

Growth factor-eluting technologies for bone tissue engineering

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Abstract Growth factors are essential orchestrators of the normal bone fracture healing response. For non-union defects, delivery of exogenous growth factors to the injured site significantly improves healing outcomes. However, current clinical methods for scaffold-based growth factor delivery are fairly rudimentary, and there is a need for greater spatial and temporal regulation to increase their *in vivo* efficacy. Various approaches used to provide spatiotemporal control of growth factor delivery from bone tissue engineering scaffolds include physical entrapment, chemical binding, surface modifications, biomineralization, micro- and nanoparticle encapsulation, and genetically engineered cells. Here, we provide a brief review of these technologies, describing the fundamental mechanisms used to regulate release kinetics. Examples of their use in pre-clinical studies are discussed, and their capacities to provide tunable, growth factor delivery are compared. These advanced scaffold systems have the potential to provide safer, more effective therapies for bone regeneration than the systems currently employed in the clinic.

Keywords Osteogenesis · Bone scaffold · Biomaterials · Stem cells · Tissue engineering

Introduction

Skeletal injuries from trauma, tumors, infections, and degenerative diseases often require a significant intervention, such as bone grafting, to facilitate healing. While 1.6 million bone grafting procedures are performed per year in the USA, the current gold standard—autologous bone grafts—is expensive, inefficient, causes donor site morbidity, and is limited in supply and size [1]. Therefore, there remains a critical need for effective alternatives to bone grafts.

Growth factors are key components in the regenerative process leading to scarless bone regeneration. A complex spatiotemporal cytokine cascade orchestrates healing following bone fracture [2]. Inflammatory cytokines cause an invasion by lymphocytes, plasma cells, macrophages, and osteoclasts. Invading macrophages clean up necrotic centers in the graft and release tumor necrosis factor (TNF), which drives increased osteoclast activity. Osteoclasts resorb fractured bone matrix, releasing incorporated insulin-like growth factor (IGF) and bone morphogenetic proteins (BMPs), and these cause osteoblastic differentiation of progenitor cells [3, 4]. Neovascularization of the fracture site occurs early in this process as endothelial cells begin sprouting angiogenesis in response to vascular endothelial growth factor (VEGF) and low oxygen tensions in the graft [5]. Endothelial cells are the primary source of BMPs within the fracture site driving osteogenesis of osteoblasts. Osteoid production by those osteoblasts begins on the outside of the fracture, creating a callus and mechanically integrating the bone [6]. Platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β), and fibroblastic

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growth factor (FGF) released from plasma cells, macrophages, and osteoblasts support cellular proliferation and differentiation [4, 7, 8]. Finally, remodeling into organized continuous bone progresses over several months according to Wolff's law.

Several of the key growth factors identified above as having critical roles during normal healing (Table 1) have been utilized in various clinical approaches to treat bony non-unions. The timing of therapeutic growth factor delivery is crucial to optimize tissue induction while minimizing adverse or inhibitory effects. However, growth factors have short half-lives and rapid clearance rates *in vivo*, particularly when delivered systemically [32, 33]. Bone tissue engineering (BTE) scaffolds have been used as a feasible treatment methodology to provide temporary mechanical support for cellular ingrowth and to actively guide tissue organization. Additionally, 3D scaffolds can localize and control the temporal delivery of protein growth factors and/or genes required for optimal *in situ* bone development and/or repair. The incorporation of growth factors into scaffolds has considerable potential to enhance healing outcomes. Here, we review growth factor-eluting technologies that can be employed to produce such scaffolds.

Growth factor incorporation strategies

A wide variety of factors—ranging from scaffold material and architecture, to the dosing and release kinetics of the incorporated growth factors—must be optimized in designing an inductive scaffold-based delivery system for BTE applications. The scaffold should exhibit suitable mechanical properties and a biodegradation rate that enables both controlled growth factor delivery and integration with host tissue. These scaffold properties are determined by the materials employed and the processing and biofactor incorporation strategies utilized. Decades of research into drug delivery materials have yielded a broad array of naturally and synthetically-derived biodegradable materials which can be employed to produce 3D scaffolds of varying architectures (reviewed in detail in [34–36]).

Controlling growth factor dosage and release kinetics is key to optimizing tissue induction while avoiding adverse or inhibitory effects [37–39]. Currently, the growth factor dosage

ranges being used are quite broad, and clinical applications typically employ supraphysiological concentrations. Although some of this dosage variation is due to differences in the animal models utilized, there remain many questions regarding optimal target doses and release kinetics. In a study comparing burst release from collagen sponges (100 % over 2 days) to polyurethane scaffolds with slow (~20 % over 19 days) or fast (60 % over 9 days) release of BMP-2 (all systems loaded with 2 μg), fast-releasing scaffolds showed the greatest *in vivo* bone formation (45 mm^3) in a rat femoral critical-sized defect model after 4 weeks, followed by the burst release collagen scaffold (30 mm^3) and the slow release scaffold (10 mm^3) [40]. This suggests that an initial burst release followed by a slow sustained release might be the best means of delivering BMP-2 for bone formation.

However, the optimal therapeutic dosage and timing of release will depend heavily on the individual growth factor(s) and the particular application. One has to account for factors such as the anatomical location, size, and nature (e.g., trauma vs. tumor re-sectioning) of the bone defect, the extent of vascularization in the surrounding tissue environment, conjunctive therapies (e.g., chemotherapy or the use of metal supports), and the health of the surrounding tissues. For example, in comparing quick and slow release of BMP-2 for induction of bone formation in orthotopic and ectopic sites in dogs, Geuze et al. found that while ectopic groups formed more bones in response to quick BMP-2 release, bone formation in the orthotopic site was independent of the release profile [38]. Furthermore, some growth factors are not effective at low doses, require co-delivery of a secondary agent to be effective, or are harmful to cells with prolonged exposure. For example, high doses and/or prolonged exposure to BMP-2 in anterior cervical spine fusion cases has resulted in high rates (23 %) of adverse effects in patients [41, 42], while failure to shut down TGF- β in reparative processes can lead to a number of fibrotic diseases [43]. Hence, in some cases, only a transient delivery of growth factor might be needed to potentiate endogenous regenerative responses [36–38].

In native tissues, growth factors are typically encrypted within the extracellular matrix (ECM) where they are protected from enzymatic and hydrolytic degradation. Once they are released from these encrypted sites, the half-life of

Table 1 Clinically applied growth factors for BTE

Factor	Action	References
Bone morphogenetic proteins (BMP)	Bone induction	[9–15]
Vascular endothelial growth factor (VEGF)	Angiogenesis	[16–20]
Platelet-derived growth factor (PDGF)	Cell proliferation and recruitment and vascularization	[7, 21, 22]
Fibroblast growth factor (FGF)	Angiogenesis, proliferation, and osteogenic differentiation	[8, 23–26]
Insulin-like growth factor (IGF)	Osteogenic differentiation	[3, 27–30]
Transforming growth factor beta (TGF- β)	Osteogenic, chondrogenic differentiation	[4, 28, 31]

growth factors in vivo is short—on the order of several minutes—due to enzymatic degradation, chemical and physical deactivation, and degradation processes such as hydrolysis, oxidation, isomerization, and aggregation [44, 45]. Therefore, one of the most critical challenges in scaffold-based growth factor delivery is maintaining native protein conformation and bioactivity throughout scaffold loading and for the duration of in vivo release [46]. The architectural design, processing, and storage of scaffold materials and the protein loading strategies must all be optimized to ensure delivery of functional growth factor for the duration of in vivo release. For example, Madurantakam and colleagues demonstrated that exposure of BMP to organic electrospinning solvents during growth factor loading affected its tertiary and quaternary protein conformations and impaired its bioactivity. In contrast using a 50 % dilution in an aqueous buffer retained the bioactivity of the incorporated BMP [47]. Similarly, degradation products of scaffold carrier polymers, such as polyesters, can increase local acidity and lead to protein denaturation or degradation, while secondary protein-polymer interactions can trigger protein mis-folding and aggregation [48]. Efforts have also been made to prevent enzymatic degradation of growth factors by tailoring scaffold pore size to reduce protease penetration into scaffold [45]. Employing a more biomimetic approach, heparin binding is commonly used to maintain and enhance BMP presentation and bioactivity and increases the half-life of BMP in culture medium nearly 20-fold [49–51].

To date, a variety of methods have been explored to incorporate drugs, protein growth factors, and DNA into 3D polymeric and composite scaffold systems and control their delivery. These approaches encompass bulk incorporation strategies and surface modification techniques, with the current trend moving towards hybrid approaches to produce tissue engineering systems capable of multiagent delivery and/or stimuli-responsive release (Fig. 1, Table 2).

Physical entrapment strategies

One of the simplest approaches to producing inductive tissue engineering systems is bulk incorporation, whereby the biofactors to be delivered are blended directly into hydrogels or within solid scaffold polymers and physically entrapped. Protein and/or DNA release kinetics from these bulk incorporation systems is typically characterized by an initial burst followed by slower release that is controlled by the diffusion and degradation rates of the matrix, which are in turn dependent on such properties as matrix, porosity, swelling behavior, polymer cross-linking density, and polymer chemistry (i.e., molecular weight, hydrophobicity/hydrophilicity, charge density). Loading efficiencies within bulk incorporation systems are generally high and are determined by factors including polymer and biofactor interactions, solubility, and concentration ratios, as well as the types of cross-linking interactions

and processing times and temperatures employed. These parameters can be specifically tuned (within limits) for various localized, controlled release applications via careful design of polymer composition and scaffold processing techniques.

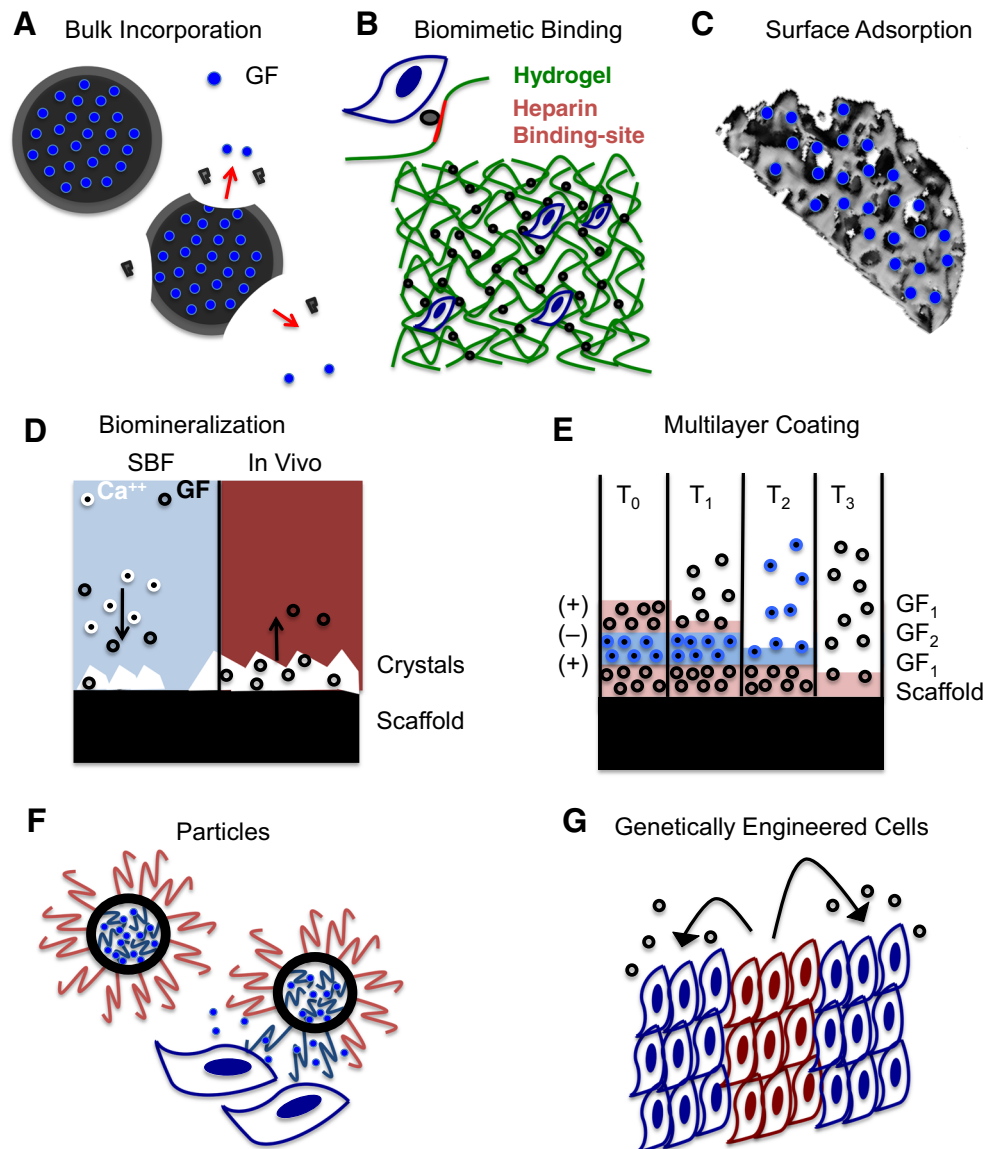
Solid scaffold polymer blending

In solid polymer or composite scaffolds, biofactors can be directly blended with core polymers via formation of polymer-solvent and biomolecule-water emulsions and subsequent freeze-drying [52], or via gas foaming [53], which eliminates the need for organic solvents that can potentially denature or degrade proteins. For example, supercritical CO₂ processing was used to incorporate rhBMP-2 within PLA scaffolds (96 µg BMP-2) yielding systems, which released low amounts (674 ng over the first 48 h, followed by 100 ng/mL per 72 h) over a period of at least 24 days. Although less than 5 % of the initially loaded BMP-2 appeared to be released cumulatively, subcutaneous implantation in a rat model resulted in bone formation at 6 weeks (~12 mm³) and persisted until at least 26 weeks [54, 55]. Growth factors can also be directly incorporated within the strands of electrospun fiber-based scaffolds via blending prior to electrospinning, emulsion electrospinning, or coaxial electrospinning (reviewed in [94]). Srouji and colleagues used coaxial electrospinning to produce scaffolds composed of core-shell fibers with an inner rhBMP-2/PEO core and outer PCL/PEG shell. They found that the degree of porosity of the outer shell determined the BMP-2 release rate, and that slower releasing scaffolds (which released 12–15 % of the loaded BMP-2 in 27 days, with 5 % released in the first 4 h) resulted in greater in vivo bone formation in a rat cranial defect model (80 % coverage vs. 55 % at 8 weeks), than faster releasing scaffolds (76 % in 27 days, with ~67 % released in the first 4 h) [56]. BMP-2 has also been successfully incorporated into a variety of electrospun scaffold architectures composed of natural and synthetic polymers, including silk, PCL, PLLA, and PLGA, as well as polymer-ceramic composite fibers which integrated hydroxyapatite nanoparticles [56–60]. These direct blending strategies are limited in the scaffold architectures that can be produced, and generally display initial diffusive burst release. Care must be also taken to ensure that processing conditions for the scaffolds do not reduce the bioactivity of the incorporated growth factors.

Hydrogel encapsulation

Hydrogel encapsulation of drugs and biomolecules is one of the simplest and most popular strategies for producing 3D-controlled delivery systems for tissue engineering. Direct physical entrapment of proteins, drugs, and DNA within hydrogels can be achieved via blending with matrix polymers prior to chemical or physical cross-linking. One of the key

Fig. 1 Methods of growth factor delivery. **a** Bulk incorporation of GFs (blue) released with degradation of the bulk (black) material (“Physical entrapment strategies”). **b** Hydrogels (green) can be modified for increased biomimetic affinity (red) to increase binding of GFs (black dots) and natural presentation to cells (blue) (“Hydrogel encapsulation”). **c** Growth factors (blue dots) can directly adsorb to the scaffold surface (gray) without specific chemical modification (“Surface adsorption”). **d** Biomineralization traps GFs (black dots) in the crystal formation (mediated by Ca^{++} in white) in simulated body fluid (SBF), and released as the crystals degrade in vivo (“Biomineralization”). **e** Multilayer coating can facilitate multiagent (GF1, black dot/GF2, blue dots) delivery and staggered release of GFs over time (“Polyelectrolyte multilayer film coating” and “Multiagent delivery”). **f** Nano and microparticles can deliver GFs (blue dots) to cells (blue) and can be functionalized with adhesion molecules (red) (“Nanoparticles and macroparticles”). **g** Cells can be genetically engineered (red) to secrete GFs (black dots) to surrounding cells (blue) “Cells as drug-eluting systems”



advantages to using hydrogels for controlled growth factor delivery is the wide array of stimuli-responsive polymeric hydrogels, which can be employed to produce on-demand release systems. However, hydrogel systems are severely limited in the scaffold architectures and mechanical properties

that can be produced and are thus often used in hybrid strategies where they are infused into other scaffold structures. Furthermore, the hydrogel cross-linking strategy employed in growth factor encapsulation must be chosen so as to minimize any chemical or physical modifications of protein structure

Table 2 Advantages and disadvantages of different drug-eluting technologies

Method	Advantages	Disadvantages	References
Bulk incorporation	Highly tunable	Processing may affect GF, burst release	[52–60]
Biomimetic binding	Increased capacity and prolonged release	Complex loading	[23, 49, 61–67]
Surface adsorption	Simple loading	Poor capacity and release kinetics	[68, 69]
Multilayer coating	Temporal release, multiagent	Processing may affect GF	[70–77]
Particles	High payloads, release control, multiagent	Complex loading	[9, 24, 78–80]
Genetically engineered Cells	Enduring, bioactive delivery	Regulatory and safety concerns	[31, 81–85]
Biomineralization	Increased osteoconductivity, biomimetic	Requires cell-mediated material degradation	[86–93]

(e.g., oxidation reactions, photodegradation, or cross-linking with polymer chains).

A variety of “smart” hydrogel systems have been developed that respond to changes in temperature, pH, mechanical forces, electromagnetic fields, irradiation, ultrasound, or the presence or absence of certain solutes by dramatically altering properties such as their swelling behavior, network structure, or degradation rate (reviewed in [95]). Similarly, biochemically responsive hydrogels, which incorporate enzymatically cleavable peptide linkage or cross-linking groups, such as matrix metalloproteinases (MMPs), result in cell-based enzymatic degradation and release of encapsulated growth factors. Holloway and colleagues developed such MMP-sensitive, cell-degradable hyaluronic acid hydrogels for BMP-2 encapsulation (100 ng BMP-2) and demonstrated that faster degrading gels (100 % mass degradation and BMP-2 release in 6 days in the presence of collagenase *in vitro*) resulted in improved bone formation in a rat cranial defect model compared to slower degrading gels (100 % degradation and release in 10 days) [96].

Chemical and affinity binding strategies

Protein growth factors can also be covalently bound or linked via biomimetic interactions to the polymers that make up the hydrogel matrix in order to more precisely control their loading, distribution, presentation, stability, and delivery. Such chemical and affinity binding strategies generally reduce burst release and prolong growth factor delivery. However, care must be taken in designing the linkage strategy so that growth factor bioactivity is preserved.

Covalent binding

A variety of covalent binding strategies can be employed to attach protein growth factors to matrix polymers thus enabling on-demand biofactor release. Such linkage strategies can be designed such that release is mediated either by hydrolysis, reduction reactions, or enzymatic degradation of the covalent bonds. For example, BMP-derived peptides functionalized with azide groups were covalently conjugated to PEG-based hydrogels via click chemistry, and the resulting system was found to induce osteogenic differentiation of bone marrow stromal cells *in vitro* [97]. In designing such covalent protein-binding systems, however, one must ensure that the linkage process does not affect the biological activity of the proteins by blocking active sites or causing denaturation, and that those growth factors which require cellular internalization for proper function are bound via cleavable linkage strategies.

Biomimetic binding interactions

In strategies which mimic the natural interactions between proteins and glycosaminoglycans (GAGs) within the extracellular matrix (ECM), electrostatic and affinity interactions can be employed to aid in hydrogel growth factor loading. In addition to retaining and delivering the growth factor over a longer period of time, one of the key advantages to utilizing such biomimetic strategies is that ECM- and GAG-bound growth factors are maintained in a more bioactive form than when diffusively released or presented via covalent tethering strategies [23, 61]. Thus, ECM binding might allow for the use of more physiologically relevant quantities of growth factors rather than the currently employed supraphysiological concentrations.

Modification of hydrogel polymers with GAGs, such as heparin, which has been shown to bind protein growth factors, including BMP-2, FGF, and TGF- β [62, 63], can increase growth factor loading, and prolong growth factor release, as well as protect encapsulated proteins from thermal degradation and proteolysis. Jeon and colleagues developed heparin-modified PLGA scaffolds which demonstrated ~99 % BMP-2 loading efficiency and prolonged *in vitro* release over at least 14 days (19 % day 1, steady rate until day 14) compared to similar unmodified PLGA scaffolds which demonstrated burst release (~100 % over 4 h). These heparin-modified scaffolds subsequently demonstrated ninefold higher bone formation at 8 weeks compared to unmodified BMP-loaded scaffolds (both loaded with 1 μ g BMP-2) in an ectopic bone formation rat hind-limb muscle model [49]. In stark contrast, Bhakta et al. found that hyaluronan-based hydrogels modified with heparin and loaded with BMP-2 (5 μ g) resulted in less *in vivo* bone formation in a similar rat hind-limb muscle model than unmodified BMP-loaded hydrogels, and theorized that it was due to a lack of early burst release (~14 % during day 1 and a total release of ~68 vs. ~26 % release on day 1 and a total release of ~84 %) [64]. These disparate results utilizing different core scaffolds (i.e., PLGA solid scaffolds vs. hyaluronan hydrogels) but the same growth factor imply that growth factor dosage and release kinetics alone may not dictate successful *in vivo* bone formation, but that scaffold architecture, porosity, and mechanical properties likely play key roles.

Another widespread biomimetic strategy for controlling biofactor delivery from hydrogels involves the incorporation of ECM peptide sequences. For example, Hubbell et al. mimicked the blood clot microenvironment that forms after skeletal fractures by replicating fibronectin subdomains and attaching them to fibrin gels, thus resulting in improved retention of growth factors, such as PDGF, FGF-2, and TGF- β , over time and improved bone healing outcomes [65, 66]. Similarly, Hamilton and colleagues screened a phage display library of peptide sequences to identify BMP-2-binding peptide sequences that they then incorporated within injectable

collagen matrices along with BMP-2, resulting in more than tenfold higher BMP-2 loading, and increased bone formation and maturity *in vivo* in a rat ectopic bone formation model, compared to collagen matrices containing only BMP-2 [67].

Surface modification strategies

In contrast to bulk incorporation strategies, surface modification techniques enable growth factor incorporation while preserving underlying scaffold architecture and properties. While surface modification strategies generally result in lower levels of growth factor incorporation, they enable delivery from a much wider variety of scaffolds and implant systems and materials, particularly those with increased mechanical strength. Techniques used to functionalize biomaterial surfaces with proteins, peptides, drugs, and/or DNA include simple surface adsorption, “grafting-to” and “grafting-from” strategies, incorporation within electrostatic layer-by-layer films, and biomineralization.

Surface adsorption

Simple surface absorption of biofactors onto scaffolds via dipping or incubation is one of the most commonly utilized techniques to produce localized delivery systems. Biofactor adsorption onto biomaterial surfaces is governed by a combination of non-specific physical forces, such as electrostatic interactions, van der Waals forces, hydrophobic/hydrophilic interactions, and hydrogen-bonding interactions, which in turn are determined by a complex interplay of factors including the species and concentrations of biomolecules present in the incubation/dipping solution, the temperature, pH and ionic strength of the solution, and material surface properties such as topography, chemistry, charge, and wettability [68]. BMP surface adsorption onto various polymeric and composite scaffold systems, particularly collagen-based matrices, has been widely investigated, with varying strategies utilized to maximize the amount of adsorbed BMPs, such as increasing incubation time, altering the pH and ionic concentration of the solution, and introducing charged functional groups to the scaffold surface [69]. Non-specific surface adsorption onto scaffold surfaces typically results in low levels of biofactor loading and poor control of release kinetics. Better control of biofactor release from scaffold surfaces, meanwhile, is more easily achieved via surface immobilization methods.

Surface immobilization

Direct immobilization of proteins and gene delivery vectors to scaffold surfaces, via covalent cross-linking or strategies involving antibody/antigen or biotin/avidin binding, has been widely studied [98, 99]. In the case of covalent surface immobilization, chemically or physically based methods are often

employed to introduce reactive functional groups onto scaffold surfaces in order to activate them for subsequent grafting. Once a scaffold surface has been chemically functionalized, via partial surface hydrolysis, oxidation, aminolysis, or plasma treatment, various reactions targeting primary amines and carboxylic acids can be utilized to immobilize protein growth factors, such as BMPs. Such surface conjugation of growth factors tends to increase protein loading and stability and prolong release compared to surface adsorption strategies. For example, when BMP-2 was immobilized onto the surface of aminolyzed PCL scaffolds via sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) cross-linking, the resulting scaffolds demonstrated increased loading efficiency (~38.5 vs. ~9%), slower release (7 vs. 27% over 15 days), and increased *in vitro* osteogenic differentiation of bone marrow stromal cells, compared to similar PCL scaffolds with surface-adsorbed BMP-2 [100]. As in the case of covalent linkage of biofactors within bulk hydrogels, covalent surface immobilization strategies must be sure to preserve protein confirmation and bioactivity and enable the release of growth factors which require cellular internalization for function.

Polyelectrolyte multilayer film coating

Deposition of polyelectrolyte multilayer films has been widely investigated for surface-based controlled release of drugs, bioactive proteins, and plasmid DNA (reviewed in [70]). A simple and versatile technique developed by Decher and colleagues [71] electrostatic layer-by-layer (LbL) deposition involves the sequential surface adsorption of alternating layers of oppositely charged polyelectrolytes (PEs) on nearly any charged substrate surface. Careful selection of the polyelectrolytes used and the layer architecture and chemistry employed enables both the tailoring of release kinetics and sequential delivery of several different proteins and/or genes [72, 73]. LbL films can be generated from a wide variety of synthetic and natural polymers, and bioactive proteins; DNA and gene delivery vectors can be incorporated within PE multilayers without any need for covalent attachment [74] and without any significant changes to their native conformation. The deposition of PE multilayers onto 3D scaffolds has enabled scaffold-based delivery of both genes and proteins [75–77]. For example, 3D printed β -tricalcium phosphate/polycaprolactone scaffolds coated with LbL films consisting of a poly (β -aminoester) (“poly 2”), chondroitin sulphate (CS), and BMP-2 resulted in a system that successfully induced *in vivo* bone formation when implanted intramuscularly in rats [76]. Meanwhile, Hammond et al. developed LbL nanolayer coatings of BMP-2 and PDGF on PLGA membranes and found that low-dose dual delivery resulted in better outcomes (healing rate, bone volume, mechanical properties, and histology) in a rat calvaria defect model than

BMP-2 delivery alone (at both 200 ng and 2 µg doses of BMP-2) [101].

Biom mineralization

Inspired by the *in vivo* process by which bone apatite crystals are formed, surface biom mineralization methods can be utilized to incorporate protein growth factors on scaffold surfaces, while simultaneously improving osteoconductivity (reviewed in [86]). Surface biom mineralization techniques involve immersing a material in simulated body fluid (SBF), which leads to formation of a calcium phosphate surface phase similar to that found in native bone [87]. Biomimetic mineralization has been used extensively to form coatings on orthopedic and dental implants, as well as on polymeric and composite tissue engineering scaffolds, resulting in enhanced osteogenic activity, and/or bone formation both *in vitro* and *in vivo* [88–91]. More importantly, biom mineralization has been used to incorporate bioactive growth factors, such as bone morphogenetic proteins (BMPs), within the formed surface coatings, thus further enhancing bone formation and tissue integration [92, 93].

Nanoparticles and macroparticles

Polymeric nano- and microparticles can be loaded with GFs via internal encapsulation, bulk mixing, or surface attachment. Such particles can then be integrated into 3D scaffold systems via the bulk- and surface-based incorporation strategies already discussed. For example, Yu et al. developed hydroxyapatite microspheres (3–5 µm diameter), which incorporated BMP-2 and VEGF by means of a layered mineral coating. By varying layer thickness, release kinetics could be tailored, with sustained release profiles of over 50 days possible [78]. Microparticles can also utilize heparin-growth factor binding, as demonstrated by Xu et al. who altered the heparin content in hyaluronic acid hydrogel particles (~1.1 µm in diameter, pore size ~24 nm) and obtained a tunable BMP-2 release system that demonstrated a constant dosage over 2 weeks and a near zero-order release profile, which induced highly efficient chondrogenesis of MSCs [79]. Beyond engineering to control release and cellular uptake rates [9, 24], these small particles can be employed to enable multifactor and/or sequential delivery, as well as spatially patterned release, thus enabling the growth of both bone and blood vessels [80].

Multiagent delivery

Natural bone development and repair involve the precise temporal and spatial orchestration of a variety of signaling cascades and cell types. Thus, current trends in scaffold-based protein and gene delivery are extending beyond controlling dosage and release kinetics to encompass multiagent delivery

and spatially controlled release. Many groups have created concentration gradients of growth factors within hydrogels using a variety of methods (reviewed in [102]), with the most common being utilization of a gradient maker which mixes two or more types of hydrogel precursor solutions (with/without the growth factor) and then subsequently cross-links them [103, 104]. Meanwhile, zonal protein delivery was demonstrated employing a scaffold composed of microspheres loaded with either VEGF or PDGF in an *in vivo* angiogenesis model, with VEGF delivery from one scaffold area resulting in formation of small blood vessels while sequential delivery of first VEGF and then PDGF in another scaffold zone led to fewer but larger and more mature vessels [105].

Many studies have highlighted the importance of multiagent delivery in enhancing bone tissue formation and vascularization within tissue engineering scaffolds. For example, dual release of low dose of BMP-2 and TGF-β3 proteins from hydrogels seeded with bone marrow stromal cells (BMSCs) induced bone formation when implanted subcutaneously in mice, while supraphysiological concentrations of either factor alone did not induce significant bone formation [106]. Meanwhile, porous PLGA scaffolds delivering both VEGF protein and poly-ethyleneimine (PEI)-condensed BMP-4 plasmid and seeded with BMSCs induced greater bone formation in critical size rat cranial defects compared to delivery of any other combination of these factors [107]. Similarly, dual delivery of covalently incorporated BMP and osteopontin-derived peptides within hydrogels enhanced *in vitro* bone marrow stromal cell mineralization, osteogenic differentiation, and vasculogenic differentiation [108]. Finally, Yilgor et al. affixed PLGA nanoparticles with release profiles of 20 days and PHBV nanoparticles with release profiles of 40 days to the surface of chitosan scaffolds to enable the sequential delivery of emulsified BMP-2 and BMP-7, resulting in increased alkaline phosphatase activity and mineralization of scaffold-seeded rat BMSCs [9, 109]. In multiagent delivery systems, the timing of growth factor release can be of even more importance, as the temporal regulation of the influx of each growth factor into the defect site can be critical to enhancing the sequential steps of bone healing.

Cells as drug-eluting systems

As cells naturally secrete GFs, osteogenic progenitor cells can be pre-seeded within scaffolds to provide an inherently bioactive supply of GFs to a defect site. Adipose-derived stem cells and mesenchymal stem cells naturally secrete relevant GFs for bone healing in response to cues from the microenvironment—such as hypoxia or ischemia—and reduce secretion once bone is healed. These cells can also be genetically engineered *ex vivo* to maintain an increased secretion profile of a specific GF through a variety of gene delivery strategies,

including transfection via nucleofection, and viral and non-viral delivery vectors [31, 82–84] (reviewed in detail, specifically for BMP gene transfection, by Wilson et al. [110]). Such cell-based therapies are limited by the tendency of the implanted cells to migrate away from the defect site or be cleared by the host.

Alternatively, inductive tissue engineering scaffolds can be utilized for material-based in situ gene delivery. Naked plasmid DNA or DNA complexed with viral, lipid-based, or polymeric delivery vectors can be integrated within 3D scaffolds using the bulk- and surface-based incorporation strategies discussed previously. A variety of scaffold-based gene delivery systems have been investigated for bone tissue engineering. For example, Elangovan et al. developed collagen sponge scaffolds with surface immobilized PEI-plasmid DNA complexes [encoding PDGF-BB] which demonstrated impressive healing over 4 weeks, recovering 50 % of lost bone volume (14-fold greater than empty control and 44-fold greater than a control scaffold) in a rat cranial defect model [85]. Using a liposomal vector in a collagen gel, Park et al. delivered the BMP-2 gene to peri-implant bone defects (defect 10 mm diameter, 7 mm depth) in a pig model. BMP-2-producing cells were present in increased number (compared to controls) at 1 week and remained at 4 weeks. Bone matrix formation was accelerated in BMP-2-treated groups at week 1 and resulted in increased osseointegration and bone regeneration [81].

Conclusion

Effective regenerative outcomes for large non-union bone defects rely on appropriate scaffold properties coupled with optimized patterns of cell and growth factor delivery. Indeed, the controlled delivery of osteoinductive and angiogenic factors that act in concert to orchestrate the formation of bone tissues remains a major engineering goal. Currently, delivery of BMPs to non-union defect sites via collagen sponge carriers is the clinical standard for growth factor-eluting scaffold technologies. Although generally successful in promoting fusion, the poor control over release kinetics and the supraphysiological BMP concentrations required to induce sufficient bone formation lead to a number of complications and adverse effects—particularly in the craniofacial area—that include heterotopic bone formation, edema, seroma, and even cancer [111, 112]. The emerging technologies described above can facilitate greater spatiotemporal control of growth factor(s) released at a bony defect site leading to safer, more effective scaffolds for use in bone regeneration.

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