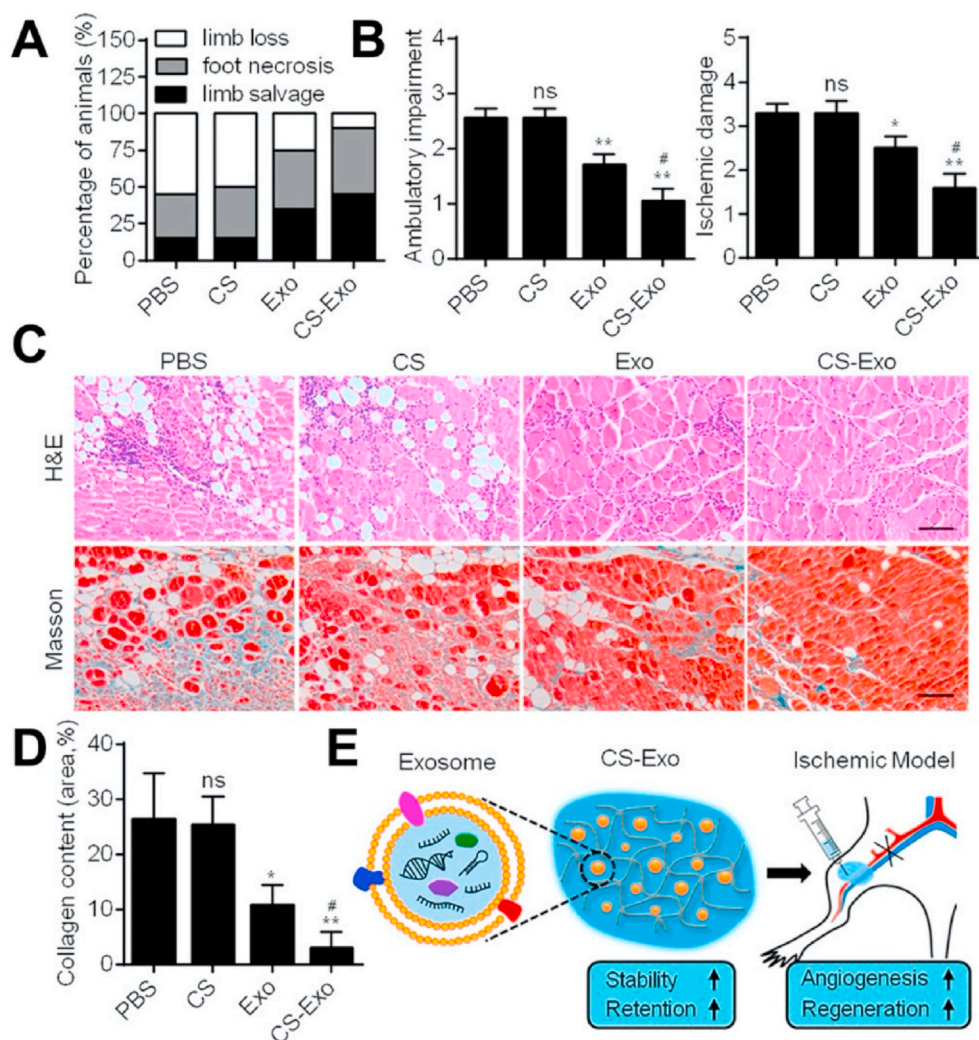


Fig. 2. Accelerated ischemic muscle recovery by treatment of chitosan hydrogel-exosomes (CS-Exo). (A) Percentage distributions of limb salvage, foot necrosis, and limb loss in each group ($n = 10$). (B) Semiquantitative clinical assessments of ambulatory impairment and ischemic damage of ischemia hindlimbs in each group ($n = 10$). (C) Representative images of muscle sections stained with H&E at day 14 and Masson's trichrome at day 28 ($n = 5$). Scale bar = 100 μm (D) Quantification of the area of fibrosis in Masson's trichrome staining sections. (E) Schematic illustration of hP-MSC-derived Exo incorporated with CS hydrogel for muscle regeneration (* $P < 0.05$; ** $P < 0.01$ vs PBS; # $P < 0.05$ vs Exo). The Figure is reproduced without modification from Ref. [40] with permission.

magnitude lower than the typical doses used in past rabbit studies.

Since hydrogel can locally release growth factors and cytokines in a spatiotemporally controlled manner at the ischemic hindlimb, it can create a niche to recruit pro-endothelial progenitor cells [63,64]. Anderson et al. have shown that local, sustained delivery of exogenous VEGF and SDF from alginate hydrogels could increase the recruitment of systemically infused endothelial progenitors to ischemic tissue, and subsequent neovascularization [41]. Late outgrowth endothelial cells (OECs) and circulating angiogenic cells (CACs, or early endothelial progenitor cells) were compared in this study which showed that CACs demonstrated greater ability to accumulate to a greater extent in the ischemic hindlimb than OECs.

3.4. Biomaterials as stand-alone treatments of PAD

3.4.1. Hydrogels

3.4.1.1. Decellularized ECM hydrogels. Decellularized ECM can be derived from different sources and widely used for tissue engineering and regenerative medicine applications [65–67]. With additional processing, decellularized ECM can form injectable hydrogels that allows for their delivery using minimally invasive approaches. The group of Kristman et al. has tested the efficacy of injectable decellularized ECM hydrogels alone as a potential therapy for treating patients with PAD [68]. They compared hydrogel derived from decellularized porcine skeletal muscle (SKM) to that derived from human umbilical cord matrix. Both hydrogels were able to produce significant improvements in hindlimb tissue perfusion. In particular,

the SKM hydrogel, which closely matched healthy tissue morphology, improved functional outcomes through stimulation of arteriogenesis and muscle progenitor cell recruitment [69–71]. To facilitate the clinical translation of the decellularized SKM hydrogel, the group further optimized the concentration of SKM in an aged mouse hindlimb ischemia model and were able to again confirm the beneficial effect of SKM hydrogel in blood perfusion in this more clinically-relevant setting [71].

3.4.1.2. Microchannel network hydrogels. The microstructure of hydrogels may guide desirable biological functions when applied to implant sites [72–74]. Lee et al. generated a gelation hydrogel with microchannel ($16.4 \pm 7.8 \mu\text{m}$) or macrochannel ($150.5 \pm 57.0 \mu\text{m}$) networks. When the gel was used to dress the defect in a mouse model of hindlimb ischemia, the hydrogel with microchannels rescued severely damaged tissues by the ingrowth of neighboring host vessels. The microchannels allowed infiltration of macrophages and specifically polarized them into pro-angiogenic phenotypes with the consequent ingrowth of functional endothelial cells, leading to increased blood perfusion (Fig. 4) [75–77].

3.4.2. Reactive oxygen species (ROS)-sensitive particles

There is a characteristic ischemic myopathy in the legs of patients with PAD [6,8,9]. The histological characteristics of myopathy are progressive myofiber degeneration and fibrosis associated with increased oxidative damage [7,78–81]. PAD induces a state of tissue ischemia and ischemia/reperfusion in which ROS are produced in levels

Table 3
Engineering delivery methods for growth factors.

Biomaterial	Growth Factor	Comment	Animal	Model	Delivery Method	Ref.
Alginate hydrogels	VEGF and IGF	In rabbits, the amount of growth factors was 2 orders of magnitude lower than the typical doses. Enhanced perfusion recovery	Female C57BL/6 J mice: Young mice (8–10 weeks old); Middle-aged (13 months old); Old mice (20 months old); Female New Zealand White rabbits (2.5–3 kg)	Mouse: Unilateral external iliac and femoral artery and vein ligation Rabbit: Ligation of the lateral circumflex artery, and the common, superficial and deep femoral arteries	The area of the ligated vessels; Muscle tissue surrounding the vessel ligation site; Mouse: 50 µL; 3 µg VEGF and 3 µg IGF; Rabbit: 500 µL; 50 µL/ injection; 20 µg VEGF and 20 µg IGF	[62]
Alginate hydrogels	VEGF and SDF	Factors were injected in the ischemic leg and were able to attract endothelial progenitors, that were systemically infused, to the ischemia site. Overcome growth factor resistance	C57BL/6 J mice for short-term recruitment studies; C. B-17 SCID mice for long-term healing	Ligation of external iliac artery and vein	Under the ligation site; 3 µg VEGF and 3 µg SDF	[41]
Nanoliposomal carrier with alginate hydrogels	Glypican-1; Syndecan-4; FGF-2	Wild-type mice or ob/ob mice (B6.Cg-Lepob/J); The ob/ob mice fed a high fat diet for 10 weeks	Wild-type mice or ob/ob mice (B6.Cg-Lepob/J); The ob/ob mice fed a high fat diet for 10 weeks	The femoral artery separated from the femoral vein and nerve, and then double ligated and the artery severed at each ligation	The region surrounding the femoral artery; 3 µg FGF-2	[48–51]
Thiolated chitosan nanoparticles	Secretoneurin	Restored perfusion at the ischemia site	Mice	The left femoral artery exposed, ligated, and excised	Thigh and calf muscles; Injection volume: 100 µL; 20 µL/injection;	[54]
PVAX nanoparticle	Neuropeptide Y ₃₋₃₆	Induced angiogenesis and arteriogenesis as well as improved functional blood flow	C57BL/6 J mice; 7–8 weeks old	Ligation of the femoral artery	57 µg Secretoneurin/mouse The thigh muscle of the ischemic hind limb Injection volume: 100 µL; 33 µL/injection;	[52]
Heparan sulphate	None	Stabilized VEGF ₁₆₅ against thermal and enzyme degradation <i>in vitro</i> Harnessed endogenous factors at injury sites	Male C57BL/6 N mice; 10 weeks old	The external iliac artery isolated, ligated twice and then transected between the two ligations	0.1 mg peptide/kg Vastus lateralis; vastus medialis, and gastrocnemius Injection volume: 60 µL; 20 µL/injection	[44,45]
Decellularized porcine skeletal muscle and umbilical cord derived matrix hydrogels	None	Significant improvements in tissue perfusion. Arteriogenesis rather than angiogenesis. Recruitment of skeletal muscle progenitors	Female Sprague Dawley rats	Removing a 2 cm segment of the femoral artery and vein	Gracilis muscle distal from the vessel ligation; A single injection of 150 µL	[69]

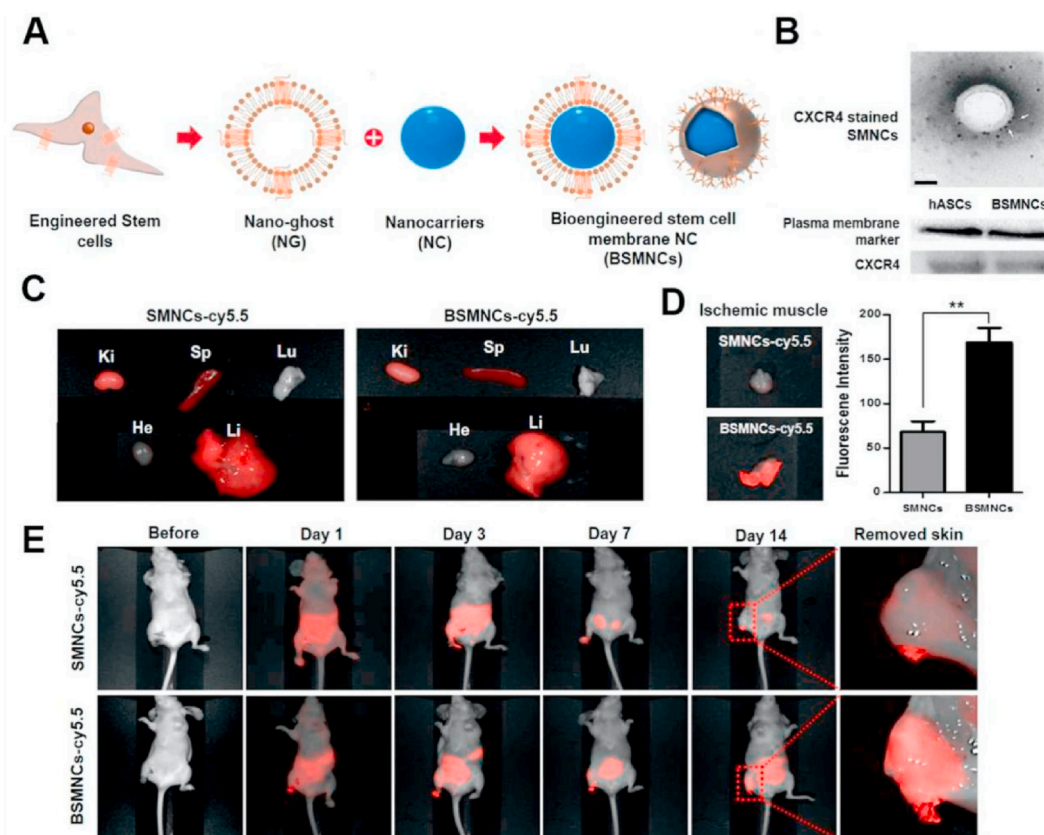


Fig. 3. Characterization of bioengineered stem cell membrane nanocarriers (BSMNCs) and time-dependent *in vivo* distribution of BSMNCs and SMNCs in murine hindlimb ischemia. (A) Schematic showing the concept and preparation of BSMNCs. (B) TEM images of immunostaining micrograph showing CXCR4 orientation on SMNCs stained with CXCR4 antibodies and a secondary anti-CXCR4 (upper). Scale bars = 50 nm. Western blot analysis showing translocation of CXCR4 from hASCs to the surface of BSMNCs (lower). (C) Biodistribution of BSMNCs and SMNCs after 14 days. Ki: kidney, Sp: spleen, Lu: lung, He: heart, Li: liver. (D) Quantitative image of *ex vivo* fluorescence intensity of ischemic induced muscles after IV injection of SMNCs or BSMNCs. (E) *In vivo* images of mice retro-orbitally injected with SMNC-Cy5 or BSMNC-Cy5 nanocarriers, before injection and 1, 3, 7 and 14 days after injection. The Figure is reproduced without modification from Ref. [59] with permission.

significantly higher than that of the cellular antioxidant potential. There are several key ROS, including hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH\cdot$), hypochlorous acid (HOCl), and superoxide anions ($O_2^{\cdot-}$). $O_2^{\cdot-}$ is capable of either reacting directly with biomolecules or producing other ROS, such as $OH\cdot$ and H_2O_2 . $OH\cdot$ can oxidize most biological molecules. H_2O_2 is not highly reactive itself, but it is an intermediate to both $OH\cdot$ and hypochlorite ($^{\cdot}OCl$) radical production. Excessive production of these ROS leads to DNA damage, protein modification, lipid peroxidation, disruption of cell signaling, and ultimately cell death. Therefore, therapeutics aiming to reduce oxidative stress have significant potential as treatments in PAD.

3.4.2.1. Poly(propylene sulfide)-based particles. Poly(propylene sulfide) (PPS) is a ROS-sensitive hydrophobic polymer. Upon exposure to ROS, it undergoes a morphological transition to more hydrophilic poly(propylene sulfoxide) and poly(propylene sulfone) [82]. Poole et al. synthesized a PPS microparticles for on-demand release of antioxidant therapeutic molecule, curcumin. PPS microparticles showed synergistic effects with curcumin for therapeutic properties. Specifically, the curcumin-loaded microspheres decreased tissue-level ROS *in vivo* and accelerated recovery in the diabetic mouse hindlimb ischemia (Fig. 5) [83]. More recently, O'Grady et al. have explored PPS as a stand-alone, locally-sustained antioxidant therapy. The investigators found PPS particles functionally improved recovery from hindlimb ischemia based on ~15–25% increases in hemoglobin saturation and perfusion in the footpads as well as earlier remodeling of vessels in the proximal limb [84].

3.4.2.2. Peroxalate ester-based nanoparticles. The group of Lee et al. designed H_2O_2 -responsive nanoparticles based on vanillyl alcohol (VA)-incorporated copolyoxalate (PVAX) [85]. PVAX is designed with peroxalate ester linked covalently in its backbone, which rapidly scavenges H_2O_2 and releases bioactive VA and CO_2 in a H_2O_2 -triggered manner. VA has antioxidant and anti-inflammatory activity, and is an active pharmaceutical ingredient in *Gastrodia elata* Blume [85]. In hindlimb ischemia/reperfusion model in mice, PVAX nanoparticles specifically reacted with overproduced H_2O_2 and exerted highly potent anti-inflammatory and anti-apoptotic activities that reduced cellular damage [86]. The investigators further loaded curcumin into PVAX (CUR-PVAX) nanoparticles as on-demand therapeutic agents for ischemic injuries. CUR-PVAX nanoparticles not only exhibited significantly higher antioxidant and anti-inflammatory activities than empty PVAX nanoparticles in vascular endothelial cells, but displayed drastic ultrasound signal in ischemic areas by generating CO_2 bubbles as contrast-enhanced ultrasound imaging agents. CUR-PVAX nanoparticles significantly suppressed the expression of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 beta (IL-1 β). The medicated nanoparticles also enhanced the expression level of VEGF and CD31, and lead to increased blood perfusion into ischemic tissues [87]. Similarly, Cho et al. have fabricated the *p*-hydroxybenzyl alcohol-incorporated copolyoxalate (HPOX) nanoparticles [88,89]. HPOX nanoparticles improved blood flow perfusion and neovascularization via VEGF expression, heme oxygenase-1 induction, endothelial nitric oxide synthase activation, and suppressed inflammatory cytokine gene

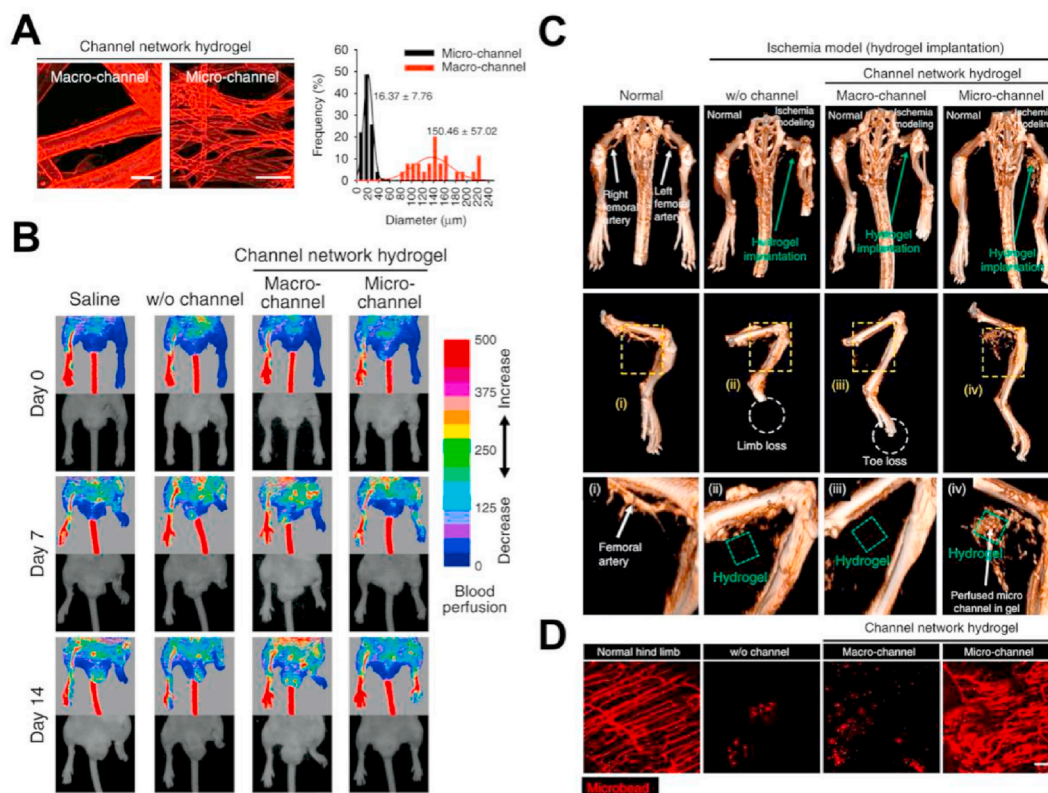


Fig. 4. Promotion of host vessel ingrowth and perfusion connection with channel networks. (A) Confocal visualization of micro- or macrochannel networks in hydrogels with their channel diameter distribution. Channels were perfused with FluoSpheres (45 nm, red). Scale bar = 100 μm. (B) Laser Doppler perfusion imaging (LDPI) of supine position in a mouse model of hindlimb ischemia with quantification of the corresponding blood perfusion ratio, compared to that of normal hindlimb at days 0, 7, and 14 post-implantation. (C) MicroCT images of arterial vasculature in ischemic tissue of mouse hindlimb at day 14 post hydrogel implantation (green dot box). (D) Confocal images of harvested hindlimb tissues post-perfusion of red FluoroSpheres. Scale bars = 100 μm. The Figure is reproduced with minor adaptations from Ref. [75] with permission. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

expression [90].

3.4.2.3. Boronated maltodextrin-based nanoparticles. The Lee group developed a unique nanoparticle system for imaging and therapy of PAD based on H₂O₂-activatable CO₂ bubble generating indocyanine green (ICG)-loaded boronated maltodextrin (ICG-BM) (Fig. 6) [91]. They generated boronated maltodextrin by making conformational changes of hydroxyls of maltodextrin with phenyl boronic ester substitutes [92]. Phenyl boronic ester of BM rapidly reacts with H₂O₂ to generate quinone methide which instantaneously reacts with nucleophilic H₂O, thus resulting in generation of 4-hydroxybenzyl alcohol (HBA). HBA is well known to exert potent antioxidant and anti-inflammatory effects in ischemic tissues. ICG is an FDA-approved agent approved for medical diagnostics with a high signal-to-noise ratio [93]. In mouse models of hindlimb ischemia, injection of ICG-BM nanoparticles in the affected legs demonstrated that the nanoparticles spontaneously react with H₂O₂ under conditions of ischemia to generate echogenic CO₂ bubbles. ICG-BM nanoparticles also significantly reduced the level of overproduced H₂O₂ and exerted highly potent anti-inflammatory and proangiogenic activities in ischemic tissues [91,94].

4. Outlook and challenges

Biomaterials have offered many benefits for regenerative medicine. In contrast to using cells alone, biomaterials can protect cells from shear forces during injection, provide structural support and protection in the recipient tissues and the ischemic environment. In addition, biomaterials may also promote cell survival and retention, and facilitate cell-

mediated tissue-matrix production, vascularization, and integration with endogenous tissue. However, biomaterials still have their drawbacks, and several parameters must be taken into account when designing biomaterials for PAD treatment.

There are several key requirements for optimal retention and delivery of a biomaterial in the intramuscular environment of the lower limb. First, the mechanically dynamic intramuscular environment requires the development of a material that a) undergoes minimal swelling once inside the target tissue; b) has mechanical properties matching those of the recipient muscle tissue and c) has high resilience to tolerate repeated compressive strain during muscle contraction without mechanical failure, which is essential for its intramuscular retention [23,95–98]. Second, the material should be porous enough to facilitate the exchange of trophic factors with the surrounding environment and allow host cell infiltration and vascular ingrowth. It is worth noting that current studies have overlooked the mechanistic role of the inflammatory activation in PAD progression. Hydrogels able to induce pro-regenerative and pro-angiogenic monocyte polarization may have clear value toward clinical applications [33,34]. Third, rapid *in situ* formation is desirable to facilitate delivery using small gauge needles that will allow for injection of the agent while it is still in liquid form with minimal injury of the recipient tissue. Finally, antioxidant and angiogenic properties will be beneficial to the management of the ischemic myopathy and the improvement of leg perfusion in PAD [99].

Challenges faced by biomaterial research are not just scientific in nature; the regulatory environment should also be considered. This is important as early attention could minimize hurdles later in the path to introducing a treatment to patient care. Given the diversity of the many biomaterial therapies, it is difficult to classify products within just one

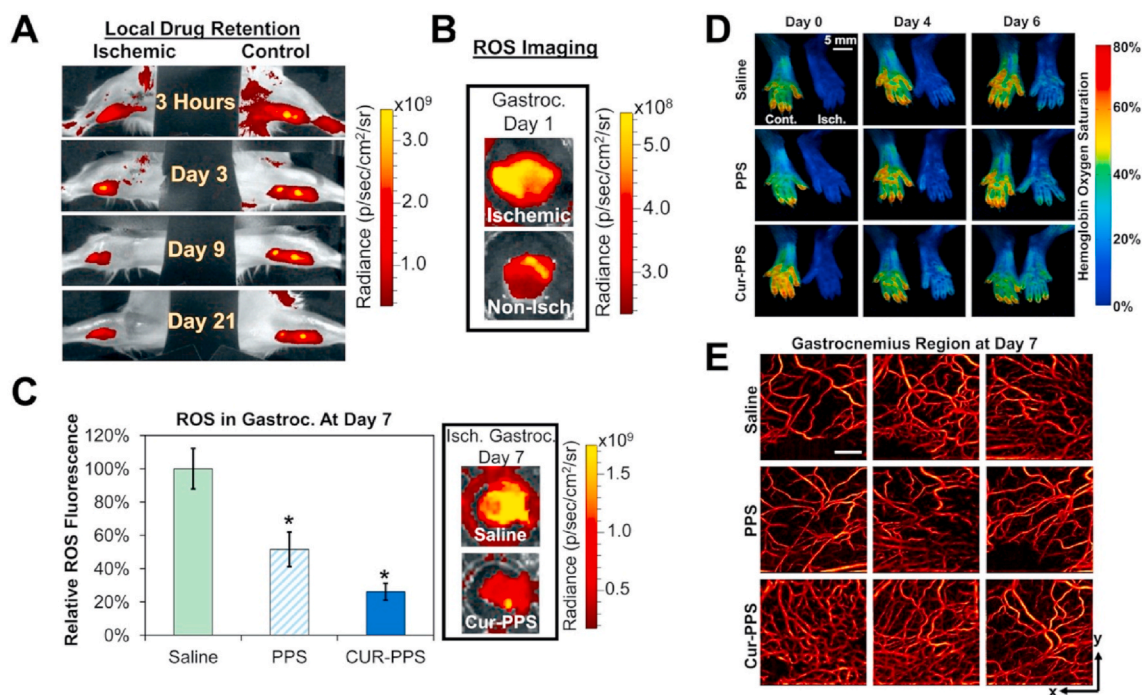


Fig. 5. Poly(propylene sulfide) (PPS) microspheres provided sustained, on demand local curcumin release and reduced tissue ROS levels, improved ischemic limb recovery with a significant increase in length of vasculature with diameters in the ischemic limb. (A) Curcumin-PPS microspheres released curcumin more rapidly in the ischemic limb in comparison to the non-ischemic control limb. (B) ROS levels in the ischemic gastrocnemius muscle were increased at day 1 post-surgery (level of ROS is 2.3-fold greater in ischemic versus control gastrocnemius). (C) Blank PPS microspheres and curcumin-loaded PPS microspheres significantly reduced ROS in gastrocnemius muscles extracted from ischemic limbs. Saline group $n = 8$, blank PPS group $n = 11$, curcumin-PPS group $n = 10$. $*P < 0.05$ relative to saline treatment. (D) Representative images from the time course of hemoglobin oxygen saturation recovery from each treatment group delivered to the ischemic limb of diabetic mice. (E) Representative images of vessel morphology from each treatment group. Scale bar = 1 mm. The Figure is reproduced with minor adaptations from Refs. [83] with permission.

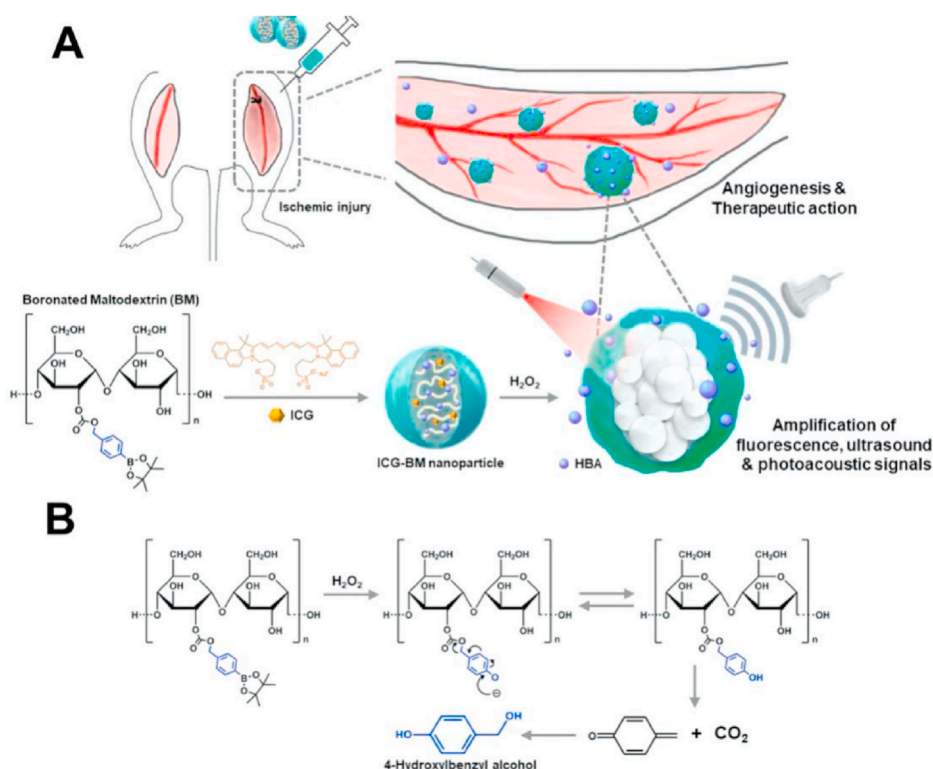


Fig. 6. Indocyanine green-loaded boronated maltodextrin (ICG-BM) nanoparticles as theranostic agents for ischemic injury. (A) A schematic diagram of H₂O₂-responsive ICG-BM nanoparticles as theranostic agents for PAD. (B) The mechanism of degradation of BM and generation of 4-hydroxybenzyl alcohol. The Figure is reproduced without modification from Refs. [91] with permission. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of FDA's regulatory arms. In 2011, the Office of Combination Products stated that any treatment that “depends, even in part, on chemical action within or on the body of man to achieve any one of its primary intended purposes, would not be a device” consequently many biomaterial therapies need to go through drug level regulatory pathways with many modalities having to undergo a three-phase, 5–10-year process, costing hundreds of millions of dollars.

Despite all these challenges, many preclinical trials have taken place, and a few have progressed to clinical trials and the results are promising. Furthermore, the field of biomaterial-based regenerative medicine has significant potential to help many patients with devastating PAD.

Author contribution statement

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Declaration of competing interest

The authors declare no conflict of interest.

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