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PEG-Maleimide Hydrogels for Protein and Cell Delivery in Regenerative Medicine

Andrés J. García
Woodruff School of Mechanical Engineering, Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology

Abstract

Protein- and cell-based therapies represent highly promising strategies for regenerative medicine, immunotherapy, and oncology. However, these therapies are significantly limited by delivery considerations, particularly in terms of protein stability and dosing kinetics as well as cell survival, engraftment and function. Hydrogels represent versatile and robust delivery vehicles for proteins and cells due to their high water content that retains protein biological activity, high cytocompatibility and minimal adverse host reactions, flexibility and tunability in terms of chemistry, structure, and polymerization format, ability to incorporate various biomolecules to convey biofunctionality, and opportunity for minimally invasive delivery as injectable carriers. This review highlights recent progress in the engineering of poly(ethylene glycol) (PEG) hydrogels cross-linked using maleimide reactive groups for protein and cell delivery.

The Need for Delivery Vehicles for Protein and Cell Delivery

Protein- and cell-based therapies represent revolutionary strategies in regenerative medicine, oncology, treatment of inflammatory disorders, and immunology[1-3] (Table 1). These next-generation therapeutics offer significant advantages over small pharmacological compounds in terms of specificity, control, and functionality. However, several challenges limit the broad application of protein- and cell-based therapeutics (Table 2). In particular, delivery considerations pose significant challenges to efficient and effective implementation.

Proteins, such as growth and differentiation factors, antibodies, and cytokines, represent important therapeutics in regenerative medicine, oncology, and other targeted therapies. For instance, protein-based therapeutics can directly promote tissue growth as in nerve regeneration, provide enzymes to digest scar tissue, recruit osteoprogenitors and induce differentiation into bone-forming cells, or promote angiogenesis/vascularization to modulate tissue healing and repair. In cancer therapeutics, protein-based interventions can modulate new blood vessel growth at tumor sites and potentially ‘starve’ cancers of nutrients, or enable targeting of tumors based on surface biomarker expression on tumors. For protein-based therapeutics, important delivery considerations include delivery route, protein stability, and dosing kinetics (target dose, residence time). For example, vascular endothelial growth factor (VEGF) is a potent inducer of angiogenesis and vasculogenesis and has emerged has a promising therapeutic to treat conditions with reduced vascular perfusion, including peripheral artery disease, coronary heart disease and myocardial infarcts, and vascularization of tissue-engineered constructs[4,5]. However, injected VEGF is rapidly cleared from tissues, resulting in reduced therapeutic efficiency. Moreover, high local concentrations of VEGF induce a potent vascularization response but these vessels are often dysfunctional and leaky and regress once the exogenous growth factor is depleted. In

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contrast, delivery of VEGF from engineered carriers leads to sustained local levels of VEGF and functional vessels that persist beyond the residence time of the delivered factor[6-8].

Cell-based therapies represent hugely promising therapeutic strategies in numerous areas of regenerative medicine and immunology. In particular, stem cell transplantation promotes therapeutic improvements in various deficiencies by providing cells that either engraft and differentiate into functional tissue constituents or secrete bioactive factors supporting host cellular activities. For example, delivery of mesenchymal stem cells and cardiomyocyte progenitors to restore cardiac function, either by secreting paracrine factors to recruit and enhance survival of endogenous cells or differentiation into contractile cells, is a promising strategy to treat myocardial infarcts[9-11]. Similarly, implantation of metabolically functional cells may provide long-term corrective function of metabolic deficiencies in ways that are superior to pharmacologic intervention. For instance, implantation of metabolically responsive, insulin-secreting cells has the potential to provide significantly better glycemic regulation than blood glucose monitoring and insulin injections[12]. Nevertheless, roadblocks to efficient cell delivery in terms of cell survival, engraftment, differentiation, and monitoring severely limit the widespread success and application of these strategies. Long term cell engraftment has been shown to correlate with enhanced therapeutic outcomes[13-15], and has also been shown to be greater in cases in which cells are delivered in an appropriate biomaterial carrier[16]. For example, bone marrow stromal cells directly injected into myocardial infarcts have poor engraftment rates (<1%) and at best modest improvements in function[17,9]. Similarly, progressive loss of transplanted pancreatic islets due to poor vascularization and engraftment significantly limit the long-term success of this promising cell therapy for type 1 diabetes[18,19]. Therefore, new classes of cell delivery vehicles that promote cell survival, engraftment, and function, including integration with host cells and tissues, are necessary to fully realize the potential of cell therapy.

Biomaterial Delivery Vehicles

Countless materials have been explored as delivery vehicles for proteins and cells. General requirements for these delivery vehicles are enumerated in Table 3, but the importance and relevance of each requirement are strongly dependent on the target application. Materials derived from natural sources, such as collagen, hyaluronic acid, alginate, and chitosan, have been extensively used in regenerative medicine and tissue engineering as protein and cell delivery vehicles. These natural materials can be biologically active, promote cell adhesion and growth, display high cytocompatibility and acceptable inflammatory profiles, and can be enzymatically or hydrolytically degraded. However, natural materials are difficult to process and manufacture into formulations with target mechanical and biochemical properties, display lot-to-lot variability, and may carry some risk of immunogenicity and pathogen transmission. Consequently, synthetic materials, such as metals, ceramics and glasses, polymers, and composites, offer significant advantages over natural materials in terms of defined composition, control over mechanical and chemical properties, manufacturability and processing. Limitations of synthetic materials include lack of bio-functionality/bio-specificity, reduced cytocompatibility compared to natural materials, and uncontrolled inflammatory host responses that often lead to foreign body reaction and fibrosis, although recent synthetic materials have been functionalized with bioactive components (e.g, peptides) to diminish or overcome these limitations. In particular, polymers provide highly tailorable synthetic materials for cell and protein delivery applications. Polymers have been extensively used as controlled delivery vehicles for proteins in various configurations, including membranes for delivery from reservoirs, biodegradable matrices that release proteins as the polymer degrades in aqueous environments, micro- and nanoparticles and micelles, and mesh networks such as hydrogels[20-23]. Polymeric systems have also advantages as cell delivery vehicles as these can be formulated as injectable carriers, tailored

to degrade at specified rates to promote replacement by repair tissue, functionalized with bioactive agents to direct cellular activities, and engineered to provide structural and biological support for cells[24,25]. Polymeric cell carriers come in diverse structural configurations including space-filling cross-linked and self-assembled networks, porous foams, micro- and nanofibrillar scaffolds, and scaffolds generated by 3-D printing and associated additive manufacturing technologies.

PEG-maleimide Hydrogel as Delivery Vehicles

An attractive class of materials for protein and cell delivery is hydrogels. Hydrogels are water-swollen physically or chemically cross-linked polymer networks that can be engineered from natural materials such as alginate and collagen or synthetic polymers such as polyethylene glycol (PEG) (Figure 1). Advantageous characteristics of hydrogels include retention of protein biological activity, high cytocompatibility and minimal adverse host reactions due to their high water content, flexibility and tunability in terms of chemistry, structure, and polymerization format, ability to incorporate various biomolecules to convey biofunctionality, and opportunity for minimally invasive delivery as injectable carriers. Excellent reviews on hydrogels for protein and cell delivery can be found elsewhere[26-32].

PEG synthetic hydrogels represent the ‘gold standard’ in this field due to their intrinsic low-protein adsorption properties, minimal inflammatory profile and history of safe in vivo use, ease in incorporating various functionalities, and commercial availability of reagents such as macromers functionalized with different reactive end groups. Various cross-linking chemistries have been pursued to create biofunctionalized hydrogel networks of PEG macromers, with Michael-type addition reactions and acrylate polymerization being the most widely used[30,33]. Cross-linking chemistry, gelation time, polymer network structure (mesh size), swelling, and degradation properties are important considerations when selecting a hydrogel for protein- and cell-delivery applications. In PEG-diacrylate hydrogels, macromers are cross-linked via free-radical polymerization of acrylate end groups. Free radicals are created either by chemical activation or UV cleavage of a photoinitiator with the added ability to spatially control incorporation of bioligands or mechanical properties through additive or subtractive photo-patterning[34,35]. Free-radical polymerization cross-linking, however, is limited by cytotoxicity (especially in the case of sensitive cells such as pancreatic islets and neurons), non-ideal network structure containing poly(acrylate) chains of various sizes, and challenges related to in situ photo cross-linking for in vivo delivery applications. In contrast, for hydrogels cross-linked by Michael-type addition, functionalized end groups on branched PEG macromers are reacted with bi-functional or branched cross-linking molecules. Michael-addition PEG hydrogels based on 4-or 8-arm PEG macromers with acrylate, vinyl-sulfone, and thiol end-groups have been extensively investigated[36-47]. Michael-type addition cross-linking avoids the use of cytotoxic free-radicals and UV light, but instead require a nucleophilic reagent, such as triethanolamine (TEA), to facilitate the addition reaction. However, hydrogels formed in the presence of high concentrations of TEA have cytotoxic effects on sensitive cell types such as endothelial cells, ovarian follicular cells, and pancreatic islets[48,49].

We have recently established maleimide groups as an alternative cross-linking chemistry for PEG hydrogels[50]. The maleimide reactive group is extensively used in peptide bioconjugate chemistry because of its fast reaction kinetics and high specificity for thiols at physiological pH. Maleimide-based cross-linking has significant advantages over other cross-linking chemistries, namely well-defined hydrogel structure, stoichiometric incorporation of bioligands, increased cytocompatibility, improved cross-linking efficiency, and reaction time scales appropriate for in situ gelation for in vivo applications[50]. Additionally, the base macromer exhibits minimal toxicity and inflammation in vivo and is rapidly excreted via the

urine[51] – important considerations in establishing the safety and translational potential of these hydrogels. We next present two examples of applications of PEG-maleimide hydrogels for protein and cell delivery.

PEG Hydrogel-based Delivery of Therapeutic Proteins for Cardiac Repair

Acute myocardial infarction caused by ischemia and reperfusion is the most common cause of cardiac dysfunction due to local cell death and inflammatory responses and fibrosis[52,53]. Protein therapeutics targeting different elements of the infarct cascade are being explored to enhance endogenous cell survival, modulate inflammation, reduce fibrosis, and promote repair[54-56]. However, direct protein injection into the myocardium has proven inefficient as the therapeutic proteins are rapidly cleared, thereby limiting the effective tissue dose. To address this delivery limitation, synthetic and natural hydrogels have been developed for controlled delivery of proteins and cells[57-68]. These therapeutic vehicles promote myocardial function and repair by supporting endogenous and transplanted cell survival and/or recruiting endogenous progenitor cells. Additionally, evidence is accumulating that natural hydrogels consisting of hyaluronic acid or decellularized ventricular extracellular matrix without exogenous therapeutic factors reduce infarct expansion and negative post-infarct remodeling possibly by providing mechanical support[69-72]. These biomaterial strategies are discussed in an excellent review[73].

We engineered hydrogels for protein delivery in order to harness endogenous cell repair to enhance myocardial repair and function[74]. PEG-maleimide hydrogels cross-linked with a protease-degradable peptide were loaded with hepatocyte and vascular endothelial growth factors (HGF, VEGF) and delivered to the infarcted myocardium of rats. The hydrogel mesh size is on the order of 35-50 nm and provides a barrier for the release of HGF and VEGF, but in the presence of proteases, the peptide cross-linkers are degraded, resulting in sustained release of HGF and VEGF. The released protein maintains equivalent bioactivity as soluble protein[51], demonstrating that this delivery vehicle supports the stability of encapsulated proteins. When delivered to the border zones following ischemia-reperfusion injury, there was no acute effect on cardiac function as measured by echocardiography. However, there was a time-dependent increase in angiogenesis, c-kit-positive stem cell recruitment, and decrease in fibrosis in infarcts treated with hydrogel co-delivering VEGF and HGF compared to direct injection of these proteins, hydrogels delivering single proteins, empty hydrogels, and untreated injured controls (Figure 2). Importantly, the dual growth factor-delivering hydrogel led to improvements in chronic cardiac function as measured by both invasive hemodynamics and echocardiography (Figure 2). These results demonstrate that dual growth factor release of HGF and VEGF from a bioactive hydrogel has the capacity to significantly improve cardiac remodeling and function following ischemia-reperfusion injury.

PEG-maleimide Hydrogels for Pancreatic Islet Delivery and Engraftment

Type 1 diabetes (T1DM) affects one in every 400 children and adolescents in the US[75]. Standard therapy with exogenous insulin is burdensome, associated with a significant danger of hypoglycemia, and only partially efficacious in preventing long term complications. Pancreatic islet transplantation has emerged as a promising therapy for T1DM[76,77]. Despite impressive initial improvements in metabolic control, few islet transplant patients maintain long term insulin independence[12,78]. Moreover, islet transplantation therapy is limited by inadequate supply of donor islets, a problem worsened by islet loss post-transplantation. Instant blood-mediated inflammatory reaction and toxic responses to immunosuppressive drugs contribute to progressive islet loss. Furthermore, inadequate vascularization of transplanted islets remains a significant cause of reduced islet viability, function, and

engraftment[79-82,77]. Therefore, there is clear need for islet delivery vehicles that promote islet survival, vascularization, and function.

Biomaterial strategies for islet transplantation (reviewed in[83,84]) have centered on (i) semi-permeable barriers for encapsulation and immunoprotection[85-90] and (ii) delivery vehicles for factors that support islet survival and/or vascularization[91-97]. We recently engineered an injectable vasculogenic, PEG-maleimide hydrogel to enhance the survival, vascularization, and engraftment of transplanted pancreatic islets in a mouse model of T1DM[51] (Figure 3). VEGF, a potent stimulator of angiogenesis, was incorporated into the hydrogel and released in an on-demand manner through protease-mediated degradation of the hydrogel network (Figure 3). The PEG-maleimide hydrogel exhibited extended in vivo release of VEGF compared to other carriers such as alginate[49]. Isolated islets encapsulated in PEG-maleimide hydrogels displayed enhanced viability and insulin secretion compared to islets encapsulated in other hydrogels, including PEG-diacrylate and collagen I[49]. This injectable hydrogel was then used to deliver islets to the small bowel mesentery, a metabolically relevant site for insulin release, in diabetic mice. Controlled presentation of VEGF and RGD cell adhesive peptides within this hydrogel significantly improved the vascularization and function of transplanted islets. Diabetic mice receiving islets transplanted in proteolytically degradable hydrogels incorporating VEGF exhibited complete reversal of diabetic hyperglycemia with a 40% reduction in the number of islets required to achieve normoglycemia[51] (Figure 3). Furthermore, hydrogel-delivered islets significantly improved weight gain, regulation of a glucose challenge, and intra-islet vascularization and engraftment compared to the clinical standard of islet infusion through the hepatic portal vein (Figure 3). This study establishes a simple biomaterial strategy for islet transplantation to enhance islet engraftment and function.

Conclusions and Outlook

Promising protein- and cell-based therapies are significantly limited by delivery considerations, particularly in terms of protein stability and dosing kinetics as well as cell survival, engraftment and function. Hydrogels represent versatile and robust delivery vehicles for proteins and cells due to the retention of protein biological activity, high cytocompatibility and minimal adverse host reactions, flexibility and tunability in terms of chemistry, structure, and polymerization format, ability to incorporate various biomolecules to convey biofunctionality, and opportunity for minimally invasive delivery as injectable carriers. Biofunctional hydrogels have shown promise in pre-clinical models for diverse regenerative medicine applications but safety and functional data in rigorous animal models are necessary to establish the translational potential of these engineering materials. Among PEG-based hydrogels, maleimide-based cross-linked hydrogels offer significant advantages over other cross-linking chemistries, including well-defined hydrogel structure, stoichiometric incorporation of bioligands, increased cytocompatibility, improved cross-linking efficiency, and reaction time scales appropriate for in situ gelation for in vivo applications.

Continued progress in material design and synthesis strategies, including the application of orthogonal chemistries and novel macromolecular materials, will accelerate the development of tailorable, multi-functional delivery vehicles. Additionally, the engineering of stimulus-triggered functionalities for spatiotemporal control of mechanical properties, degradation and protein release kinetics, and cell-instructive activities will yield materials that better mimic tissues and promote the integration of transplanted or recruited endogenous cells with host tissues. Advances in immunology and stem cell biology will lead to the identification of potent biomolecules, such as immunoregulatory cytokines and cell recruitment factors, which can be integrated into delivery vehicles to generate immunomodulatory and reparative

materials to harness endogenous repair. Finally, the combination of advanced imaging modalities for in vivo tracking of delivered proteins and cells and powerful transgenic animal models will provide rigorous platforms to evaluate engineered delivery vehicles.

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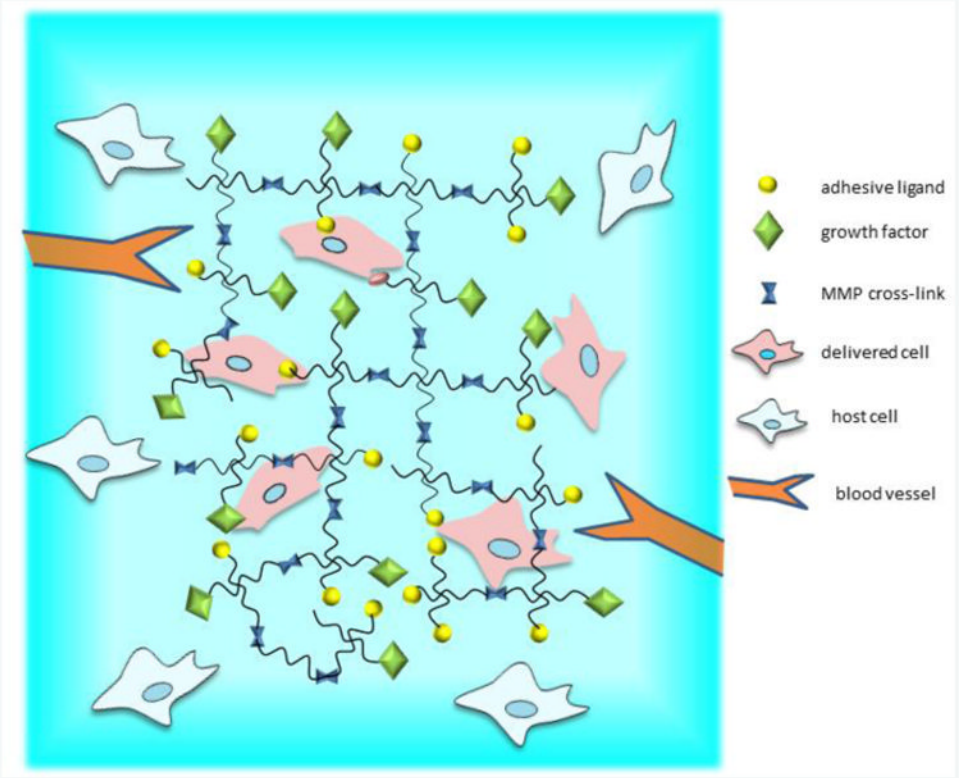

Figure 1.
Biofunctional hydrogels for protein and cell delivery. Hydrogel network is functionalized with bioactive molecules, including cell adhesive peptides, protease-degradable cross-links, and growth factors. Hydrogel is designed to promote host cell interactions and blood vessel ingrowth to promote integration of transplanted donor cells.

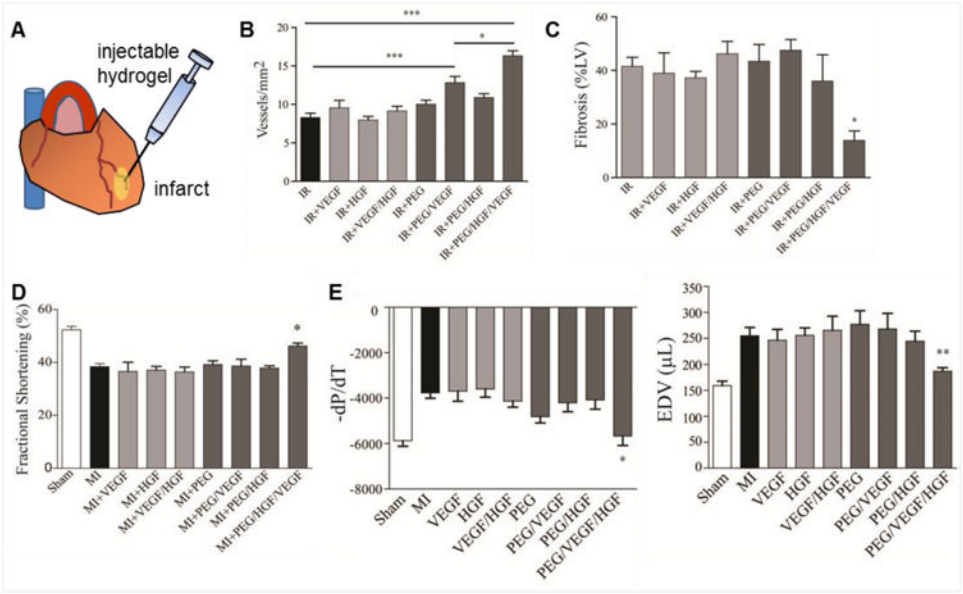

Figure 2.

PEG-maleimide hydrogels for protein delivery to myocardial infarcts. (A) Hydrogels cross-linked with a protease-degradable peptide and loaded with HGF and VEGF were injected into the infarcted myocardium. Infarcts treated with hydrogel co-delivering VEGF and HGF (B) enhanced angiogenesis (*p<0.05, ***p<0.001) and (C) decreased fibrosis (*p<0.05) at 21 days post-treatment compared to direct injection of these proteins, hydrogels delivering single proteins, empty hydrogels, and untreated injured controls. Dual growth factor-delivering hydrogel led to improvements in (D) fractional shortening (*p<0.05) and (E) hemodynamics (dP/dT = change in pressure over time, EDV = end-diastolic volume, *p<0.05, **p<0.01) at 21 days post-treatment. Adapted from Ref. 74.

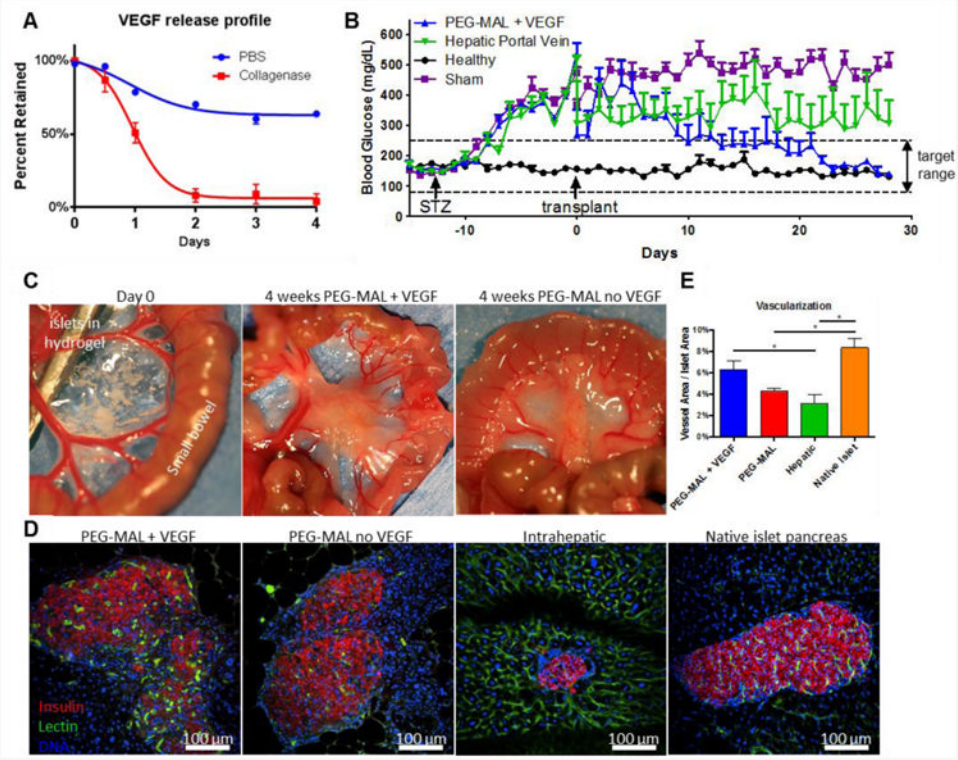

Figure 3.

Vasculogenic PEG-maleimide hydrogels for pancreatic islet cell engraftment and function. (A) VEGF release profile from collagenase-degrading gels or gels treated in PBS as measured by ELISA showing on-demand release of VEGF. (B) Random daily blood sugar levels in streptozotocin-induced diabetic mice transplanted within syngeneic islets (400 islets). Only islets delivered within PEG-maleimide hydrogels with VEGF restored normoglycemia ($p<0.001$). (C) Transplant site in the small bowel mesentery at day 0 and at 4 weeks demonstrating significant remodeling of the PEG-maleimide hydrogel. (D) Islet graft explants (4 weeks) with patent vascular structures stained with IV-perfused FITC-lectin (green), DAPI (blue), and immunostained for insulin (red). (E) Quantification of vascular area normalized to islet area $p<0.05$). Adapted from Ref. 51.

Table 1

Representative protein- and cell-based therapies or clinical trials and associated delivery vehicles.

Application	Protein	Cell	Delivery Vehicle
spinal fusion	BMP-2 (Medtronic)		collagen sponge
myocardial infarct		bone marrow-derived stem cells (Amorcyte) MSC (Osiris)	Saline
rheumatoid arthritis, Crohn's disease & other inflammatory disorders	adalimumab TNF-α antibody (Humira, Abbott)		Saline
stroke		neural stem cells (ReNeuron)	Saline
diabetes		porcine β cells (Living Cell Tech)	Alginate
cartilage		autologous chondrocytes (Genzyme)	Collagen
breast cancer	trastuzumab HER2 antagonist antibody (Herceptin, Genentech)		Saline
diabetes	insulin lispro (Humalog, Eli Lilly)		Saline
leg ulcer		fibroblasts/keratinocytes (Organogenesis)	cell-derived matrix
critical limb ischemia		MSC (Stempeutics)	Saline

Table 2

Considerations for protein and cell therapeutics.

Protein	**Cell**
selection of therapeutic due to complex underlying biology	autologous, allogenic donor cells, including ex vivo manipulations
delivery route & vehicle	delivery route & vehicle
bioactivity & stability	cell dose, survival, & engraftment
dosing & clearance kinetics	mechanism of action: paracrine/trophic support vs. direct functional support
host response	host response
manufacturing, including expression, purification, sterilization	manufacturing, including sterilization
regulatory aspects, safety & monitoring	regulatory aspects, safety & monitoring

Table 3

General requirements for protein and cell delivery vehicles.

Protein Vehicle	**Cell Carrier**
delivery route	delivery format (e.g., injectable vs. pre-formed)
protein loading capacity	vehicle structural, mechanical, biochemical properties to support target cell activities
protein bioactivity & stability after encapsulation	immunoisolation considerations
release mechanisms & kinetics to match desired pharmacokinetics	cell loading & cytocompatibility
host response to protein & vehicle	host response & integration, including vehicle degradation to allow tissue ingrowth
vehicle residence time & clearance	Vascularization
manufacturing, including sterilization	manufacturing, including sterilization
regulatory aspects, safety & monitoring	regulatory aspects, safety & monitoring