

Sustained Growth Factor Delivery in Tissue Engineering Applications

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(Received 5 September 2013; accepted 29 November 2013; published online 7 December 2013)

Associate Editor Tzung Hsiai oversaw the review of this article.

Abstract—The beautifully orchestrated complexity of the temporal spatial growth factor gradients during embryogenesis offer a striking contrast to systemic bolus administration that lack tissue specificity and sustained protein localization, often requiring supraphysiological protein doses to produce the desired therapeutic dose. These attributes may be responsible for clinically observed dangerous tissue overgrowth, inflammation, and even tumor formation. Growth factor delivery within an implanted scaffold is a very attractive way to modulate cell behavior. For short term delivery, proteins can be non-specifically adsorbed to the material surface or simply entrapped within the bulk scaffold. For more sustained delivery, many researchers have turned to the ever increasing list of covalent immobilization methods that have profound applications in purification, biosensing, imaging, and drug discovery by tethering proteins, nucleic acids, carbohydrates, synthetic polymers, small molecules, nanotubes, and even whole cells. This review focuses on the use of covalent immobilization to achieve sustained growth factor delivery for tissue engineering. Covalent immobilization techniques will be reviewed in terms of design, protein bioactivity/stability, efficiency, and spatiotemporal distribution. Further, the biological response to sustained growth factor delivery will also be covered, such as cell interaction, cell responsiveness, proliferation, differentiation, extracellular matrix production, and tissue regeneration. This focused review is anticipated to inform investigators on the selection of optimal immobilization strategies for their specific applications.

Keywords—Sustained release, Controlled release, Drug delivery, Growth factor immobilization, Conjugation, Biomaterials.

INTRODUCTION

Tissue regeneration is an intricate, biological process that depends on tightly regulated signaling molecules at exact times, locations, and concentrations. Early attempts at growth factor delivery from carriers exhibited

limited modulation of release characteristics and no protection from detrimental conditions in the environment.⁵³ Growth factors are often added to the scaffold surface *via* electrostatic interaction and ionic complexation, and the corresponding release depends on protein-surface interactions that are governed by surface charge, surface roughness, and surface energetics.²² For more sustained release, bioactive molecules can be physically encapsulated within the scaffolding material and in microparticles. Concerns related to protein stability and immunogenicity from various drug delivery approaches are comprehensively reviewed by Jiskoot *et al.*²⁵

Covalent immobilization methods must be assessed in terms of gradients, spatial distribution and density, conjugation efficiency, dose dependence, downstream signaling, heparin/affinity-based delivery, dual delivery, and cleavable linkers. For the purpose of this review, 100% or less delivery over the course of 30 days is considered successful sustained release, based on the timescale required for repairing different tissues.^{7,21,61} For example, tissues such as bone and skin require weeks to months for complete extracellular matrix (ECM) deposition, full tissue strengthening, angiogenesis, and reinnervation.⁶¹ Further, ligament healing is reported to take 6 weeks to 3 months based on clinical evidence,²¹ and cartilage healing in canine defects is characterized by a proliferative phase at 1.5 months followed by a remodeling phase lasting 3–6 months.⁷

EMERGING RESEARCH USING COVALENT IMMOBILIZATION

Polymer scaffolds can be functionalized to interact with bioactive molecules (Table 1), and the resulting covalent immobilizing hinders free diffusion and

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TABLE 1. Summary of select covalent immobilization schemes used in tissue engineering.

Signaling molecule	Material	Linker chemistry	Application
RGD or IKVAV ⁴⁹	PLA-PEG	Avidin/biotin-NHS	heart or nerve
GRGDSP ⁴⁷	Acrylamide-PEG	Sulfo-SMCC	Blood vessels
RGD ^{1,58}	Alginate	EDC/NHS	Muscle or bone
RGDS ¹³	PEG	NHS	Skin
RGD and FGF-2 ¹⁴	PEG	NHS	Blood vessels
NGF ²⁶	p(HEMA)	EDMA	Nerve
NGF and NT-3 ⁴⁴	p(HEMA)-PLL	EDMA	Nerve
BMP-2 ⁷⁴	PCL	Sulfo-SMCC	Bone
BMP-2 or FGF-2 ⁸	Chitosan	EDC/NHS	Bone or skin
BMP-7 peptide ^{35,36}	Hydroxyapatite	Aminosilane	Bone
IFN- γ ^{31,32}	Methacrylamide chitosan	Thiol, maleimide-streptavidin/biotin	Nerve
Ephrin-A1 ⁴³	PEG	NHS	Blood vessels
EGF ³⁹	PMMA- <i>g</i> -PEO	NPC	Bone
RGD or EGF ⁶⁶	Chitosan	NHS	Cartilage
BMP-2 ³³	PLGA	NHS	Bone
BMP-2 ²⁷	Silk fibroin	EDC/NHS	Bone
TGF- β 1 ³⁸	PEG	NHS	Blood vessels
FGF-2 ³⁴	HA	Heparin, NaBH ₃ CN	Skin
FGF-2 ⁶³	HA-gelatin-chitosan	Heparin, EDC/NHS	Cartilage
FGF-2 ²⁰	Chitosan-alginate	Heparin, EDC/NHS	Skin
BMP-2 ⁶⁵	Hydroxyapatite	Heparin, EDC/NHS	Bone
BMP-2 ³	HA-PEGDA	Heparin, thiol	Bone
BMP-2 ¹⁵	Gelatin-CaP-PHBV	Heparin, EDC/NHS	Bone
PDGF ³⁰	Gelatin-PCL	Heparin, EDC/NHS	Blood vessels
EGF and FGF-2 ^{28,48}	PLLA	Heparin, EDC/NHS	Nerve
SDF-1 α ⁷³	PLLACL	Heparin, EDC/sulfo-NHS	Blood vessels
FGF-2 ⁷⁰	PLGA	Heparin, EDC/NHS	Blood vessels
BMP-2 ²⁴	PLGA	Heparin, EDC/NHS	Bone
FGF-2 ⁶⁹	Collagen	Heparin, EDC/NHS	Blood vessels
FGF-2 ⁷¹	Pluronic	Heparin, APMMA	Blood vessels
TGF- β 3 and dexamethasone ⁴⁶	PLGA	Heparin, N/A	Cartilage
VEGF, Ang-1, KGF, and PDGF ¹⁶	HA-PEGDA	Heparin, thiol	Blood vessels
VEGF and FGF-2 ⁴⁵	Collagen	Heparin	Blood vessels
VEGF and RGD ⁵⁰	MMP-cleavable-PEG	Maleimide	Blood vessels

Acronyms: Arg-Gly-Asp, RGD; Ile-Lys-Val-Ala-Val, IKVAV; polylactide, PLA; polyethylene glycol, PEG; *N*-hydroxysuccinimide, NHS; Gly-Arg-Gly-Asp-Ser-Pro, GRGDSP; sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, sulfo-SMCC; *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide, EDC; Arg-Gly-Asp-Ser, RGDS; fibroblast growth factor, FGF; nerve growth factor, NGF; poly(2-hydroxyethylmethacrylate) [p(HEMA)], EDMA, ethylene dimethacrylate; neutrotrophin-3, NT-3; PLL [poly(L-lysine)], bone morphogenetic protein, BMP; polycaprolactone, PCL; interferon- γ , IFN- γ ; epidermal growth factor, EGF; poly(methyl methacrylate)-*graft*-poly(ethylene oxide), PMMA-*g*-PEO; 4-nitrophenyl chloroformate, NPC; poly(D,L-lactide-co-glycolide), PLGA; transforming growth factor- β , TGF- β ; hyaluronic acid, HA; polyethylene glycol diacrylate, PEGDA; calcium phosphate, CaP; poly(hydroxybutyrate-co-hydroxyvalerate), PHBV; platelet-derived growth factor, PDGF; poly(L-lactide), PLLA; stromal cell-derived factor-1 α , SDF-1 α ; poly(L-lactide-co-caprolactone), PLLACL; *N*-(3-aminopropyl) methacrylamide, APMMA; not available, N/A; vascular endothelial growth factor, VEGF; angiotensin-1, Ang-1; keratinocyte growth factor, KGF; matrix metalloproteinase, MMP.

prolongs growth factor release.²⁹ For covalent immobilization, conjugation chemistry is important to consider when designing the proper functional groups to target and protect. Functional groups such as hydroxyl-, amino-, or carboxyl groups are introduced to each other through blending, copolymerization, chemical solutions, or physical treatment.⁶⁴ Recent developments in chemical bioconjugation methods and click chemistry are extensively reviewed by Jabbari,²³ Lutz *et al.*,³⁷ and Binder *et al.*⁵ Conjugating signaling molecules to a scaffold using specified linker chemistry holds some advantages over noncovalent immobilization. Namely, the options for linking, lifetime, and

controlled release of growth factors within a scaffold may be improved. Though, when a growth factor is chemically conjugated to the scaffold through a specific functional group, that blocked functional group may compromise the protein bioactivity. Further, the efficacy of this conjugation reaction dictates how much initial protein is required, and the conjugation efficiency may be insufficient using some covalent immobilization techniques. When further considering covalent linkers, linker spacing and arm length need to be optimized to maintain active proteins that are not sterically hindered, and in the case of cleavable linkers, allow protease intrusion. Longer linkers of 7–30 atoms

or 30 Å with greater spacing tend to allow the growth factor a significant degree of mobility to remain bioactive and maintain binding affinity, due to less steric hindrance, and this also provides easier access to cleavable sequences by proteases, leading to greater release.^{4,12,59} It is important to note that covalent immobilization of a signaling molecule prevents its internalization by cells, thus lengthening the protein's active lifetime as an extracellular trigger until the linker bond is broken or the scaffold is degraded. However, some signaling molecules need to be internalized to activate downstream signaling. Moreover, permanent presentation of a ligand that continuously stimulates cells can also lead to serious problems such as tissue overgrowth.

Still, conjugation chemistry is truly modular and can be used to design custom linkages between surfaces and proteins. It allows researchers to choose qualities of interest and couple these building blocks using controlled reactions. Bifunctional chemical linkers can be bound on one end to a growth factor and on the other end to a scaffold. The tether in between can be composed of a variety of polymers and can contain a number of desired components such as peptides, fluorescent tags, cytokines, and proteins.

Conjugating growth factors to a stabilizing molecule incorporated in the linker chain may also extend the protein's lifetime. For example, dextran- and polyethylene glycol (PEG)-based conjugates have been shown to stabilize proteins by increasing the circulation half-life time *in vivo*, namely by increasing the protein hydrodynamic radius which protects it from renal clearance, proteolysis, and immune system recognition.^{9,42} Further, the retention time of dextran-based conjugates once internalized within a cell is prolonged.⁹ Both stabilization and retention can be modified by choosing different polymer lengths and branching architectures.⁹ Conjugating polymers like dextran or PEG to various growth factors may help maintain the growth factor in its active state for longer periods of time.

Gradients, Spatial Distribution, and Density

Growth factors can be tethered to scaffolds in concentration gradients, spatial distributions, and various densities to direct cell adhesion, migration, and differentiation of progenitor cells. This points to the importance of growth factor patterning in ECM maintenance and equilibrium. Cells can be organized into complex structures based on cues from both bioactive molecule arrangement and ECM architectural features.⁶ Specific ligand distribution required for tissue regeneration differs between growth factors and can even be distinct for the same growth factor during various phases of tissue formation. Biomaterials engi-

neered to present ligands in patterns may be critical for regulating desired cell interactions and responses.

Integrin-binding peptide sequence fragments are often conjugated to materials to assist cell adhesion. Often polymer spacers are covalently attached to solid surfaces to serve as a way to immobilize growth factors or peptide fragments at defined concentrations while minimizing loss of bioactivity due to steric hindrance. Using avidin-biotin affinity binding, biotinylated ligands, either fibronectin fragment Arg-Gly-Asp (RGD) or laminin fragment Ile-Lys-Val-Ala-Val (IKVAV), were patterned with nanometer precision on block copolymer polylactide-poly(ethylene glycol) (PLA-PEG) matrices *via* flexible PEG chains, spatially confining aortic endothelial cells or PC12 nerve cells to the RGD or IKVAV micropatterned lines, respectively.⁴⁹ Leukemia inhibitory factor (LIF) and stem cell factor (SCF) were immobilized using PEG spacers in wide range of concentrations, and this method lends itself to tethering regulated amounts of signaling molecules at specific densities.⁵¹ In another example, poly(methacrylic acid) (PMAA) linkers immobilized in a gradient on a substrate were functionalized with RGD to induce cell adhesion as the ligand gradient increased.¹⁹

Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) was conjugated to an acrylamide-PEG-based interpenetrating network at various surface densities using the heterobifunctional crosslinker sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), and endothelial cell adhesion and spreading increased with GRGDSP density, activating extracellular signal-regulated kinase (ERK).⁴⁷ Alginate was modified with RGD using carbodiimide chemistry to determine the effect of ligand density on proliferation and differentiation of skeletal myoblasts,⁵⁸ and osteoblast-seeded RGD-alginate gels formed bone *in vivo* at 16 and 24 weeks.¹ Arg-Gly-Asp-Ser (RGDS) was covalently immobilized on PEG hydrogels in gradients at different concentrations using photopolymerization, and human dermal fibroblasts aligned along the RGD gradient and migrated toward increasing concentration.¹³ In a similar example, both RGDS and linearly graded basic fibroblast growth factor (bFGF or FGF-2) were covalently tethered on photopolymerizable PEG gels, and smooth muscle cells (SMCs) aligned and migrated along the growth factor gradient in the direction of increasing bFGF concentration.¹⁴ Immobilized epidermal growth factor (EGF) was micropatterned in a gradient on polystyrene, and Chinese hamster ovary (CHO) cells with overexpressed EGF receptor (EGFR) preferentially grew on high EGF density regions.¹⁰

Chemotactic and haptotactic agent concentration gradients have been shown to guide axons, and this model was translated to a poly(2-hydroxyethyl-methacrylate) [p(HEMA)] implant with nerve growth

factor (NGF) immobilized in a gradient, which stimulated PC12 neurite growth toward greater NGF concentrations.²⁶ Moreover, immobilized NGF and neurotrophin-3 (NT-3) concentration gradients showed synergistic effects in guiding dorsal root ganglion neurons.⁴⁴ Such implants with spatially distributed growth factors may be critical to enhancing axonal guidance and regenerating the injured nerve.

Conjugation Efficiency

To decrease the cost and waste associated with lost protein, the conjugation efficiency between growth factors and carriers is a priority. Efficiency is especially important because larger amounts of initial protein do not necessarily correspond with larger amounts of conjugated or loaded protein. Thus scaling up is not always the answer. One study found that conjugation efficiency of bone morphogenetic protein-2 (BMP-2) immobilized on polycaprolactone (PCL) scaffolds was greatly improved from 10 to 40% compared to BMP-2 that was physically adsorbed onto PCL.⁷⁴ Either BMP-2 or FGF-2 were covalently loaded onto chitosan films using carbodiimide chemistry, and BMP-2 covalent coupling efficiency was dramatically improved from ~25 to ~64% compared to adsorption, while FGF-2 coupling efficiency remained ~50% for either loading method. After 3 weeks, ~80% of either covalently immobilized growth factor remained, while only ~20% of adsorbed protein was retained.⁸ Greater loading efficiency of BMP-7 derived peptide was exhibited when covalently grafted onto nano-hydroxyapatite using aminosilane chemistry vs. the peptide being non-specifically adsorbed. Peptide-functionalized nano-hydroxyapatite was dispersed in poly(D,L-lactide-co-glycolide) (PLGA), resulting in a two-phase sustained release over 3 months.^{35,36} Photopolymerizable methacrylamide chitosan was thiolated and conjugated with maleimide-streptavidin in order to specifically bind biotin-conjugated rat interferon- γ (rIFN- γ). Nearly 100% of the biotin-rIFN- γ conjugated to the scaffold, whereas when the scaffold was not functionalized with streptavidin, ~80% of nonspecifically adsorbed biotin-rIFN- γ was lost.³²

Dose Dependence

Growth factors usually influence cell behavior at very low concentrations around 10^{-9} to 10^{-11} M.¹⁷ Moreover, the elicited response is typically biphasic, with low concentrations insufficient to activate cells and high concentrations excessive for saturated receptors.¹⁸ Because the role of morphogens is often dose dependent, spatial control is intrinsically achieved

since only tissues within a certain distance from the release point contact active growth factor concentrations.¹¹ Thus, the local concentration in the microenvironment, not the total dose of delivered growth factor, dictates the degree of cellular response.¹¹

Ephrin-A1, a ligand critical for vascular development and angiogenic remodeling, was covalently modified and photopolymerized onto PEG hydrogels, stimulating endothelial cell adhesion in a positively correlated, dose-dependent manner as measured by cell number and area, similar to polystyrene with pre-adsorbed ephrin-A1. Moreover, ephrin-A1-immobilized PEG stimulated the formation of endothelial tubules with luminal diameters between 5–30 μm .⁴³ Neural stem/progenitor cells (NSPCs) were induced down the neuronal lineage most effectively with the single growth factor IFN- γ , compared to brain-derived neurotrophic factor (BDNF) and erythropoietin, and neuronal differentiation of NSPCs on IFN- γ -immobilized methacrylamide chitosan scaffolds exhibited dose dependence on IFN- γ , with the most effective dose occurring at the highest tested concentration.³¹

Downstream Signaling

Physical presentation of growth factors to cells, whether in immobilized or soluble form, directly affects cell function from the onset of ligand-receptor binding to the activation of downstream signaling. It is important to look at cell signaling pathways when assessing the cellular response to delivered growth factors. It must be ascertained whether protein conjugation has masked active binding sites necessary for bioactivity. The level of cell responsiveness, such as the level of growth factor receptor expression, is important for cell-protein interactions. Cell binding to growth factors and resulting downstream signaling must remain intact. A bind-and-lock strategy was used to orient vascular endothelial growth factor (VEGF) in its bioactive state through its heparin-binding domain before the addition of a secondary functional group covalently coupling VEGF onto the heparin-functionalized surface. Covalently bound VEGF phosphorylated VEGF receptor-2 (VEGFR-2) in cells and altered human umbilical vein endothelial cell (HUVEC) morphology to be less stretched and polarized than HUVECs receiving soluble VEGF.²

Like many proteins, EGF is unstable in physiological fluids and has a very short half-life.⁶⁰ Further making EGF ineffective as a therapeutic agent is that human EGF requires many hours of continuous exposure to be functional and too high of concentrations may induce receptor downregulation.⁶⁰ This highlights the need for sustained delivery of EGF in order for it to be an effective and viable treatment in tissue regeneration.

When contacting immobilized EGF on polystyrene, keratinocytes expressed high levels of EGFR, low ERK1/2 and Akt phosphorylation, decreased proliferation, and increased migratory and aligned phenotype. However, keratinocytes in the presence of soluble EGF displayed low EGFR, high ERK1/2 and Akt phosphorylation, and exhibited proliferative rather than migratory behavior.⁵² EGFR signaling is known to assist cell survival and may have a role in bone development and homeostasis. Scaffolds designed to promote survival and proliferation of aspirated marrow cells were tethered with EGF to sustain its local delivery, and tethered EGF increased human bone marrow cell differentiation into osteogenic colonies over soluble EGF, as assessed by alkaline phosphatase (ALP) staining.³⁹ *N*-hydroxysuccinimide (NHS)-activated chitosan covalently reacted with EGF promoted chondrocyte proliferation and increased glycosaminoglycan content.⁶⁶

BMP-2 localized on PLGA scaffolds using a heterobifunctional PEG spacer enhanced bone formation when bone marrow-derived mesenchymal stromal cells were seeded on constructs and implanted into bilateral, full-thickness rabbit cranial defects.³³ BMP-2 was also immobilized on silk fibroin matrices using carbodiimide chemistry, improving osteogenic differentiation of human bone marrow stromal cells.²⁷

Fibroblast differentiation into myofibroblasts during soft tissue healing is mediated by transforming growth factor- β 1 (TGF- β 1).⁶⁹ Surfaces functionalized with aldehyde and epoxy groups were covalently immobilized with TGF- β 1 while maintaining the ability to induce normal human dermal fibroblast differentiation into myofibroblasts.⁴¹ While scaffolds containing peptide adhesion substrates improve cell adhesion, this modification often compromises ECM production and requires additional growth factors to counteract the decrease in matrix synthesis. Covalently bound adhesive ligands and TGF- β 1 within PEG hydrogels synergistically increased vascular SMCs to increase matrix production over soluble TGF- β 1 or tethered TGF- β 1 alone.³⁸

Heparin/Affinity-Based Delivery

In affinity binding, a substrate specific to the protein of interest is conjugated to a scaffold. Protein affinity toward that substrate receptor, along with total receptor capacity, drive the delivery and release. Heparin, a highly sulfated glycosaminoglycan, can be physically or covalently immobilized on a scaffold and presented for secondary associations with heparin-binding growth factors. The basic heparin-binding domains on growth factors interact electrostatically with the acidic sulfate and carboxylic acid moieties on

heparin. Heparin protects proteins from degradation, and growth factor release in this system is moderated by enzymatic degradation of the scaffold.⁶⁴

Delivered growth factors can be stabilized by the physical structure and chemical composition of the carrier. Hyaluronic acid, a stabilizing polymer, was conjugated to heparin, which has inherent binding sites for the FGF family of proteins, and rapidly bound FGF-2 was released upon enzymatic digestion in a fully functional state as seen by fibroblast growth and with increased stability and activity over free form FGF-2.³⁴ Hyaluronan was also crosslinked with gelatin and chitosan into a porous scaffold upon which heparin was then covalently immobilized using carbodiimide chemistry. bFGF was bound to heparin by affinity force, and heparin-bFGF-ternary scaffolds provided a favored environment for chondrocyte viability.⁶³

Heparin-functionalized chitosan-alginate scaffolds, created using *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) carbodiimide chemistry, increased bFGF binding efficiency 15-fold over that of bare chitosan-alginate.²⁰ Hydroxyapatite scaffolds vacuum-coated with a thin film of collagen type I were immobilized with heparin using EDC/NHS, reducing the release of loaded BMP-2 approximately four-fold.⁶⁵ Thiol-modified hyaluronic acid hydrogels were crosslinked with polyethylene glycol diacrylate (PEGDA) and thiol-modified heparin, attenuating the release of BMP-2 and maintaining ALP activity by mesenchymal precursor cells for up to 28 days.³ Calcium phosphate/poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) nanocomposite microspheres were laser sintered into intricate scaffolds based on computer-aided design (CAD) models and computed tomography (CT) scans, and after gelatin coating heparin was immobilized using EDC/NHS so that BMP-2 could be affinity bound onto the scaffold, thus increasing ALP and osteocalcin expression by mesenchymal stem cells.¹⁵ Heparin was conjugated to PCL/gelatin scaffolds using EDC/NHS chemistry, while allowing the negatively charged sulfonic groups on heparin to remain free to trap platelet-derived growth factor (PDGF) *via* electrostatic interaction, which promoted SMC proliferation and infiltration.³⁰

Differentiation of human embryonic stem cells into neural cells is dictated by EGF and bFGF, although EGF has been shown to be more potent in inducing neuronal and glial markers and cell extensions.²⁸ EGF and bFGF were adsorbed onto nanofibrous PLA but did not enhance axon growth beyond the biophysical cues inherent in electrospun aligned nanofibers. When EGF and bFGF were immobilized onto poly(L-lactide) (PLLA) nanofibers using covalently functionalized

heparin, axon growth was $>100 \mu\text{m}$ longer than with simply adsorbed growth factor.²⁸ Heparin was also functionalized on PLLA nanofibers using homobifunctional PEG, allowing the heparin to then bind laminin and bFGF, and these immobilized factors in addition to aligned nanofibers synergistically improved neurite extension and dermal fibroblast migration in wound healing.⁴⁸ Heparin was similarly functionalized on poly(L-lactide-co-caprolactone) (PLLACL) nanofibers, followed by immobilization of stromal cell-derived factor-1 α (SDF-1 α), and anastomosed vascular grafts recruited endothelial cells and smooth muscle cells, accelerating endothelialization and improving patency.⁷³

Covalently coupled heparin on PLGA was engineered to release 50% of loaded bFGF over 30 days with maintained bioactivity as demonstrated by HUVEC proliferation and blood vessel formation in the PLGA subcutaneous implant.⁷⁰ Covalent loading of heparin onto PLGA was increased over three-fold by using star-shaped vs. linear PLGA polymer for scaffolds. Bioactive BMP-2 was continuously delivered up to 30 days from heparin-PLGA and induced nine-fold greater bone formation and four-fold greater calcium content *in vivo* than BMP-2 released without heparin modification.²⁴

PLGA was functionalized with diamino-PEG to combat uncontrolled, non-specific protein adsorption, and the PEG end amine group was further coupled to heparin to provide a substrate for growth factor tethering that permitted the natural binding and presentation of bioactive, matrix-sequestered molecules.⁵⁷ Collagen matrices crosslinked and heparinized with EDC/NHS bound and released bFGF with variable kinetics depending on the molar ratio between EDC and heparin carboxylic acid groups.⁶⁹ Vinyl-conjugated heparin and terminally di-acrylated Pluronic were photo-crosslinked with bFGF into a hydrogel, and the resulting sustained release activated HUVEC proliferation *in vitro* and produced greater neovascularization *in vivo* as seen by dense capillaries than carriers without heparin.⁷¹

Dual Delivery

Because tissue development is a complex process involving numerous growth factors, tissue engineered constructs designed for multi-protein delivery are likely more effective for regeneration and more clinically applicable. Due to current challenges with single factor delivery, only a few studies have successfully explored dual growth factor delivery using covalent immobilization. A thorough review by Chen *et al.*¹¹ provides recent studies utilizing noncovalent delivery of multiple growth factors. With careful design and

consideration of complexity, properly timed and controlled release kinetics of multiple signaling molecules can increase construct utility and mimic natural repair mechanisms. Growth factors play key roles at different stages in the tissue development and repair process, and orchestrating the delicate balance may improve regeneration and should be strongly considered in carrier design. Simultaneous or sequential delivery of multiple growth factors can be achieved by using different methods of immobilization or by changing polymer degradation rates through molecular weight and ratio formulations.

PLGA microspheres loaded with dexamethasone and immobilized with TGF- β 3 through heparin simultaneously released both molecules with approximately zero order kinetics, leading to dramatic lacunae phenotype formation by mesenchymal stem cells.⁴⁶ With the aim of therapeutic angiogenesis, PDGF was encapsulated in PLGA microspheres with different degradation rates which were embedded within a bulk PLGA-alginate scaffold containing VEGF *via* surface association. Dual delivery led to the rapid formation of mature vascular networks with >100 vessels/ mm^2 in a non-obese diabetic (NOD) mouse femoral artery and vein ligation model.⁵⁵

Elicited angiogenesis was also targeted with *in situ* crosslinked PEGDA-hyaluronan-based hydrogels delivering VEGF, angiopoietin-1 (Ang-1), keratinocyte growth factor (KGF), and PDGF *via* covalently bound, thiol-modified heparin. Gels injected into mouse ear pinnae showed vascularization with all combinations of growth factors, as quantified by microvessel density.¹⁶ VEGF and FGF-2 dually incorporated within acellular, porous heparin-collagen scaffolds led to the most mature and highest density of blood vessels when subcutaneously implanted in rats, with no hypoxic cells after 3 weeks.⁴⁵

Despite the benefits of dual delivery, some combinations of growth factors have been shown to be detrimental and in some examples inhibited bone formation and ingrowth, although conflicting studies using the same growth factor combinations exist.^{40,54,56,62,68,72} Inhibitory effects may be due to improper growth factor doses or kinetics, lack of mechanical stimuli, or significant tissue trauma caused by the creation of or implantation into the bone defect model.⁶⁸ This points out that tissue regeneration mechanisms may require still more precise spatial and temporal control beyond sequential delivery to mimic complex biological processes.

Cleavable Linkers

In contrast to covalent conjugation schemes that provide permanent presentation of a growth factor,

growth factors can be released from the carrier as demanded by the tissue repair process upon encountering cues from the *in vivo* environment. Since proteases like matrix metalloproteinase (MMP) and plasmin are present in wound and chronic degeneration environments as well as remodeling environments, they make desirable targets for triggering growth factor release. Peptide sequences that act as substrates for specific enzymes can be covalently immobilized on scaffolds and render the linker both biologically responsive and cleavable. Peptide sequence linkers are cleaved, freeing downstream growth factors that promote tissue formation into the environment as demanded by cells. This leads to a “release as needed” system. As cells produce proteases to assist ECM breakdown during either degeneration or remodeling, appropriate growth factors are released to counteract degeneration or support remodeling. Such enzyme-sensitive delivery mechanisms are mediated by the local release of enzymes from active cells, and release is governed by enzyme concentration. A cleavable conjugation scheme allows for a sustained release of growth factor into the environment and also for protein internalization by cells.

Alternatively, cleavable peptide sequences can be embedded in the matrix itself within the polymer backbone. Growth factors that are covalently or noncovalently immobilized within the matrix are released upon cleavage of peptide sequences. This approach to growth factor release is accompanied by a loss in overall scaffold mechanical properties during enzymatic cleavage but allows improved cell infiltration as the scaffold degrades. In this type of cleavable scaffold scheme, release is impacted by enzyme concentration and bulk scaffold degradation rate, followed by poorly controlled diffusion. However, as diffusion-based strategies become more sophisticated, such as multi-polymer delivery systems and platforms that manipulate polymer diffusivity and degradation characteristics, they can be employed to reduced burst release and sustain multi-week kinetics. Recently, a bioartificial hydrogel scheme to induce vascularization included an MMP cleavable crosslinker bifunctionalized with two PEG-acrylates, mono-PEG-acrylated RGD and VEGF, and all three PEG components were photopolymerized under ultraviolet light with a photoinitiator. Degradable hydrogels implanted subcutaneously in Lewis rats released VEGF gradually and increased blood vessel density, and implants in a mouse ischemic hind-limb model demonstrated strongly encouraged reperfusion.⁵⁰

DISCUSSION

As the field of tissue engineering aims to tackle more ambitious regeneration endeavors, more sophisticated

advancements in controllable, biomimetic cell signaling are required. Advances in growth factor delivery using covalent immobilization offer more precise spatiotemporal regulation than ever before. Chemical conjugation of biological molecules allows a much longer growth factor lifetime and also the possibility of biore sponsive release mechanisms. Enzyme-mediated cleavage of scaffolds and tethers is a promising way for growth factors to stimulate local cells within the carrier and later enter the injury site upon demand by the environment. Although not covered in this review, gene therapy in the form of DNA and RNA release systems,⁶ DNA-based coatings functionalized with growth factors,⁶⁷ and therapeutic transgenes encoding tissue-specific transcription factors or soluble growth factors¹¹ may be used to initiate and sustain tissue repair.

Growth factor delivery is moving toward the direction of multiple growth factor systems with spatial and temporal control to stimulate different healing responses tailored to specific tissues.¹¹ Examples include engineering osteochondral constructs, complex multi-functional tissues, and implants requiring angiogenesis to sustain growth of the tissue of interest. The field of tissue engineering will surely benefit from emerging developments in covalent methods of growth factor delivery that allow for more refined control of regeneration stimuli.

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