REVIEW

Novel biomaterial strategies for controlled growth factor delivery for biomedical applications

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Growth factors (GFs) are soluble proteins secreted by cells that have the ability to regulate a variety of cellular processes and tissue regeneration. However, their translation into clinical applications is limited due to their short effective half-life, low stability, and rapid inactivation by enzymes under physiological conditions. To maximize the effectiveness of GFs and their biologically relevant applicability, a wide variety of sophisticated bio-inspired systems have been developed that augment tissue repair and cellular regeneration by controlling how much, when, and where GFs are released. Recently, protein immobilization techniques combined with nanomaterial carriers have shown promise in mimicking the natural healing cascade during tissue regeneration by augmenting the delivery and effectiveness of GFs. This review evaluates the latest techniques in direct immobilization and relevant biomaterials used for GF loading and release, including synthetic polymers, albumin, polysaccharides, lipids, mesoporous silica-based nanoparticles (NPs), and polymeric capsules. Specifically, we focus on GF-encapsulated NPs in functionalized microporous scaffolds as a promising alternative with the ability to mimic extracellular matrix (ECM) hierarchical architectures and components with high cell affinity and bioactivity. Finally, we discuss how these next-generation, advanced delivery systems have been used to enhance tissue repair and regeneration and consider future implications for their use in the field of regenerative medicine.

INTRODUCTION

Growth factors (GFs) as powerful therapeutic agents for tissue regeneration

GFs are soluble proteins secreted by cells that have the ability to regulate a wide range of cellular behaviors, such as proliferation, migration and differentiation, by binding to specific transmembrane receptors on target cells.[1] The chemical identity, duration, concentration and sequence of multiple GFs and the particular extracellular matrix (ECM) in which they are applied can have a significant effect on cell fate and the responsiveness of wound healing.[2,3] In particular, the context in which GFs are used, for example, alone or in various combinations, has shown great therapeutic potential in preclinical models and clinical applications. Some of the more widely studied GFs in tissue regeneration and repair are those involved in angiogenesis and osteogenesis. These include vascular endothelial growth factors (VEGFs), fibroblast growth factor (FGF) and placental growth factors (PlGFs), which are involved in angiogenesis and play a crucial role in the repair of most tissues;[4–6] insulin-like growth factors (IGFs), transforming growth factor beta (TGF-β), bone morphogenetic proteins (BMPs), particularly BMP-2, BMP-4 and BMP-7, which are the most extensively used osteogenic proteins for inducing new bone formation in bone defects and ectopic sites;[7–10] and platelet-derived growth factor-BB (PDGF-BB), which plays a pivotal role in the formation of periodontal tissue and the recruitment of osteoblast progenitors and stem cells.[6,11] To date, the only GFs that have been clinically approved by the Food and Drug Administration (FDA) are BMP-2 and BMP-7 for lumbar spine fusion and bone fracture and PDGF-BB for the enhancement of granulation tissue formation.

Clinical limitations of GFs

As summarized above, a number of GFs have shown great therapeutic potential in clinical trials, and some have been approved for specific biomedical applications. However, only a relatively small subset has achieved commercial success due to safety and cost-effectiveness issues. A major limitation of natural GFs is their short effective half-life, low protein stability, and rapid deactivation of their specialized properties by enzymes at body temperature. As an example, FGF-1 presented intrinsically low stability, with an active half-life of only 1 h in serum at 37 °C,[12] whereas the half-life of basic fibroblast growth factor (bFGF) is 3 min[13] and that of VEGF is only 50 min[14] following intravenous injection. Moreover, direct injection of GFs in high enough doses in order to reach and sustain a sufficiently high local concentration to be effective may result in side effects *in vivo*. Clinical doses of BMP-2 in lumbar spinal arthrodesis is associated with an increased risk of new cancer development.[15] Similarly, VEGF has been shown to trigger edema and systemic hypotension in cardiovascular

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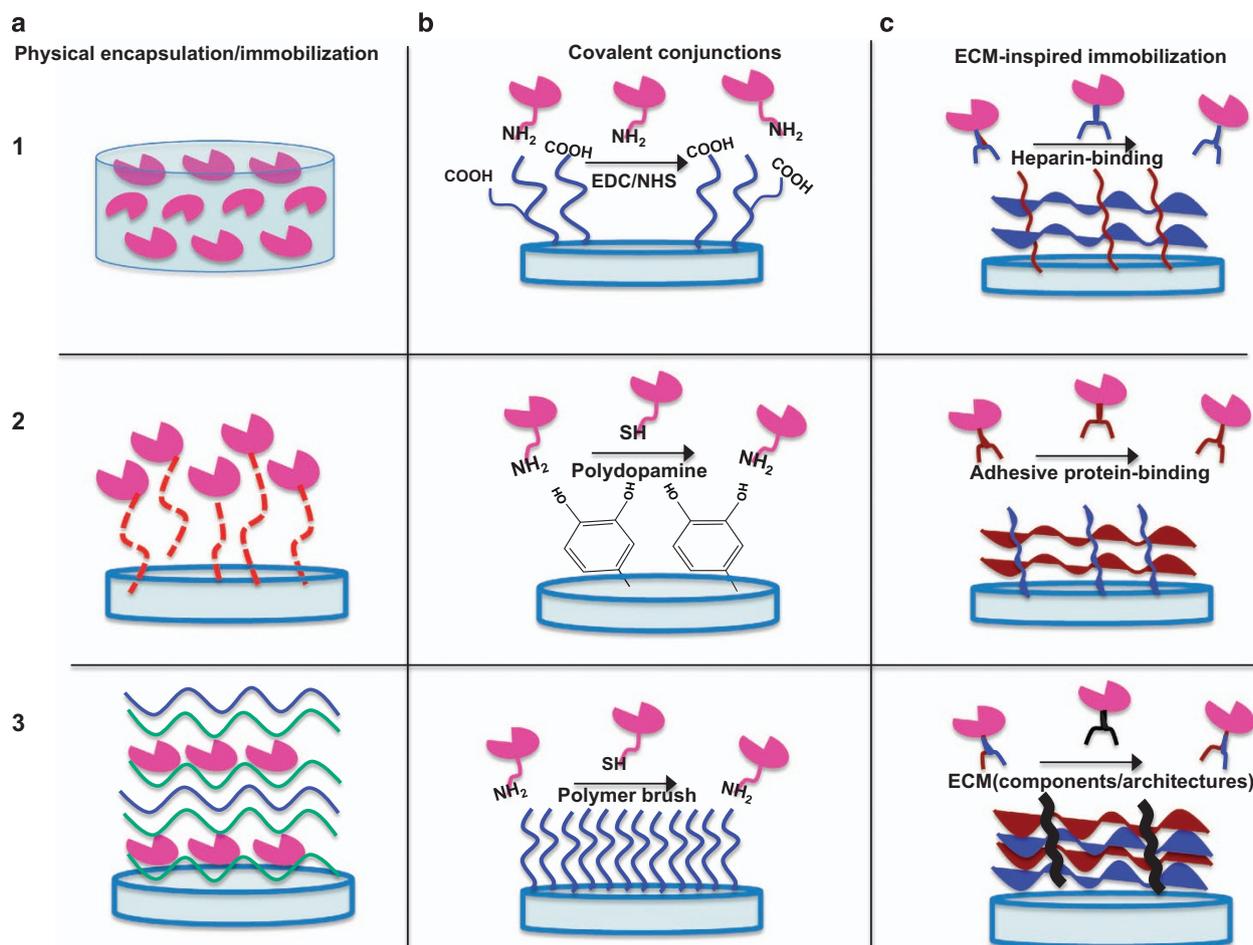Received 15 March 2017; revised 13 June 2017; accepted 10 July 2017

Figure 1 The direct approaches for the immobilization/encapsulation of growth factors (GFs) to biomaterials. (**a**) Physical immobilization techniques. (**b**) Nonselective covalent immobilization of GFs through their functional residues. (**c**) Extracellular matrix (ECM)-inspired immobilization reactions used for the orientation of GFs on the surfaces of biomaterials.

and peripheral applications, raising serious concerns for its use in bone engineering.[16] In addition to serious side effects, the lack of cost-effective methods for producing recombinant GFs at vastly supra-physiological levels is another major obstacle that may limit their biomedical applications.

Advanced drug delivery systems in tissue engineering

Collectively, the safety and cost-effectiveness issues of GFs mainly derive from their initial burst release kinetics and rapid clearance at injured sites when they are administered without appropriate spatio-temporal control. The short half-lives of the GFs, their relatively large size and their potential toxicity at high systemic doses suggest that conventional delivery techniques are not well suited to the clinical administration of GFs. To address these limitations, more sophisti-cated delivery systems that allow for controlled, precise, sustained, and localized release of these proteins have been developed, enabling optimal doses and spatial/temporal gradients in localized sites for effective tissue regeneration.[17] Recently, nano-delivery systems have been developed that are capable of providing an artificial ECM for cell attachment and penetration while maintaining a 3D network on which tissue regeneration can be facilitated.[18,19] In this review, we will focus on the most important aspects of these delivery mechanisms. First, we will discuss how GFs can be grafted onto appropriate biomaterials using advanced techniques, namely, directive

immobilization. Second, we will highlight examples of biodegradable NPs, particularly synthetic polymers, albumin, polysaccharides, lipids, mesoporous silica-based nanocarriers, polymeric capsules and other nanocarriers for GF loading and release. Third, we will present examples of functionalized GF-encapsulated NP scaffolds and discuss their use in tissue regeneration enhancement in recent years. Finally, we will provide perspectives on rational design, which pools resources to accelerate the production of clinically available devices for regenerative engineering.

IMMOBILIZATION AND RELEASE OF GFS IN DIRECT APPROACHES FOR BIOMEDICAL APPLICATIONS

For direct GF delivery, three distinct strategies have been pursued in the past few years for biomaterial presentation of GFs in tissue engineering: physical, covalent, and bioaffinity GF immobilization.[20] There are three types of direct binding of GFs in the carrier matrix, as summarized in (Figure 1). Table 1 presents a detailed description of some of the most relevant studies on direct factor delivery systems for biomedical applications.

Physical encapsulation/immobilization of GFs

Physical encapsulation of GFs in the delivery system. The simplest method to encapsulate GFs into a 3D polymer matrix is to mix the factors within the polymers before their gelation or solidification

Table 1 Direct approaches for controlled release of GFs on various substrates for biomedical applications

Substrates	GFs	Immobilization approaches	Biological effect	Biomedical applications	Reference
PLGA scaffolds	VEGF	Physical encapsulation	The VEGF released from the polymer scaffolds was over 70% active up to 12 days	Bone regeneration	21
Alginate-sulfate/alginate scaffolds	VEGF, PDGF, TGF-β1	Physical encapsulation	The triple factor-bound alginate-based porous scaffolds promoted vascularization in vivo	Vascularization	22
Gelatin hydrogel	bFGF	Physical encapsulation	The bFGF loaded hydrogel has stronger regenerative effects on acute vocal fold scarring than that of direct injection of bFGF	Vocal fold regeneration	125
Diblock copolymer matrix	Hepatocy-te growth factor (HGF)	Physical adsorption	The controlled HGF release promoted ECM synthesis and vocal fold regeneration	Vocal fold regeneration	126
PLA porous films	NGF	Encapsulation	This novel strategy significant promoted guided neurite extension from PC12 cells	Peripheral nerve regeneration	127
HA, TCP and neutralized glass ceramic	BMP-2, bFGF	Physical immobilization	Both GFs lose their biological activity after their initial burst on the surfaces of these inorganic carriers in vitro	Bone regeneration	23
Polydioxanone fiber	VEGF	Physical immobilization	VEGF treatment promoted new blood vessels and connective tissue formation	Pulp regeneration	128
Glass slides	BMP-2	LbL self-assembly	The BMP-2 being trapped in the LbL films and remaining their bioactive for more than 10 days	Bone regeneration	129
TCP/HAP granules	BMP-2	LbL self-assembly	LbL films-coated organic granules sequestered significant amounts of BMP-2, and enhanced the osteoinductivity of scaffolds	Bone regeneration	130
Anodized titanium	BMP-2, bFGF	LbL self-assembly	LbL films were capable of sustained release of both GFs over 25 days, and increased bone growth	Bone regeneration	131
Polycaprolactone/β-TCP scaffolds	BMP-2, VEGF	LbL self-assembly	LbL films that sequester of BMP-2 VEGF in different ratios in degradable films	Bone regeneration	28
Gelatin based coatings	Nerve growth factor (NGF)	LbL self-assembly	The coatings can be applied to neural electrodes for sustained delivery of NGF to increase neuron density	Alzheimer's disease treatment	132
PLGA scaffolds	VEGF	Carbodiimide crosslinking	Immobilized VEGF enhanced endothelial cell proliferation	Angiogenesis	133
Fibrin microthreads	HGF	Carbodiimide crosslinking	Myoblast proliferation increased significantly on stiffer, crosslinked, matrix, regardless of the amount of HGF	Skeletal muscle regeneration	134
Polyethylene terephthatalate (PET) surfaces	BMP-2	Carbodiimide crosslinking	BMPs grafted to PET surface promoted osteogenic differentiation of pre-osteoblastic cells	Bone regeneration	135
3D printed polycaprolactone (PCL) scaffolds	BMP-2	Mussel-inspired chemistry	This grafting realized sustained release of BMP-2 and enhanced cell proliferation and osteoconductivity of the scaffolds	Bone regeneration	136
Titanium surfaces	BMP-2	Mussel-inspired chemistry	The immobilization of BMP-2 induced stem cell to osteoblast and mineralization on titanium surfaces	Bone regenerationi	137
Collagen-GAG (CG) matrix	BMP-2, PDGF	Benzophenone (BP) photolitho-Graphy	Both factors could be covalently grafted to the CG matrix in defined patterns	Regenerative medicine application	138
Gelatin-based hydrogel	VEGF	Covalent immobilization	The gelatin-based drug-releasing hydrogel can induce capillary-like tube formation and axonal	Peripheral nerve regeneration	139
Chitosan collagen hydrogels	TGF-β	Covalent conjunction	The biofunctional hydrogel with controlled release of GF promoted chondrogenesis.	Cartilage regeneration	140
Fibrin matrices	PDGF	Covalent immobilization	PDGF conjugated fibrin matrix effectively increased tissue perfusion and induced the growth of a mature neovasculature	Ischemic tissue regeneration	141
Titanium surfaces	BMP-2	Reductive amination reaction	Immobilized BMP-2 at a surface density (>50 ng/cm2) significantly promoted the osteoblast functions	Bone regeneration	38
PLGA microspheres	BMP-2	Bioaffinity tethering	BMP-2-immobilized PLGA microspheres significantly enhanced ALP activity, calcium deposition of MG63 cells	Bone regeneration	142
Synthetic polymer matrix	FGF	Bioaffinity interaction	The EGF release promoted epithelization, collagen deposition, and granulation tissue formation.	Dermal regeneration	143
Thermoresposi-ve surfaces	Epidermal growth factor (EGF)	Affinity interaction	Heparin-decorated thermoresponsive surfaces facilitated the effective binding of EGF, which enhanced the functions of cultured hepatocytes	Liver disease treatments	47
ECM proteins	VEGF, PDGF TGF-β, FGF	Bioaffinity interaction	ECM would significantly enhance GF capacity to induce wound healing and bone repair	Wound healing  and bone regeneration	144
Hyaluronic acid hydrogel	BMP-2	Affinity interaction	BMP-2 immobilized hydrogel promoted formation of ectopic bone with better production of collagen fibers compared to delivering the BMP-2 in non-functionalized hydrogel	Bone regeneration	47
Titanium surfaces	BMP-2	ECM-inspired approach	The ECM-inspired microarchitecture and its immobilized BMP-2 with both cell affinitive and high GF loading capacity synergistically enhance the activity and osteogenetic differentiation of stem cells	Bone regeneration	50

Abbreviation: GFs, growth factors.

(Figure 1a1). This approach has the advantages that the optimized properties of scaffolds are not substantially affected by the presence of factors, and the bioactivity of GFs can be retained during their incorporation. For example, Murphy *et al.*[21] have developed VEGF-encapsulated poly(lactic-co-glycolic acid) (PLGA) scaffolds by introducing the GF into a gas foaming/particulate leaching process and showing that the VEGF released from the polymer scaffolds was over 70% active for up to 12 days. Freeman *et al.*[22] have synthesized alginate-sulfate/alginate scaffolds that incorporated VEGF, PDGF-BB and TGF-β1, which were bound to alginate-sulfate with an affinity similar to heparin, thus enabling the sequential delivery of three distinct GFs. The results demonstrated that vascularization within the triple factor-bound, alginate-based porous scaffolds was superior to that found in scaffolds consisting only of a single growth factor following subcutaneous implantation in rats. To our knowledge, such direct GF encapsulation can be achieved by impregnation, diffusion of the pre-formed matrix in very small volumes of GF solutions or immersion of the matrix in a GF solution. However, this incorporation approach is inefficient since only a small fraction of GFs can be bound in this system; in addition, the GFs have unpredictable release profiles.

Absorption of GFs on the surfaces of the matrix. Over the last two decades, the physical immobilization of GFs onto various matrices has attracted much attention because it is easy to accomplish under mild conditions at room temperature; however, this approach can frequently result in the inefficient retention of stable soluble protein and poorly controlled delivery (Figure 1a2). When Ziegler *et al.*[23] directly immobilized BMP-2 and bFGF onto synthetic bone implants (HA, TCP and neutralized glass ceramic) utilizing the physical approach, they found that both GFs lost their biological activity after their initial burst on the surfaces of these inorganic carriers *in vitro*. The BMP-2 released from the inorganic carriers during the initial period led to a significant increase in the activity of ALP and the proliferation of human primary osteogenic sarcoma cells and these effects were attributed to the unbound bioactive protein. However, cells cultured in protein released from carriers after 24 h of adsorption did not increase the expression of ALP or other osteogenic activity. These studies underscore the need for more advanced methods that strengthen the immobilization of GFs to various implant surfaces or their encapsulation within carriers for long-term retention of osteoinductive activity.

Reyes *et al.*[24] developed brushite cement and brushite/PLGA composite systems that controlled the release rate of PDGF, TGF-β1 and VEGF to enhance bone regeneration. PDGF and TGF-β1 were directly adsorbed onto pure brushite cement implants, whereas VEGF pre-loaded PLGA microspheres were incorporated in brushite scaffolds. *In vivo* results indicated that PDGF and TGF-β1 were delivered more rapidly from brushite cement implanted in intramedullary defects in rabbit femurs than VEGF. Approximately 40% of the PDGF and TGF-β1 was released in the first 24 h with release rates of both GFs dropping to ~ 5.5% per day during the next 6 days. A total release of 90% of the GF was achieved after three weeks. In contrast, VEGF pre-encapsulated in PLGA microspheres and incorporated in brushite implants showed a more controlled release profile. A steady release of 7% per day was detected within the first week, and the release dropped by 1.2% per day during the following three weeks. A total release of almost 80% of the VEGF was accomplished within two months. Notably, the three types of observed GF release profiles showed a high correlation between *in vivo* and *in vitro* conditions. Therefore, the incorporation of GF-loaded polymeric microspheres in scaffolds could

enable a sustained and more stable release of GFs from scaffolds *in vivo* compared to other configurations.

Layer by layer self-assembly. Although the direct adsorption of GFs on substrates was the first approach for the delivery of multiple GFs, it proved to be difficult in regulating the release rates of multiple GFs in a programmable manner (Figure 1a3). Therefore, alternative strategies can be employed to address this situation by incorporating multiple strata from the top to the bottom of the structure, such as bilayered or tetralayered coatings, for the spatial and temporal control of the delivery of multiple drugs within the healing tissue. The method, known as the Layer-by-Layer (LbL) approach, frequently relies on electrostatic interactions between oppositely charged polyelectrolytes and GFs to deposit functional polymer coatings on surfaces of varying composition and shape.[25,26] Particularly relevant to protein delivery, the LbL approach takes advantage of facile aqueous baths for the construction of functional coatings; these baths have the potential to preserve fragile, soluble protein activity compared to typical methods that utilize harsh organic solvents for the fabrication of protein delivery systems.

The use of multi-layer biotechnology allows the design of GF vehicles with optimal release rates to the mimic GF profiles normally present during various types of tissue regeneration. For example, Hammond *et al.*[27] first prepared tetralayer architectures by the LbL technique to capture and deliver GFs on the microgram-scale for a multi-week period, which resulted in defectively stimulating host cell behaviors *in vivo*. The LbL architecture was created on a 3D scaffold (on glass surface) that was repeatedly dipped (100 times) with tetralayer units consisting of (1) poly(β-aminoester) (positively charged polymer), (2) chondroitin sulfate (negatively charged polysaccharide), (3) BMP-2 (positively charged protein) and (4) chondroitin sulfate. *In vitro* results showed that 80% of the BMP-2 was released over 2 days (with minimal burst release) and that a sustained release of the remaining 20% of encapsulated GFs occurred over a period of approximately 2 weeks. In a follow up study, LbL films were constructed with tunable BMP-2 and VEGF delivery in degradable {poly(β-amino ester)/polyanion/growth factor/polyanion} tetralayer repeat films, where the polyanion used for BMP-2 and VEGF adsorption are poly(acrylic acid) and chondroitin sulfate.[28] No burst release of either GF was found in this system; the release of BMP-2 was sustained over a period of 2 weeks, whereas VEGF was discharged from the layers only during the first 8 days. *In vivo* results indicated that the mineral density of ectopic bone induced by BMP-2/VEGF LbL films was ~ 33% higher than that of single GF entrapped layers. Another study recently utilized laponite clay as interlayer barriers in LbL films, which acted as a dual-purpose biomimetic microenvironment that sequentially released an antibiotic, followed by the release of BMP-2 for orthopedic implant applications.[29] These studies suggested that the LbL approach could serve as a promising delivery system for the precisely controlled release of multiple drugs by employing components with different hydrolytic degradation rates.

Covalent conjunctions
Covalent immobilization (Figure 1b) of GFs to biomaterials can eliminate the possibility of initial burst release and has emerged as a promising approach for improving the stability and persistence of GFs when delivered into cells or tissues.[30] Covalent immobilization is warranted when the binding biomolecules are incapable of adsorption on the substrate surfaces or when the GF adsorbs by weak physisorption forces due to improper orientation and conformation. By contrast, covalently immobilized GFs most often involve chemical/

enzymatic reactions between proteins and functionalized surfaces, offering important control of the amount, orientation, retention, and distribution of GFs in solid matrices, achieving GF delivery that is both localized and sustained. When the GF is chemically bound to the biomaterials, its desorption rate is controlled by enzymatic or hydrolytic cleavage of the chemical bond that grafts it to the matrix, facilitating the establishment of multiple release models for GFs, such as linear, pulsatile, or sequential protein release profiles. In addition, covalent conjunction can prolong GF availability and reduce the amount of protein required, thereby potentially increasing the therapeutic efficiency of biomaterials for enhanced tissues regeneration at reduced costs.

Carbodiimide coupling immobilization. Carbodiimide coupling chemistry presents one of the most popular approaches for covalently grafting GFs to various substrates due to its simplicity, high conjugation ratio, low cost and mild reaction conditions (Figure 1b1).[31] Among these coupling reagents, the prevalent crosslinking molecule is similar to 1-ethyl-3-(3-dimethylamminopropyl) carbodiimide (EDC) for bioconjugation purposes. EDC mediates the conjugation reaction between carboxylic acid groups and amino groups, producing the formation of stable intermolecular amide bonds in aqueous environments at physiological pH.[32] Carbodiimide coupling reactions may engage the amino groups in the lysine residues and N-terminus of GFs. However, proteins may lose their bioactivity during immobilization due to damage in the active sites and bioactive functional groups from the chemical binding method.

Mussel-inspired bioconjuctions. Observation of the natural phenomenon of the adhesion of mussels to a variety of material surfaces has led to an important advance in the area of biomedical science, specifically in the development of new drug delivery systems (Figure 1b2). Scientists have long noted that mussels can strongly attach to a diverse variety of substrates due to the formation of 3,4-dihydroxy-L-phenylalanine (DOPA), which is the major active site of the extraordinarily robust adhesion near the plaque-material interface.[33,34] On the basis of these investigations, Messersmith and his group found that dopamine can be easily deposited on virtually all types of organic and inorganic substrates to form a polydopamine layer with a molecular structure highly similar to that of DOPA.[35] The primary advantages of polydopamine, as seen with mussels, are its robust and strong adhesion properties to virtually all types of surfaces in mild conditions, regardless of the surface's specific chemical properties, and the formation of reversible and noncovalent interlayers with high affinity and stability.[36]

Previous studies utilizing the reactivity of polydopamine films to immobilize GFs onto a large number of substrates provide a promising approach for the effective loading and sustained release of GFs, which ultimately improves tissue repair and implant integration. Polydopamine mainly tethered GFs to substrates through covalent bonds between the amino groups of GFs and the quinone groups (the catechol groups in dopamine can be oxidized into quinone groups) of polydopamine via Michael-type addition reactions and Schiff-base reactions. For example, Cho et al.[37] developed polydopamine decorated poly(l-lactide) nanofibers for BMP-2 delivery, and surface chemical analysis confirmed that the successful tethering of BMP-2 was mediated by polydopamine films and that ~90% of the BMP-2 was stably attached to the polymer surfaces for >28 days. The results suggest that this polydopamine modification method can be a useful tool for grafting biomolecules onto substrates, leading to improved bioactivity and accelerated tissue regeneration.

Other chemical coupling. Several other types of reactive groups with different chemistries have also been used to covalently attach GFs to biocompatible substrates. For instance, the surfaces of titanium alloy substrates were first functionalized with oxidized dextran, and BMP-2 was covalently linked to dextran-modified surfaces through a chemical conjugation between the amino-acid groups of proteins and the aldehyde groups of oxidized polysaccharides.[38] Similarly, Leipzig et al.[39] immobilized PDGF-AA or BMP-2 to a photopolymerizable methacrylic chitosan polymer through a streptavidin linker to guide neural stem cell differentiation *in vivo*. Another example demonstrated the use of parylene coatings in the sustainable immobilization of GFs via a maleimide–thiol coupling reaction under mild conditions.[40] The site-specific approach defined in this study produced preferential bonds without damaging the tertiary structure of the GFs or compromising their biological activities.

The use of flexible, thin polymer brushes to covalently immobilize GFs has received considerable attention because their controlled polymer densities and nanometer size can facilitate better biological responses (Figure 1b3). Psarra et al.[41] recently synthesized a poly (acrylic acid) polymer brush with a high grafting density to bind hepatocyte growth factor (HGF) and bFGF with a physisorption or chemisorption approach. Both GFs showed high loading efficiencies onto poly(acrylic acid) brushes based on their initial concentrations. The results indicated that the BMP-2-conjugated polymer brush could enhance adhesion and differentiation in terms of the alkaline phosphatase activity of MC3T3 cells on Ti surfaces. Therefore, polymer brushes biofunctionalized with GFs can be a promising method for creating enhanced cellular activity. In summary, while a variety of covalent conjugation approaches have been developed, a potential disadvantage of these methods is that GFs may lose a part or most of their bioactivity after covalent coupling.

ECM-inspired GF delivery systems

Heparin-based binding approach. Taking inspiration from the natural interactions between ECM and GFs, bioaffinity tethering approaches (Figure 1c1) have been designed for optimal delivery systems in a highly spatio-temporal controlled manner, further mimicking ECM functions. The ECM is a highly dynamic microenvironment that regulates multiple cellular processes; in addition, it acts as a reservoir for GFs due to their ability to bind multiple molecules with high affinity. In particular, many growth factors, such as BMP-2, BMP-7, VEGF, PDG and FGF-2, interact specifically with the heparin sulfate of the ECM.[5,42] Therefore, a number of biomaterials have been decorated with heparin or heparin sulfate-mimetic molecules, sequestering the heparin-binding ability of GFs to improve their delivery. For example, Jha et al.[43] developed a series of heparin-functionalized hyaluronic acid-based hydrogels and investigated the effect of heparin molecular weight (MW) and its relative concentration on the loading efficiency and retention behavior of TGFβ1. The results demonstrated that high MW heparin facilitated TGFβ1 loading and retention and exhibited the slowest release kinetics, which was primarily due to its higher affinity for TGFβ1 compared to low MW heparin. More importantly, GF binding to high MW heparin decorated hydrogels and induced more robust differentiation of stem cells into endothelial cells, which further stimulated vascular-like network formation within the hydrogels.

Adhesive protein-based binding. On the other hand, several GF-binding motifs are present within ECM adhesive proteins, such as collagen, fibronectin, fibrinogen and vitronectin (Figure 1c2).[44–46] In an ectopic bone defect model in the rat, hyaluronic acid hydrogel

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functionalized with the fibronectin fragment was designed for the delivery of BMP-2, and it promoted the formation of twice as much ectopic bone with a better production of collagen fibers compared to BMP-2 delivered via non-functionalized hyaluronic acid hydrogel.[47] In a critical-sized calvarial defect model in the rat, dual-delivery of BMP-2 and PDGF-BB with the adhesive fibronectin fragment was able to accelerate bone formation at low doses, whereas the growth factors delivered without the adhesive proteins produced no significant effect.[48] The two aspects of retained engineered GFs, along with natural biological adhesive proteins, may produce synergistic effects in nervous system reconstruction and repair.

ECM components and hierarchical structure-based binding. In addition to the bioaffinity of ECM components, the architecture of ECM microenvironments also plays a pivotal role in directly modulating GF activity and cell fate during tissue repair (Figure 1c3). Eap *et al.*[49] constructed biomimetic ECM nanostructures for the immobilization of BMP-2 by assembling polysaccharides (chitosan) on collagen nanofibers. Both *in vitro* and *in vivo* evaluations confirmed that this sophisticated strategy enhanced the safety and efficacy of the therapeutic matrix in terms of bone formation compared to the more simplistic, traditional approaches based on immersion of the substrates with GFs. To comprehensively simulate ECM functions, our group has developed bioadhesive microporous architectures that mimic the functions of ECM through self-assembling polydopamine microcapsules and chitosan, which not only enhance osteoblast functions but also but also facilitate BMP-2 immobilization and provide sustained release in local sites (Figure 2).[50] More importantly, the bioadhesive microarchitecture and its immobilized BMP-2 with both cell affinitive and high GF loading capacity synergistically enhanced the activity and osteogenetic differentiation of stem cells. Once bound to the ECM, the bioactivity and release kinetics of immobilized GFs are dependent on their binding-affinity, underlying micro/nano structures, and the use of multiple immobilization approaches.

NANOCARRIERS FOR GF ENCAPSULATION AND RELEASE FOR BIOMEDICAL APPLICATIONS

Synthetic polymer NPs

Biocompatible NPs have received considerable attention for drug delivery due to their small dimensions, high surface area to volume ratio, high drug loading efficiency, and ability to quickly respond to environmental stimuli, such as temperature, pH, magnetic field or ultrasound (Table 2; Figure 3a). Synthetic polymers include polylactide (PLA), polyglycolide (PGA) and PLGA copolymers, which are well-suited for NP fabrication to control drug release because of their established safety profile, long history of clinical applications, and well-understood degradation pattern. Among the different synthetic polymers developed to fabricate polymeric NPs, PLGA is one of the most successfully used synthetic polymers due to the following desirable properties: (1) FDA approval for clinical use in humans as a drug delivery system, (2) well-described formulations and methods of production adapted to various types of drugs, ranging from small molecules to macromolecules and (3) ability to protect drugs from degradation and the possibility of sustained release.[51] Various methods include emulsion-solvent evaporation (single and double emulsion), phase separation, emulsification-solvent diffusion, solvent displacement, dialysis, spray drying and self-assembly, which have been extensively applied in the successful synthesis of PLGA NPs.[52,53] The most common method used for the preparation of PLGA NPs is the emulsification-solvent evaporation technique. However, this

method is primarily used for the encapsulation of hydrophobic drugs. A modification of this procedure called the double or multiple emulsion technique has become the favored protocol for encapsulating hydrophilic compounds and proteins.[54]

In addition, GFs directly adsorbed on hydrogels or scaffolds tend to exhibit rapid release in the short-term due to the open pore architecture and exposure of GFs to the bio environment, which weaken the bioactivity of GFs. As an indirect approach, GF-loaded NPs provide an effective approach to controlled GF release from scaffolds (Table 3).[55] Shoichet *et al.*[56] developed a novel delivery system that encapsulated EGF in PLGA NPs, followed by EGF polymeric particles dispersed in a hyaluronan methylcellulose (HAMC) hydrogel. The PLGA NPs effectively controlled EGF release within the first week and led to tissue repair in a mouse stroke model. Moreover, it has been reported that a poly(ether)urethane/polydimethylsiloxane/fibrin-based scaffold containing PLGA NPs loaded with VEGF and bFGF enhanced granulation tissue formation and maturity as well as collagen deposition when compared to other groups.[57] One problem concerning PLGA is its poor affinity under physiological conditions, resulting in inadequate protein retention. Employing natural polymers or chemical crosslinking methods can enhance drug-loading ratios. In this delivery system, bFGF and VEGF were first encapsulated in PLGA-based NPs by a modified solvent diffusion method using heparin and human serum albumin as stabilizing agents.

Protein-based NPs for GF delivery

Albumin NPs have received considerable attention as drug delivery vehicles because they have several advantages (Figure 3b). First, the albumin molecule contains multiple drug binding sites, which leads to the high binding capacity of various drugs for albumin NPs. Specifically, due to their high content of charged amino acids (for example, lysine, arginine, glutamate and aspartate), albumin-based NPs are capable of loading either positively or negatively charged molecules by electrostatic forces without the addition of other accessory compounds.[58,59] Second, their surface properties are well tolerated by charged polymers, which broaden the application of albumin NPs in biomedical areas. Third, albumin-based NPs are biodegradable, easy to fabricate, and reproducible under mild conditions by desolvation (coacervation),[60] emulsification,[61] and self-assembly techniques.[62] It should be noted that desolvation is the most widely used method for the preparation of biomolecule-encapsulated albumin NPs.

In the coacervation process, albumin-based NPs are prepared by a continuous dropwise addition of ethanol to an albumin solution under continuous stirring until the solution becomes slightly turbid. During the addition of ethanol into the aqueous solution, albumin is phase separated due to its diminished water-solubility.[60] The BSA concentration, pH value of the aqueous solution, rate of ethanol addition, and ethanol to water ratio (the final volume ratio of ethanol added to the starting aqueous BSA solution) play key roles in determining the particle size and polydispersity of the resulting NPs.[63] Since morphologically formed albumin particles are not sufficiently stabilized after dispersion in water, conventional albumin NPs prepared by this method often employ glutaraldehyde (GA) to stabilize them in which the amino moieties in lysine residues and arginine moieties in the guanidino chains of albumin are solidified by a covalent reaction with the aldehyde-group of glutaraldehyde.[64]

However, glutaraldehyde reacts with the free amines on albumin and possibly with any amines on the loaded biomolecules, which could adversely affect drug bioactivity. The potential toxicity of
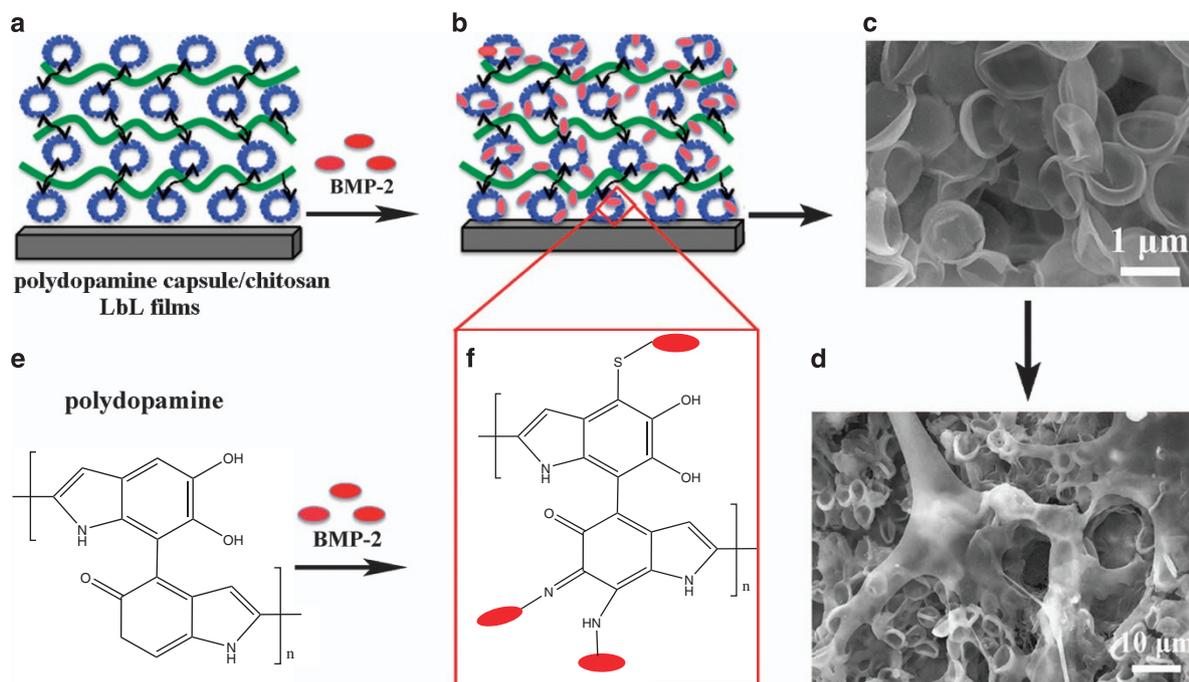
Figure 2 Schematic illustration of formation of polydopamine capsules/chitosan LbL films and subsequent loading and release of BMP-2 for enhanced cell adhesion. In detail, polydopamine capsules and chitosan self-assembly (**a**), BMP-2-loaded LbL films (**b**) Scanning electron microscopic (SEM) image shows polydopamine capsule-based architectures have highly interconnected micro-pore structures throughout the surfaces (**c**), BMSCs adhesion on LbL porous films (**d**), the structure of polydopamine molecules (**e**), the reaction mechanisms between polydopamine and BMP-2 in this system (free amino groups and thiol groups of BMP-2 and the catechol groups of polydopamine react with each other to form Schiff-base and Michael-type addition covalent bonds) (**f**).

glutaraldehyde is also a problem for *in vivo* delivery in this approach. As an alternative to glutaraldehyde, coating the protein NPs with cationic polymers provides a superior approach to stabilizing the particles and protecting the bioactivity of the encapsulated drug against enzymatic degradation.[65] BMP-2 could be encapsulated in branched polyethylenimine (PEI) coated-BSA NPs by a conversion process, which resulted in an encapsulation efficiency that was typically > 90% in all NPs.[66] *In vitro* release studies showed that the PEI concentration used in this delivery system efficiently controlled the release of BMP-2 in a gradual and sustained release pattern over a 10-day period. Furthermore, the higher PEI concentration realized a slower release of BMP-2 with a minimal burst release. It is hypothesized that the PEI coatings physically reduced BMP-2 diffusion through the NP surfaces; PEI stabilized the particles and protected the NP structure from disintegration. However, the osteoinductive activity and cytotoxicity of BMP-2 encapsulated in PEI coated-BSA NPs was a significant issue, and the PEI coatings would likely decrease BMP-2 activity. In addition, biocompatible polymers, such as PEI-polyethylene glycol (PEG)[67] PEGylated multilayers[68] and poly-L-lysine (PLL),[65,69] are widely used to coat BSA NPs to effectively reduce their toxicity and enhance the bioactivity of their encapsulated drugs.

Polysaccharide-coated BSA NPs have been extensively employed as biomolecule reservoirs to construct biomimetic microenvironments for bone regeneration. Our previous publication demonstrated that chitosan and chitosan/oxidized alginate-coated BSA NPs were assembled on Ti surfaces to form biomimetic nanostructures (Figure 4).[70] The nanostructured architectures exhibited a sustained release of BMP-2 and vancomycin over a long period, which was ascribed to the polysaccharide coatings that stabilize the BSA NPs in their bioenvironments. Bone marrow stromal cell (BMSC) culture tests indicated that the BSA NP-based architectures intrinsically facilitate the attachment, proliferation, and differentiation of BMSCs. Incorporating BMP-2 into the nanostructured architectures significantly promotes the osteogenetic differentiation of BMSCs, which reveals the synergistic effects of nanostructured architectures and BMP-2 on cell behaviors. Li *et al.*[71] developed a new electrospun nanofiber scaffold that incorporated chitosan-coated BSA NPs for the controlled dual delivery of BMP-2 and dexamethasone (DEX). BMP-2-loaded BSA NPs were prepared in aqueous solution by the conversation technique and then stabilized by a chitosan layer though electrostatic interaction. Both the BMP-2-encapsulated BSA NPs and DEX were then incorporated into poly(ε-caprolactone)-poly(ethylene glycol) nanofibers through an electrospinning process. The dual-drug-loaded nanofiber scaffold showed a sequential release model, which promoted calvarial defect repair in bone tissue engineering. Our research also confirmed that chitosan-decorated BMP-2-encapsulated (BSA) NPs immobilized on graphene oxide/chitosan scaffolds significantly enhanced bone marrow stromal cell functions and induced bone tissue regeneration.[72]

Polysaccharide-based NPs

Polysaccharides are comprised of multiple monosaccharide units joined together by glycosidic linkages (Figure 3c).[73] Polysaccharides are the most attractive and promising natural polymers for the preparation of drug-encapsulated NPs due to their abundance in nature, low toxicity, high stability, low cost, biocompatibility, and presence of various functional groups on molecular chains. In particular, hydrophilic groups, such as amino, hydroxyl, or carboxyl groups, endow polysaccharides with multiple charges in aqueous solution. Among the widely investigated polysaccharides, chitosan is the most used cationic polymer, whereas alginate, heparin, chondroitin sulfate and hyaluronic acid are negatively charged.

Table 2 Select synthetic, natural and inorganic nanocarriers for controlled growth factor delivery in biomedical applications

NPs	GFs	Method	Biological effect	Biomedical applications	Reference
Synthetic polymer NPs	VEGF, PDGF, bFGF	Double emulsion	The system allowed spatiotemporal control of GF release, which promoted angiogenesis	Cardiovascular regeneration	70
Chitosan coated-BSA NPs	BMP-2	Modified desolvation	The chitosan coatings improved the stability of BSA NPs and realize the sustained release of BMP-2 for a long term	Bone regeneraiton	71
Heparin/chitosan/poly(γ-glutamic acid) NPs	FGF-2	Polyelectrolyte complex	Approximately 85% of the bound FGF-2 was released from the polysaccharide-based NPs within 48 h. After 48 h, there was very little GF release up to 72 h	Ischemic tissue regeneration	72
Biopolymer- coated liposomes	FGF-2	Modified emulsification-ultrasonication	The entrapment efficiency was 72 %, and the system realized the sustained release of EGF	Regenrative medicine application	73
Nanostructured lipid carriers (NLC)	EGF	Emulsification ultrasonication based method	The EGF-NLC promoted wound healing associated with number of arranged microvasculature, fibroblast functions, collagen deposition in full-thickness excisional model	Wound healing	74
Inorganic NPs	EGF	Copre-cipitation	The system showed receptor-mediated targeted delivery and high diagnostic potency	Therapy of brain tumor	75
Chitosan- functionalized MSNs	BMP-2	Templated sol-gel method	The initial burst release and following sustained release of BMP-2 is favorable to promote osteoblast differentiation and ectopic bone formation	Bone regeneration	76
Protein nanocapsules	VEGF	Aqueous assembly	The sustained released VEGF from nanocapsules resulted in enhanced vascular formation in vitro and in vivo	Angiogenesis	77
PLGA nanocapsules	BMP-2	Free-radical polymerization	Compared to the direct use of native BMP-2, sustained release of BMP-2 from the nanocarriers successfully enhanced bone formation with better bone quality and reduced the side effects	Bone regeneraiton	78

Charged polysaccharides can form physically crosslinked NPs in the presence of oppositely charged crosslinkers, which are either small molecules or polymers. Since ionically crosslinked NPs are readily prepared in mild conditions without the use of organic solvents at room temperature, they are promising agents for encapsulating more fragile drugs (for example, peptides and proteins) without significant loss of therapeutic function. Among polysaccharides, chitosan has been the most extensively investigated IC-NP as a carrier of small molecular drugs, proteins, and plasmid DNA, and it is prepared in the presence of the polyvalent anion tripolyphosphate (TPP). For example, Rajam et al.[74] developed EGF- and FGF-incorporated chitosan/TPP NPs by the ionic gelation method for the sustained release of dual GFs. The drug loading capacity and loading efficiency of EGF or FGF in chitosan/TPP NPs were approximately 30 ± 2.5 ng mg^{-1} of particles and $91 \pm 1.5\%$, respectively. The cumulative release of EGF and FGF from the delivery systems was approximately 83% and 84%, respectively, on day 35, without burst release in the initial stage, which greatly improved the attachment and proliferation of fibroblasts. Furthermore, the chitosan-based NP delivery system prepared by the ionic gelation method can also accommodate the multiple release model by chemical modifications. In another study by Butko et al.[75], bFGF was incorporated into chitosan/TPP and N-succinyl-chitosan/TPP NPs by ionotropic gelation, and both types of NPs exhibited a similar entrapment efficiency of bFGF (~60%). The N-succinyl-chitosan/TPP NPs presented a larger and faster release of bFGF (18%) for up to 7 days in comparison with chitosan/TPP NPs, which could be attributed to the higher swelling of N-succinyl-chitosan/TPP NPs at pH 7.4 in this study.

Recently, polysaccharide-based NPs prepared by a mild polyelectrolyte complex technique have received considerable attention for GF release due to their simple process and high affinity for GFs. Alonso and co-workers[76] have prepared hyaluronic acid/chitosan NPs for the multifunctional delivery of two pro-angiogenic growth factors: VEGF and PDGF-BB. The resultant NPs had an average size of 200 nm and were able to efficiently encapsulate both growth factors, reaching association values of 94% and 54% for VEGF and PDGF, respectively. In vitro release studies indicated that PDGF-BB was released from the polysaccharide-based NPs in a sustained pattern over 1 week, whereas VEGF was completely released within 1 day.

However, chitosan-based NPs have also shown some disadvantages for GF release and tissue regeneration. In particular, chitosan has poor solubility in physiological conditions due to its strong intermolecular hydrogen bonding, thereby greatly restricting its biomedical application. Consequently, Tan et al.[77] have prepared polysaccharide NPs utilizing the electrostatic interaction between N,N,N-trimethyl chitosan chloride (TMC) and low-molecular-weight heparin (LMWH) to achieve the sustained release of VEGF. In this delivery system, TMC has a higher solubility than that of chitosan in physiological conditions, which increases its applicability in tissue repair. In addition, LMWH can lead to less bleeding, and it exhibited a higher affinity for VEGF than that of normal heparin. These VEGF-loaded LMWH-TMC NPs showed a spherical morphology with an average size of 146.5 nm, and they were integrated in injectable nanofibrous hydrogel scaffolds in order to further decrease the burst release of VEGF at lower levels and increase the sustained release of VEGF in the longer term. The scaffolds significantly preserved the bioactivity of VEGF, thereby strongly promoting the proliferation and differentiation of adipose-derived stem cells for soft tissue engineering applications.

Heparin-based NPs have shown strong affinity and sustained release functions when coupled with various GFs, making them great candidates for emerging biomedical applications.[78] For example,
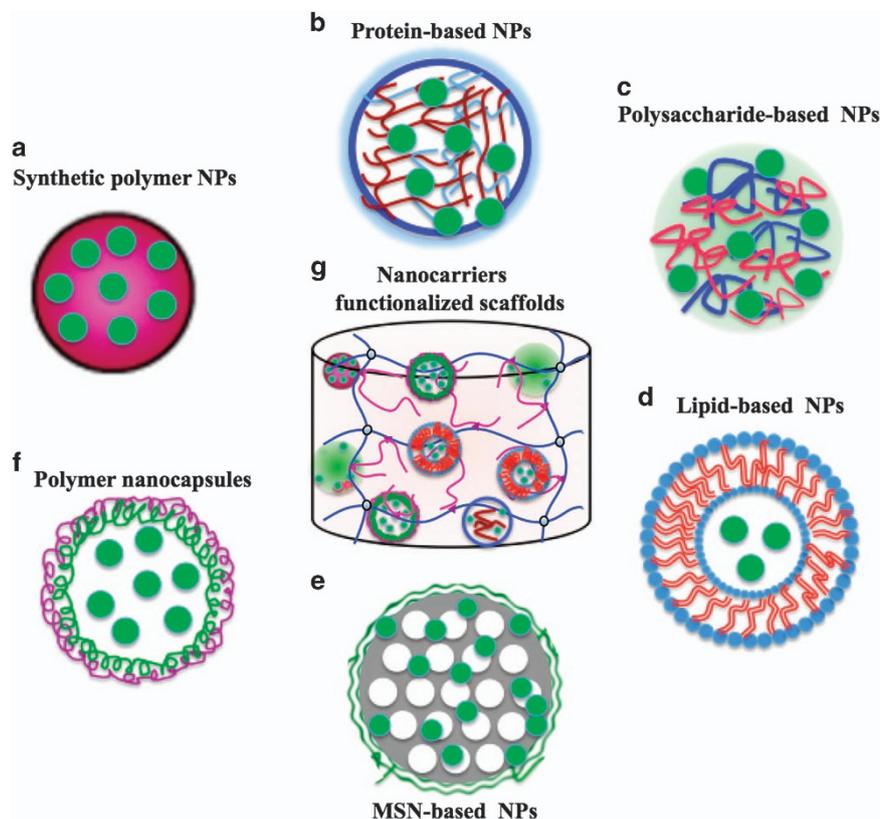
Figure 3 Multiple nanocarriers for growth factors (GFs) encapsulation and release (**a**–**f**), GFs encapsulated nanocarriers functionalized biomaterials (**g**).

Tang *et al.*[79] developed heparin-functionalized chitosan/poly(γ-glutamic acid) (γ-PGA) NPs by a simple polyelectrolyte self-assembly method for the controlled release of bFGF. The NPs were formed through electrostatic interaction of the cationic chitosan and anionic γ-PGA and heparin, and the loading efficiency of bFGF increased significantly with the increase of encapsulated heparin in the particles. In another study, the role of heparin-based nanocomplexes encapsulating two GFs in the enhancement of osteoblast function were investigated.[80] Approximately 80% of the PlGF-2 and 90% of the BMP-2 was stably retained in the nanocomplexes over a long period of time. *In vitro* biological assays indicated that the dual delivery of PlGF-2 and BMP-2 appears to have a synergistic effect on bone tissue regeneration in comparison to the delivery of either GF alone.

In addition, chondroitin sulfate is an anionic polysaccharide that is structurally similar to heparin, and it has a high binding affinity with positively charged GFs. In our previous study,[81] BMP-2-encapsulated chitosan/chondroitin sulfate NPs were prepared through a simple and mild polyelectrolyte complexation method without involving harsh organic solvents or high temperature (Figure 5). NPs in the 482–698 nm mean size range were prepared, and the loading efficiency of BMP-2 in the particles was ~91%. These NPs were physically immobilized on biphasic calcium phosphate (BCP) scaffolds for the sustained release of growth factors. The protein-encapsulated NP on functionalized scaffolds exhibited a sustained release of protein over 40 days, whereas protein that was directly adsorbed onto the BCP scaffolds showed a burst release rate in the initial period. The long-term sustained release of protein could be ascribed to the slow degradation of the polysaccharide NPs and the stable binding force between the NPs and the surfaces of scaffolds. BMP-2 encapsulated NPs on BCP scaffolds induce more ectopic bone formation than bare BCP scaffolds, which indicated the combination of a polysaccharide-based drug delivery system and porous scaffolds as a promising artificial bone substitute in clinical applications.

Lipid-based nanocarriers for controlled GF release
Lipid-based NPs were developed as advanced carriers for drug delivery due to their excellent amphiphilic properties, which originated from one or more phospholipid bilayers and facilitated their entrapment of lipophilic or hydrophilic drugs (Figure 3d).[82,83] Lipid-based NPs are generally regarded as biocompatible and non-toxic because they are prepared with phospholipids derived from mammalian cell membranes.[84] Three main configurations of lipid-based NPs are liposomes, solid lipid particles (SLN), and nanostructured lipid carriers (NLC).[85] Liposomes are closed carriers formed by bilayers of hydrated phospholipids that enclose an aqueous core, which endows liposomes with amphiphilic properties and the ability to trap both hydrophobic and hydrophilic drugs. Liposomes can protect the activity of biomolecules against a range of environmental conditions, including extreme pH, ionic strength and temperature. Therefore, several liposomes have been developed as EGF carriers for wound healing and bone repair.[86,87] Furthermore, magnetic liposomes with incorporated BMP-2 or TGF-β1 were prepared for enhanced bone or cartilage formation after topical injection.[88,89] The NPs had an average size of ~ 100 nm with an encapsulation efficiency of approximately 20% for BMP-2 and TGF-β1 in the liposomes. The results confirmed that both types of encapsulated GFs from the magnetic liposomes seemed to be cumulatively released at the target site over a prolonged period.

Nevertheless, liposomes have also shown several disadvantages, such as forming particle aggregates under aqueous conditions and spilling

Table 3 Controlled release of GFs from a scaffold/nanocarrier composite for tissue regeneration

NPs	GFs	Scaffolds	Biological effect	Biomedical applications	Reference
Heparin coated PLGA NPs	bFGF, VEGF	Fibrin based-scaffolds	The combined delivery system allows GFs to be maintained in the wound site and avoids the burst release of GFs in the initial phase	Wound healing	69
Polysaccharid-e coated BSA NPs	BMP-2	PCE nanofibers	The BMP-2-loaded nanofiber scaffold showed a sustained release behavior for long term, which promoted calvarial defect repair in bone tissue engineering	Bone regeneration	91
Sulfated-chitosan based NP	BMP-2,	Gelatin sponge	This system showed an initial burst release of BMP-2 in first 24 h and a slow release for 21 days to 77.8±3.6%. The BMP-2 release significantly enhanced peripheral vessel and new vessel formation	Bone regeneration	101
Liposome	EGF	Chitosan gel	EGF-encapsulated multilamellar vesicle liposomes were fabricated to preserve the activity of biomolecules, and the encapsulation efficiency of EGF in the liposomes was about 58%	Wound healing	102
MSNs	NGF	Collagen hydrogel	The MSN showed a high loading capacity and sustainable release of NGF for over a week. When the NGF-loaded MSN was further incorporated in a collagen gel, the novel delivery system promoted neuritogenesis while preserving the cell viability	Neurogenesis	103
PLGA, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanocapsules	BMP-4, PDGF, IGF-I	Chitosan scaffolds	It was found that simultaneous fast release of PDGF and BMP-4 led to highest proliferation rate of human mesenchymal stem cells	Bone regeneration	104

Abbreviations: GFs, growth factors; NPs, nanoparticles; PCE, poly(ε-caprolactone)-poly(ethylene glycol); PHBV, poly(3-hydroxybutyrate-co-3-hydroxyvalerate).

their contents in the short-term, as unsaturated lipids are susceptible to enzymatic dissociation.[90] To address these issues, polymer coatings on liposomes provided an effective way to stabilize the particles and offered the desired release kinetics of encapsulated drugs. In addition, a three-dimensional LbL structure composed of xanthan and galacto-mannan biopolymers was assembled on dioctadecyldimethylammo-nium bromide liposome for EGF delivery,[91] and it showed an entrapment efficiency of 72%. The bilayer of biopolymer-coated liposomes (165 nm) increased the sustained delivery of EGF up to 5 times at a first order rate of 0.005 min^{-1}. The use of these natural polysaccharide LbL coatings could be useful for improving biocompatibility, given the GF release profile of liposomes. In addition, EGF-encapsulated multilamellar vesicle liposomes were fabricated to preserve the activity of biomolecules, and the encapsulation efficiency of EGF in the liposomes was ~ 58%.[92] The drug-loaded liposomes were further incorporated in chitosan gel to stabilize the structures of particles for enhanced wound healing. Overall, liposomes have a relatively low loading efficiency for GFs due to the incompatibility of lipids with proteins, which is the major obstacle in the widespread use of liposomes for GF delivery.

SLNs containing a solid lipid core were found to be an alternative carrier to liposomes due to their high stability *in vivo*. However, their low drug loading is an important drawback that could compromise future applications of this vehicle. Fortunately, recent investigations on lipid-based NPs (especially NLCs) have helped to improve SLNs and liposomes. NLCs with liquid lipid cores (solid lipids and oils) had an increased number of imperfections in the solid core matrix, thus facilitating the encapsulation of an increased amount of biomolecules while preserving the long-term stability of the nanocarriers.[82,93] For instance, EGF-loaded SLN (332 nm) and NLC (348 nm) have been developed through the emulsification-ultrasonication technique for the treatment of chronic wounds.[94] Characterization of the vehicles indicated that the encapsulation efficiency of EGF-loaded NLC was significantly higher than that of EGF-SLN. Moreover, the encapsulation process did not comprise the biological activity of EGF, as these lipid-based NPs improved the proliferation of keratinocytes and fibroblasts compared with free EGF. These data confirmed that NLC improved GF binding to the carriers and enabled sustained release of the GFs. Finally, these findings revealed that the EGF-lipid-based NPs, particularly EGF-NLC, could promote healing characteristics and maturation in terms of chronic wounds and re-epithelization grade. Recently, gelatin NLCs were prepared for the sustained release of bFGF[95] and NGF[96] to enhance functional recovery in hemiparkinso-nian rats and recovery in spinal cord injury, respectively. Altogether, the long-term stability, high loading efficiency, improved controlled release, and drug safety administration demonstrated by the use of NLCs makes them ideal candidates for drug delivery systems in clinical applications.

Mesoporous silica NPs as GF carriers

Mesoporous silica NPs (MSNs) or nanoporous silica NPs, have received considerable attention for biomedical applications because of their high specific surface area, controllable particle size, large pore volumes, accessible surface modifications and superior biocompatibility (Figure 3e).[97–99] For example, BMP-2 peptides were grafted onto MSNs by covalently bonding through an aminosilane linker, and DEX was then incorporated into the channel of MSNs to construct dual delivery systems.[100] The dispersibility and stability of MSNs in phosphate-buffered saline or cell medium were significantly improved by drug functionalization. *In vitro* results showed that the drug-loaded MSNs significantly promoted the osteogenic differentiation (ALP
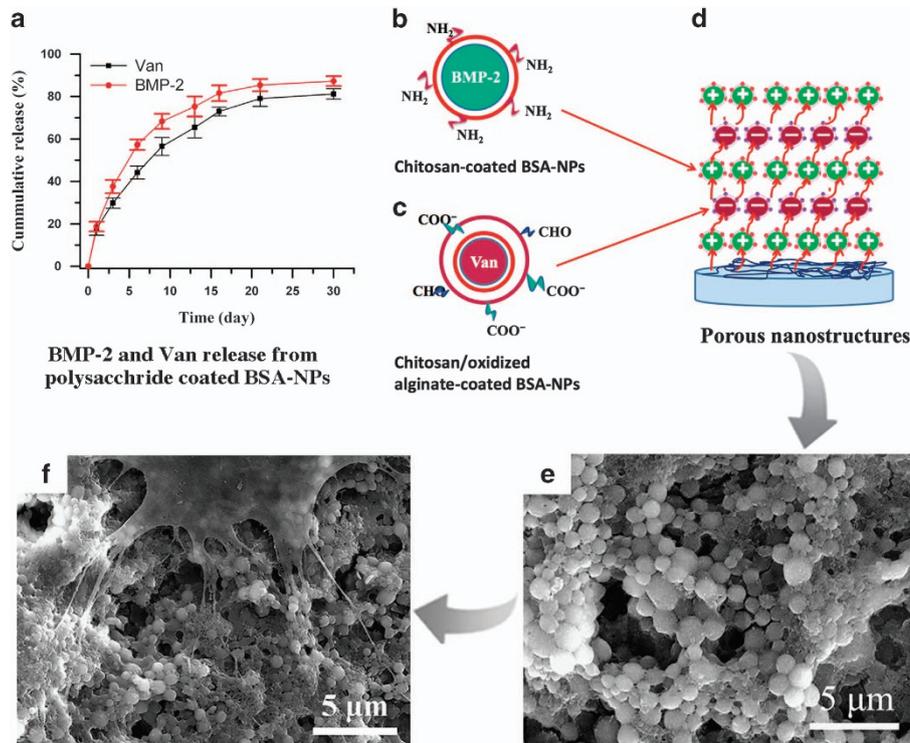
Figure 4 Schematic of bovine serum albumin (BSA)-based nanopaticle (NPs; BSA-NPs) assembled on titanium (Ti) surfaces for promoting cell adhesion. BMP-2 and vancomycin (Van) have sustained release up to 30 days from polysaccharide-coated BSA-NPs (**a**); Chitosan coated BMP-2 loaded BSA-NPs were prepared by a modified desolvation method (**b**); Chitosan/oxidized alginate coated Van loaded BSA-NPs were obtained by the same method (**c**); Cationic BSA-based NPs and anionic NPs were alternatively deposited on the Ti surfaces in a layer-by-layer technique (**d**); Surface morphologies of nanoporous structures by assembly of BSA-NPs on Ti surfaces (**e**); SEM image of BMSCs on nanostructured architectures (**f**). Bone marrow stromal cells (BMSCs) on nanostructured surfaces have close contact with the BSA-NPs, and present long thin pseudopodia similar to the size of NPs.

activity, calcium deposition) of BMSCs. Moreover, the MSNs-based dual delivery system synergistically induced effective osteoblast differentiation and ectopic bone formation *in vivo*, indicating that the MSNs have potential applications as bimolecular reservoirs in bone tissue engineering. It has also been reported that increasing the pore diameter of MSNs increases the drug delivery rate.[101] Neumann et al.[102] have prepared BMP2-functionalized nanoporous silicon NPs (NPSNPs) using a coupling method via an aminosilane linker. The NPs have an average size ranging from 30 to 50 nm (pore size ~ 2.8 nm), and the loading efficiency of BMP-2 loaded-NPSNPs was ~ $0.99\,\mu g\,mg^{-1}$. *In vivo* results confirmed that even BMP-free amino-modified NPSNPs show a certain osteoinductive effect, which, however, is significantly stronger with immobilized BMP-2 and can be further enhanced by supplementation with dexamethasone. Collectively, silica-based NPs are suitable as potential carriers for the controlled delivery of GFs in the area of bone tissue regeneration.

The surface decoration of MSNs with suitable polymers directly affects the drug release mechanism by enhancing drug diffusion resistance, which is an effective approach for achieving sustained drug release from MSNs.[103] Previous studies have synthesized PEG-decorated MSNs with an encapsulated trypsin inhibitor, a model protein molecule for GFs.[104] *In vitro* results indicated that protein release from the MSNs without the thin PEG film exhibited an initial burst followed by constant release, whereas the initial burst release of protein was completely inhibited in the case of the PEG-coated MSN system, and the release mechanism was found to have a zero-order release pattern over the entire period. Furthermore, recent

developments in polymer coated-MSNs have enabled the programmable delivery of GFs by exploring stimulus-responsive polymers. Taken together, the surface modification of MSNs with functional polymers may enhance clinical drug outcomes by preserving their bioactivity and optimizing their release mode.

The combination of MSNs and porous scaffolds seems to be a promising platform for drug delivery in tissue engineering. Mehrasa et al.[105] have fabricated PLGA/gelatin scaffolds containing MSNs by an electrospinning method; *in vivo* results demonstrated that the introduction of MNPs into the porous scaffolds improved the attachment, proliferation and formation of cellular processes of PC12 cells. Furthermore, the incorporation of MSNs into 3D porous scaffolds provided a potential way to encapsulate and deliver multiple drugs due to their biocompatibility and large-capacity storage of biomolecules.[106,107] Lee et al.[108] developed a new delivery system based on a collagen hydrogel containing NGF-loaded MSNs in neural tissue engineering. The MSN showed a high loading capacity and sustained release of NGF for over a week. When the NGF-loaded MSN was further incorporated in a collagen gel, the cumulative release amount of NGF was slower, and it followed the same long-term sustained release patterns. The novel delivery system of NGF-loaded MSNs, in combination with collagen hydrogels, promoted neuritogenesis while preserving cell viability, confirming that MSN embedded-porous scaffolds could also be extended to other GFs. Collectively, MSN-based delivery systems have been shown to be attractive candidates as GF carriers for tissue regeneration due to their excellent biocompatibility and large porosity.
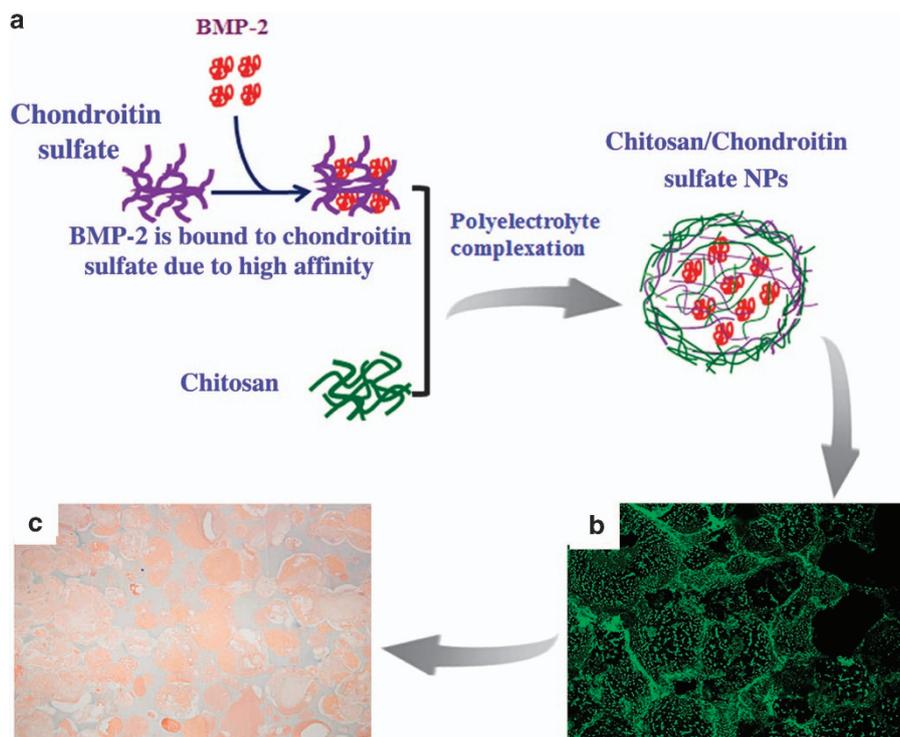
Figure 5 Schematic of the preparation process of BMP-2 encapsulated chitosan/chondroitin sulfate NPs and immobilization of the NPs on porous biphasic calcium phosphate (BCP) scaffolds for the study of ectopic bone formation. (**a**) Preparation of BMP-2 encapsulated chitosan/chondroitin sulfate NPs (**a**), FITC labeled chitosan/chondroitin sulfate NPs uniformly distributed on the BCP scaffold, as revealed by a confocal laser scanning microscope (CLSM) (**b**), Immunohistochemical staining for collagen type I on deparaffinized sections of BMP-2 encapsulated NPs functionalized BCP scaffolds: extensive immunoreactive area (brown) is observed (**c**).

Polymer nanocapsules for controlled GF release

Nanocapsule-based delivery systems have been extensively studied for biomedical applications because they offer a number of advantages, including low polymer content, high drug encapsulation efficiency, and polymeric shell protection against enzyme degradation (Figure 3f).[109,110] Segura *et al.*[111] have synthesized VEGF-loaded protein nanocapsules by assembling a thin polymer layer around the protein core by *in situ* free-radical polymerization. The introduction of peptides as crosslinkers to the polymer nanocapsules realized the cell-demanded release rate of VEGF by mediating the catalytic hydrolysis of polymer shells via extracellular proteases. The sustained release of VEGF from nanocapsules significantly enhanced vascular formation *in vitro* and *in vivo*. Recently, BMP-2 loaded degradable protein nanocapsules were developed via in situ polymerization to increase effective osteogenisity and reduce side effects.[112] Compared to the direct use of native BMP-2, the sustained release of BMP-2 from nanocarriers successfully enhanced bone formation with better bone quality while reducing the side effects associated with inconsistent bone formation and inflammatory edema in traditional spinal cord fusion surgery. The BMP-2 nanocarriers provided a safe and more effective GF therapy for bone tissue regeneration than native BMP-2. It has been reported that a combination of bioactive VEGF encapsulated the inner core of PLGA nanocapsules (diameter < 200 nm) and superparamagnetic iron oxide NPs (SPIONs) embedded in PLGA nanoshells was synthesized as a therapeutic strategy to enhance angiogenesis.[113] The PLGA nanocapsules showed high encapsulation efficiencies for both VEGF and SPIONs and exhibited sustained VEGF release over 14 days. *In vitro* studies confirmed that GF bioactivity in the polymer nanocapsules significantly increased the proliferation of

human microvascular brain endothelial cells upon the release of VEGF. In addition, VEGF-loaded magnetic PLGA nanocapsules could be guided in pro-angiogenic treatments under an external magnetic field.

Biomimetic strategies capable of the sequential and spatiotemporal release of GFs and other signaling molecules in local sites may lead to improved cell functions and may accelerate effective tissue repair. For this purpose, biodegradable chitosan scaffolds containing various GFs loaded-nanocapsules with different degradation rates were prepared for enhanced bone tissue regeneration.[114] In particular, faster degrading PLGA capsules (190–615 nm) and slower degrading poly (3-hydroxybutyrate-co-3-hydroxyvalerate) nanocapsules (255–712 nm) were separately loaded with BMP-4, PDGF and IGF-I and subsequently incorporated into chitosan scaffolds for the tunable release of multiple GFs in biometric microenvironments. The morphology of chitosan scaffolds with a sponge-like open porous microstructure had a pore size in the range of 100–200 μm. It was found that the dual, fast release of PDGF and BMP-4 achieved the highest proliferation rate of human mesenchymal stem cells and enhanced mineralization *in vivo*.

Other nanocarriers for controlled GF release

Recently, several NPs, such as inorganic, mussel-inspired or stimulus-responsive NPs, have received considerable attention for protein delivery due to their stable properties and versatile functions arising from their ease of handling and suitable surface chemistry.[36,115–118] Shevtsov *et al.*[119] developed EGF incorporated dextran-coated super paramagnetic iron oxide NPs (SPION) as a diagnostic agent for increasing magnetic resonance imaging contrast. The EGF functionalization increased the retention of NPs in tumor sites and exhibited

high magnetic resonance contrast potential. However, these NPs were prone to accumulating at a specific site and their long-term toxicology should be further investigated. It should be noted that various inorganic nanoparticulate materials for drug delivery were associated with long-term toxicity, raising serious concerns. Among the many inorganic delivery systems, calcium phosphate (CaP) NPs received special emphasis because of their superior biocompatibility and biodegradability and because CaP is the major constituent of bone and tooth. Ding et al.[120] exploited hydroxyapatite (HA) NPs and silk fibroin to achieve effective loading and the programmable, sustained release of BMP-2. Their studies demonstrated that the combined system provided preferable microenvironments for the tunable release of BMP-2 and significantly enhanced bone regeneration. However, synthesis of GF-loaded CaP NPs and the maintenance of particle stability are critical challenges for biomedical applications.[118]

Temperature-sensitive drug delivery is among the most widely investigated stimulus-responsive nanocarriers, and these nanocarriers can be combined to form dual-drug delivery systems in programmable concentrations for specific times. Poly(N-isopropyl acrylamide) (PNIPAM) and its derivatives have become the most extensively studied synthetic polymers in biomedical applications due to their lower critical solution temperatures, which are near physiological conditions [121] and are therefore suitable for mediating GF release in vivo. Kose et al.[115] have synthesized PNIPAM and PLGA nanocarriers for the encapsulation of IGF-I and TGF-β1, respectively.

Taking inspiration from adhesive mussel proteins that facilitate the adsorption of multiple biomolecules and cell behaviors, our group recently developed polydopamine NPs with a uniform size distribution (~260 nm) and a high affinity toward a variety of soluble proteins and peptides to decorate various scaffolds, including bio-ceramic, metallic, and polymer-based scaffolds (Figure 6).[122] Interestingly, the biodegradable polydopamine NPs enhance BMP-2 adsorption on porous TCP scaffolds, and they realized the sustained release of BMP-2 in vitro for up to 30 days on scaffolds. Furthermore, polydopamine with immobilized BMP-2 synergistically enhances the adhesion, proliferation and osteogenetic differentiation of BMSCs and induces ectopic bone formation in rats. In our previous study, we developed EGF-loaded polydopamine NPs/PNIPAM hydrogels to enhance cell affinity, adhesive strength, and tissue adhesiveness.[123] The results confirmed that incorporating polydopamine in the hydrogel helps to graft and sustain the release of EGF, which was ascribed to the high affinity of polydopamine to proteins via covalent-based reactions. In summary, the GF-grafted polydopamine NPs combined with porous scaffolds exhibited high cell affinity and bioactivity in vivo, which might have potential applications for tissue regeneration.

CONCLUSIONS AND PERSPECTIVES

When delivering GFs to promote tissue regeneration, the most important issue is to obtain optimal concentrations and gradients of the suitable GFs for precise spatial/temporal demands. To date, significant progress has been made in understanding fundamental information about the dynamic microenvironment and the biological properties of GFs to further develop effective GF-based delivery systems for the spatiotemporal regulation of protein delivery. A wide variety of direct and indirect approaches have been employed in recent decades, such as covalent conjunctions, ECM-inspired binding, and NP-based delivery systems, to mimic ECM environments to facilitate tissue repair. Most of the delivery systems presented in this review demonstrated enhanced stability and therapeutic potential of the GFs upon immobilization when compared to traditional regenerative medicine and drug delivery systems. It should be noted that

NP-based GF delivery systems have demonstrated several advantages due to their high surface area, nanostructures, and precise control of multiple proteins that play pivotal roles in the regulation of cellular adhesion, proliferation, differentiation and extracellular matrix production. Specifically, the nanotoxicology and related toxicity issues such as cytotoxicity, ecotoxicity and genotoxicity are of crucial importance to enhance their potential applications in clinical trials. The details of toxicity evaluation of different GF nanocarriers are provided in Supplementary Information. Furthermore, these nanocarriers can be combined with microporous scaffolds via advanced nanotechnology techniques in order to mediate GF release kinetics in a predesigned manner to increase tissue healing and regeneration (Figure 3g).

A major challenge to the successful delivery of GFs in the clinic is that considerable amounts of biomolecules are required during tissue regeneration. Spatially controlled GF presentation will continue to play a critical role in tissue engineering strategies and will be coupled with biomaterials that permit tunable release profiles to achieve true spatiotemporal regulation during delivery. Importantly, for this approach to realize its full potential, continued efforts to understand native biological signaling pathways during tissue development and healing will be necessary. This requirement includes recapitulating the concentrations and spatial and temporal distributions of bioactive factors during these processes, accounting for the effects of cellular heterogeneity, the construction of native tissue at high resolution and their carriers on target cells. Such knowledge could serve as engineering design criteria to specifically guide the release of GF from the delivery system. Thus, the delivery system should not only release the appropriate GFs at accurate doses and kinetics; it should also offer insights on multiple GFs and their native microenvironments during tissue healing in vivo. Once these elements are taken into account, the expanding variety of GFs can hold great promise for future therapies in clinical regenerative medicine.

GF delivery from a tremendous number of biomaterials has provided satisfactory results in bone fractures and fibular defects, improvements in Alzheimer's disease and periprosthetic fractures, and advances in peripheral nerve regeneration, dermal healing, and skeletal muscle regeneration. Significant advances have optimized the delivery of GFs to target tissues and facilitated their progression through clinical trials to reach the market. In addition, an osteoconductive β-TCP matrix/PDGF-BB delivery system was developed to reach a long duration of PDGF-BB for dental therapy in clinical applications by utilizing innovative tissue engineering and drug encapsulation principles. However, supraphysiological and high doses of these GFs are required for therapeutic benefits, and in some cases, these doses have triggered certain side effects, including increased ectopic tissue formation, wound complications, radiculopathy, or cancer risk, from systemic exposure to the administered GFs.[124] In addition, although sophisticated release profiles can now be achieved for research purposes and some commercially available GF products have been used in humans, clinical trials on release technology and the results of many such systems have been largely unsatisfactory or at least suboptimal. These limitations are primarily due to their simple design, poor drug release mechanism, biomaterial selection and matrix/carrier fabrication method, which make it is hard to mimic natural tissue regeneration.

Taking these points into account, it is clear that further research is necessary regarding the programmable release of growth factor delivery and smart biomaterial design. A combination of growth factors (BMP/VEGF, BMP-2/BMP-7) and their release profiles in different biomaterials has the potential to improve tissue regeneration
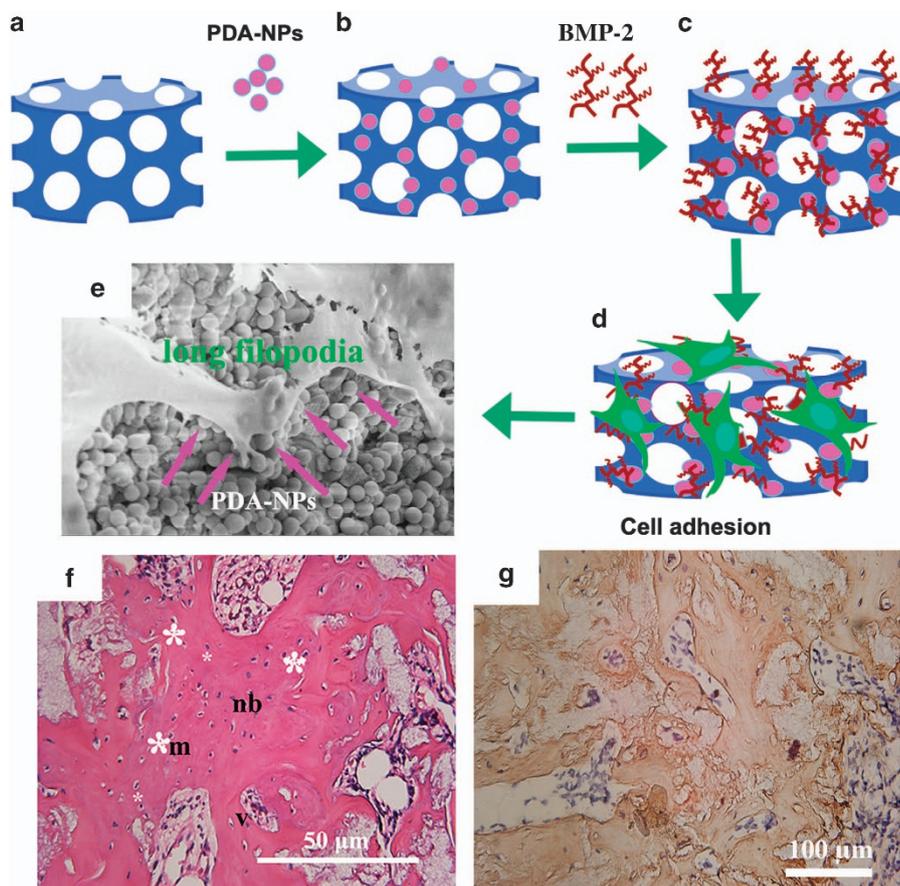
Figure 6 Polydopamine NPs functionalized on porous scaffolds for BMP-2 immobilization and enhanced bone tissue regeneration. (**a**) Porous TCP scaffolds; (**b**) polydopamine NPs immobilized on scaffolds; (**c**) BMP-2 adsorption on polydopamine NP-modified scaffolds (polydopamine-NP-TCP); (**d**) *In vitro* cell culture on porous scaffolds; (**e**) Attachment and spreading of BMSCs on polydopamine-NP-TCP scaffolds after 1 day; (**f**) Hematoxylin and eosin (H&E) staining micrographs of polydopamine-NP-TCP scaffolds retrieved after 12-week implantation; (**g**) Immunohistochemical staining for collagen type I on deparaffinized sections of polydopamine-NP-TCP scaffolds retrieved after 12-week implantation. m, material; nb, new bone; v, vessel.

in vivo. Thus, multiple GF release plays an essential role in the recruitment, proliferation, and functional activities of MSCs during the early phases of wound repair and the promotion of tissue regeneration. Second, while a number of GFs have been demonstrated to be efficient at high doses in multiple biomedical applications, safety and precision medical treatment have driven research towards the development of novel delivery systems that enable dose reduction and optimize the concentrations of the right GFs at the right time. Therefore, smart nanomaterials that are sensitive to physiological or exogenous stimuli have been widely applied to deliver GFs at the right time and/or place. Indeed, the development of smart materials, mainly based on ECM-like biomaterials, appears to provide an attractive alternative for delivering multiple GFs in a better-controlled fashion in the future.

As the field of tissue regeneration moves towards the design of multifunctional platforms to better recapitulate the complex process of natural tissue repairing, combinations of bioactive proteins with appropriate biomaterial carriers will direct innovation. To date, biomaterial-based GF delivery systems derived from synthetic, natural, or inorganic materials in various structures can provide differential immobilization efficiency and release kinetics, which enables the most successful release of multiple GFs with biological activity in the local environment. These new methods of biomaterial synthesis are still in the initial stages of lab research and development, lacking consistent

clinical data. Future research directions for this type of biomaterial in tissue repair and regeneration should be focused on a comprehensive understanding of biomaterial-tissue interactions and the optimization of their chemicophysical properties for long-term performance. Therefore, the elegant synthesis of biomaterials in terms of biological compatibility and structural components that mimic native tissue repair through the integration of multiple areas, including biochemical stimuli (such as GFs, adhesive proteins, genes or functional groups) and physical cues (micro/nano-architectures, stimuli-responsive prosperities, stiffness, shear stress, hydrophobic and hydrophilic properties), is important for the development of next-generation biomedical devices. Finally, exploration of the potential of GF-based delivery systems for clinical use strongly depends on more researchers and multidisciplinary approaches that combine engineering, biomaterials, medicine, and the technical expertise of medical specialists. Close cooperation among experts in materials science, tissue engineering, chemistry and medicine may eventually bridge the gap between current research and clinical applications, and it is likely that multiple GF delivery strategies will provide many therapeutic benefits in the treatment of complex wounds and pathologies in the near future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the NPG Asia Materials website (http://www.nature.com/am)