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Expert Review

Nanoparticulate Systems for Growth Factor Delivery

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Abstract. The field of nanotechnology, which aims to control and utilize matter generally in 1–100 nm range, has been at the forefront of pharmaceutical development. Nanoparticulate delivery systems, with their potential to control drug release profiles, prolonging the presence of drugs in circulation, and to target drugs to a specific site, hold tremendous promise as delivery strategies for therapeutics. Growth factors are endogenous polypeptides that initiate intracellular signals to regulate cellular activities, such as proliferation, migration and differentiation. With improved understanding of their roles in physiopathology and expansion of their availability through recombinant technologies, growth factors are becoming leading therapeutic candidates for tissue engineering approaches. However, the outcome of growth factor therapeutics largely depends on the mode of their delivery due to their rapid degradation *in vivo*, and non-specific distribution after systemic administration. In order to overcome these impediments, nanoparticulate delivery systems are being harnessed for spatiotemporal controlled delivery of growth factors. This review presents recent advances and some disadvantages of various nanoparticulate systems designed for effective intact growth factor delivery. The therapeutic applications of growth factors delivered by such systems are reviewed, especially for bone, skin and nerve regeneration as well as angiogenesis. Finally, future challenges and directions in the field are presented in addition to the current limitations.

KEY WORDS: drug delivery; growth factor; nanoparticulate systems; tissue regeneration; tissue targeting.

INTRODUCTION

The increasing need for more efficient and less invasive treatment of damaged or diseased tissues is stimulating development of new technologies in the field of regenerative medicine and tissue engineering. By applying combinations of biomaterials, cells and bioactive molecules, regeneration of damaged or diseased tissues can be facilitated, ultimately leading to functional replacement of these tissues. Growth factors are polypeptides secreted by a wide range of cell types and transmit signals to regulate cellular activities, such as migration, differentiation and proliferation. By binding to specific receptors present on the surface of target cells, growth factors elicit cellular actions and can initiate a cascade of biological events to stimulate the regenerative process. In tissue engineering strategies, the local presence of growth factors at the defect tissue site to trigger healing and regenerative processes is of key importance to successful outcomes (1–3). Since growth factors generally display a short biological half-life in circulation, usually several minutes (4–6), and they undergo rapid degradation *in vivo*, bolus injection or systemic/local infusion would result in lower availability of growth factors than the physiological requirement for tissue repair (1). As a result, repeated administration may lead to undesirable systemic effects and toxicity due to the non-specific distribution and accumulation of growth factors throughout the body. In addition, growth factors poorly pass through biological barriers which are mainly composed of lipid membranes, because of their poor diffusivity and low partition coefficient in the lipid phase (7). Therefore, for an efficacious clinical outcome, a delivery system for growth factors is necessary to guide the growth factor to the site of action and prevent the rapid dispersal from the site.

Delivery systems for growth factor have been designed in a variety of configurations, and fabricated from diverse types of synthetic and natural materials. Growth factors were incorporated in the system during or after the fabrication by covalent or non-covalent means; the latter includes simple adsorption, electrostatic interaction, or complexation. Growth factors were delivered in the format of protein itself (the focus of this review), genes encoding the protein (8) or cells secreting the protein (2) (the latter two were considered beyond the scope of this review). Among the existing delivery strategies, particulate delivery systems are distinguished from others by their reduced size, with the range of 1–1,000 μm for microparticles and <1 μm for nano-sized particles (9), and have been used for the regeneration of different tissues (10).

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Compared to microparticles, nanoparticles (NP) may offer improved transport properties and pharmacokinetics profiles *in vivo* after systemic administration, since they can penetrate deeper into tissues through fine capillaries and epithelial lining, allowing more efficient delivery of therapeutic agents to target sites (11). Moreover, nano-scale dimensions impart remarkable physiochemical properties to NP systems that are able to optimize some of the most fundamental properties, including solubility, diffusivity, biodistribution, release characteristics and immunogenicity, and most importantly, the ability to target to uniquely identified tissue with minimal distribution to normal tissues (12–14). Such properly designed site-specific targeted NPs will open the possibilities of addressing the failure of traditional therapeutics.

A recent survey indicated that ~26 NP agents have been approved for clinical use (15), and numerous NP systems are currently or soon-to-be under clinical testing (16,17). NP systems for growth factor delivery include liposomes, polymeric NPs, micelles, dendrimers, and other miscellaneous particulates. A wide diversity of materials have been utilized for NP preparation, with polymeric materials being the most widely investigated (7,14). Owing to such advantageous properties as biodegradability, biocompatibility with physiological systems, natural abundance and suitability for chemical modifications, NPs made from natural polymers have been extensively investigated. Albumin, for example, was formulated into Abraxane™ NP carrier for the anticancer drug pacilitaxel (17). Our group recently reviewed advances in the NP systems designed for delivery of various drugs (18). This review is an update on NP delivery systems with special focus on growth factor delivery. An overview of design criteria for growth factor delivery will be briefly discussed, followed by presentation of various NP delivery systems, including lipid- and polymer-based systems, and other miscellaneous systems. The most appealing aspect of the NP systems, namely targeted delivery, will be summarized. Therapeutic applications of growth factors delivered by NPs will be reviewed in the context of bone, skin, and nerve regeneration, as well as angiogenesis. Finally, the future challenges and directions from the authors' opinions will be provided.

GENERAL CONSIDERATIONS FOR NANOPARTICULATE SYSTEMS IN GROWTH FACTOR DELIVERY

Since growth factors action during regeneration is both temporally and spatially controlled, there is a considerable need for optimizing the mode of growth factor delivery, so that the local presence of a bioactive growth factor can be maintained at therapeutic concentrations for a prolonged time period, while reducing its adverse impact on systemic tissues. Several critical issues need to be addressed on this aspect. First, being highly labile molecules, the integrity of growth factors may be compromised by environmental factors, resulting in denaturation or deactivation. The NP fabrication process needs to be operated under mild conditions; harsh organic solvent, high temperature or pressure, and extreme pH or ionic strength values should be avoided. Meanwhile, since growth factors are highly water-soluble, the interaction between phases should be taken into consideration if the incorporation process relies on the affinity of growth factor to the lipophilic phase of an emulsion or for the polymer (7). When growth factor incorporation into NPs is performed by covalent means, the bioactivity of the modified growth factor should be examined carefully for any undesirable changes in intrinsic activity.

Secondly, the physiochemical properties of the NPs, the size and size distribution, surface charge and the nature of NP substance, are likely to determine *in vivo* fate of the growth factors delivered. It is generally recognized that 20~200 nm particulates are suitable for systemic delivery of therapeutics; the larger size particles suffer from quick uptake by the reticuloendothelial system (RES) and rapid clearance from the circulation, whereas the smaller size will tend to cross the fenestration in the hepatic sinusoidal endothelium, leading to a hepatic accumulation instead of long circulation times (13). Neutral and hydrophilic surfaces display lower opsonization than the charged and hydrophobic particles (19). To achieve the long-term *in vivo* circulation and reduce the clearance by the RES, surface modification with polyethylene glycol (PEG) for the "stealth" effect is an established method (20,21). The materials for constructing the NPs should be biocompatible, non-immunogenic and biodegradable. The latter property is also critical for growth factor release, given the low diffusivities of the growth factors in physiological systems (22).

Thirdly, targeting ability of NPs for site-specific delivery of growth factors is paramount when the growth factor is delivered systemically. Targeted delivery concentrates the growth factor at the site of action and potentially reduces the undesired effects at normal tissues. Though a small quantity of growth factor is usually required to generate a cellular response, the short half-life and rapid degradation *in vivo* may prevent non-targeted growth factors from reaching the site of action and obtaining the efficacious results. The targeted delivery can be fulfilled by: (1) stimuli-induced targeting based on physical properties, such as magnetic induction between magnetic NPs and a magnet at the site of action; and (2) cell- or tissue-specific targeting based on strong affinity of a ligand to target receptacle, such as Arg-Gly-Asp (RGD) peptide to specific cellular receptor or the mineral affinity of bisphosphonates (BPs) to mineral phase of bone (23).

Finally, it is essential to design NPs with optimal and controllable growth factors release profiles to meet the temporal and spatial demands for the growth factors. Specifically, the tissue of interest needs to be exposed to growth factors for a sustained period of time, and at desired concentrations for a successful outcome in tissue repair (2). The release kinetics of growth factor from NPs may be affected by several factors, such as the degradation rate of NPs, the physiological diffusivity of a growth factor, the loading mode of growth factor and other possible factors (18). A better protection against environmental factors and more tunable control is achieved if loading is performed by encapsulation, rather than adsorption onto the NPs.

Successful combinations of these factors in the design of NPs are likely to lead to enhanced therapeutic outcomes, while reducing the undesired effects at normal tissues.

TYPES OF NANOPARTICULATE SYSTEMS FOR GROWTH FACTOR DELIVERY

When delivered by a NP system, the *in vivo* fate of growth factor will be determined by the physiochemical

properties of the NPs rather than those of the protein. The possibility of tailoring both the internal and surface structures of NPs will serve well for the delivery. NP delivery systems reported to-date including lipid-, polymer-based systems, and other miscellaneous systems (Fig. 1). Growth factors such as basic fibroblast growth factor (bFGF, or FGF-2), bone morphogenetic proteins (BMPs), transforming growth factor-β (TGF-β, including TGF-β1, TGF-β2, and TGF-β3), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), nerve growth factor (NGF), and insulin-like growth factor (IGF), have been already employed in tissue engineering, and their delivery by NP systems is discussed below (Table I) with the size of typical NP systems summarized in Fig. 2.

Lipid-Based Systems

Liposomes, solid lipid particles (SLN), and lipid nanocapsules (LNC) are different configurations of lipid-based NPs (24). Liposomes are closed vesicles formed by bilayers of hydrated phospholipids which enclose an aqueous core (25). This structure enables liposomes to entrap both hydrophilic and hydrophobic drugs: water-soluble drugs are entrapped in the aqueous core, while oil-soluble drugs reside within lipid bilayers. Liposome preparation techniques include sonication, reverse phase evaporation, freeze-dried rehydration, detergent depletion and high-pressure homogenization (26). SLNs contain a core of solid lipids, while LNCs contain a liquid lipid core. Surfactants are often added for stabilization of particles as a shell. Due to the nature of the core, the loading efficiency of hydrophilic growth factors in SLNs is low, since growth factors tend to partition in the water phase during preparation. Loading onto preformed SLNs by adsorption procedure is generally adopted to circumvent this problem (27). The main established techniques for SLNs production are high-pressure homogenization and microemulsion-based technique (27), while the formulation process of LNCs is typically based on phase inversion temperature method plus the temperature recycling treatment (28).

Since lipid-based systems can be prepared with phospholipids that naturally occur in the mammalian cell membranes, they are generally regarded as biocompatible and non-toxic. The systems can also be tailored with respect to

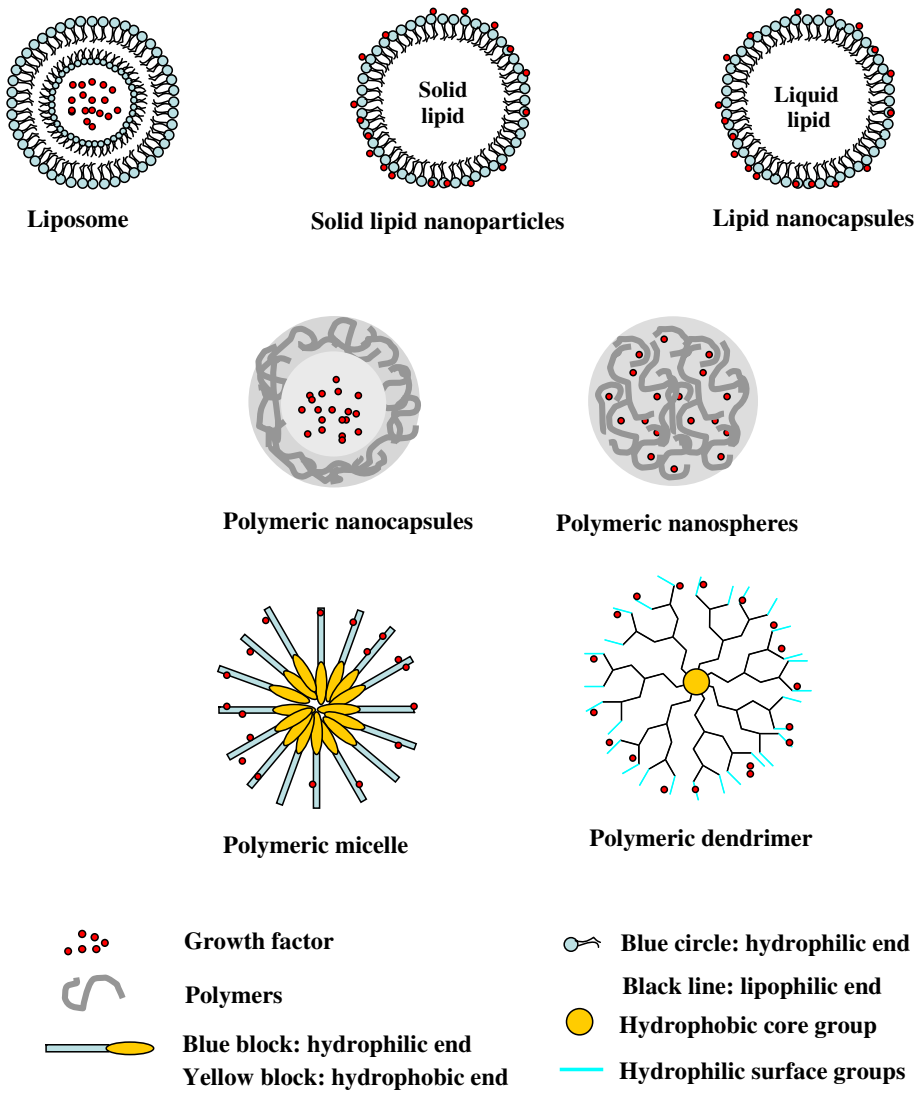


Fig. 1. Schematic representation of different NP systems.

Table I. Summary of *In Vitro* Investigations of NP Systems for Growth Factor Delivery

Growth factor	NP system	EE (%)	Significant observations	Reference
bFGF (FGF-2)	PCL-PEG-PCL	61%	After an initial burst of ~60%, bFGF was released for 8 days with 70% of total bFGF release	(50)
	Tetronic®-PCL-heparin micelle	50–70%	bFGF was released for over 2 months due to specific interaction with heparin	(72)
	Chitosan/dextran sulfate/PLL	N/A	Nanocapsules were made by layer-by-layer method. bFGF activity was retained in the capsules	(67)
	Chitosan/ tripolyphosphate	27%	~70% of bFGF was released at 24 h. bFGF integrity was not affected by the encapsulation procedure	(63)
NGF	Streptavidin (strep-QDs)-conjugated NGF	N/A	QD conjugation adversely affected the NGF activity	(84)
	[a]PBCA coated with polysorbate-80	N/A	20-fold higher cellular uptake by the coated NPs than the uncoated	(53)
BMP-2	PEI-coated BSA	>90%	BMP-2 release was controlled by PEI coating concentrations. PEI coating reduced the initial burst release compared to the uncoated NPs. BMP-2 activity was retained.	(62)
	PLL-coated BSA	>90%	BMP-2 activity was retained	(61)
	Calcium phosphate NPs in PLGA MPs	N/A	A sustained release of BMP-2 for over 7 weeks in a reasonably linear profile	(82)
	Dextran	83%	Released BMP-2 stimulated proliferation and differentiation of rabbit bone marrow stem cells	(68)
KGF-2 (FGF-10)	Dextran sulfate/polycations	78%(chitosan) 68% (PEI) 75% (PLL)	≥80% of the encapsulated KGF-2 was released over 11 days for all formulations. The released protein enhanced the proliferation of EC, compared to free protein solution.	(65)
EGF	Polystyrene/Poly (methacrylic acid)	~100%	EGF was released as a pseudo-zero order pattern after initial burst effect of 50%	(55)
	G5 PAMAM dendrimers	N/A	The PAMAM-EGF conjugate stimulated cell growth to a greater degree than free EGF	(77)
	PEG-coated DPPC/ CHO liposomes	<20%	Enhanced EGF resistance to proteases, higher permeability of EGF across Caco-2 cells	(29)
	DPPC/LPC liposomes	N/A	Increased osteoclast recruitment and enhanced teeth movement	(30)
IGF-I	PLGA	22–43%	Preparation methods affected the IGF-I release	(42)
BMP-7	Core/shell NPs composed of liposome (CHO and DDAB) /sodium alginate/chitosan	<20% for uncoated liposome; >80% after coating	Burst release was reduced by layer-by-layer coating of liposome, and up to >85% of BMP-7 was released over 4 weeks. The preosteoblast differentiation was enhanced.	(37)
TGF-β3	Heparin/PLL	N/A	Promote neocartilage formation after 4-week cultivation with mesenchymal stem cells	(107)
VEGF	Polycations/dextran sulfate	83%(chitosan) 48% (PEI) 53% (PLL)	75% of VEGF was released in a 10-day period for all NP formulations	(64)
	PLGA/Pluronic F-127/heparin	N/A	A linear, sustained release of VEGF without burst release was achieved for 37 days with 85% of the loaded VEGF being released	(43)
	Lecithin/F-127	N/A	Sustained released of VEGF was affected by the lecithin amount in the system	(36)
	[a]PLGA-PEG/PLGA-alendronate	N/A	NPs displayed high affinity to hydroxyapatite	(96)
	[a]CHOL-TOE-BP liposome	N/A	Liposome displayed high affinity to hydroxyapatite	(97)

[a] No growth factor was encapsulated

EE encapsulation efficiency; *N/A* not available; *PCL* poly(ε-caprolactone); *PEG* polyethylene glycol; *PLL* poly-L-lysine; *QDs* quantum dots; *PBCA* poly(butylcyanoacrylate); *PEI* polyethylenimine; *BSA* bovine serum albumin; *PLGA* poly(DL-lactide-*co*-glycolide); *MP* microparticle; *NP* nanoparticles; *G5 PAMAM* the 5th generation of polyamidoamine dendrimer; *DPPC* dipalmitoylphosphatidyl-choline; *CHO* cholesterol; *LPC* lysophosphatidylcholine; *DDAB* dimethyldioctadecyl-ammonium bromide; *Pluronic F-127* poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock polymer; *CHOL-TOE-BP* cholesteryl-trisoxyethylene-bisphosphonic acid

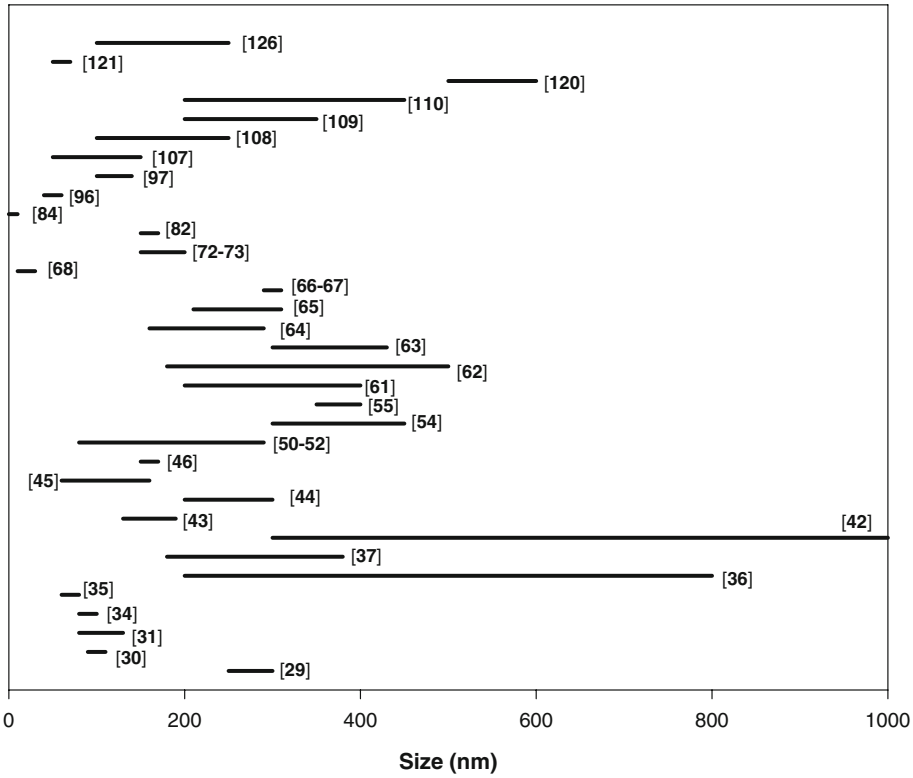


Fig. 2. Size distribution of various NP systems used for growth factor delivery. The lines shows the range of NP sizes reported in the publication, indicated by the reference number shown next to the line.

physical and chemical composition of the vehicle, as well as the delivery requirements, such as coating with antifouling agent PEG for an extended half-life in the circulation and engineered to bear with targeting moieties. Lipid-based systems, especially liposomes, are an extensively studied and well established carrier system for various drug delivery; several liposome-based NPs have been approved for human use as anti-cancer reagents or vaccine delivery (16). Recently, it was reported that recombinant human EGF was encapsulated in PEG-coated liposomes for oral delivery (29). The PEG-coated liposomes prepared from dipalmitoylphosphatidylcholine (DPPC, ~300 nm) enhanced the resistance of EGF against protease degradation, demonstrated higher permeability of EGF across Caco-2 cells and improved the bioavailability (given by increased plasma AUC, the area under the concentration-time curve) of EGF as compared to PEG-coated phosphatidylcholine (PC, ~250 nm) liposomes or EGF alone. EGF was also encapsulated in ~100 nm liposomes prepared from a mixture of DPPC and lysophosphatidylcholine (LPC) for osteoclast recruitment during tooth movement in rats (30). The EGF encapsulated liposomes induced greater osteoclast recruitment as compared to EGF solution after local injection into the root furcation of the molar, as a result of the increased time of EGF performance at the application site. Magnetic egg phosphatidylcholine (EPC) liposomes were prepared with the addition of magnetite particles for BMP-2 (31), and TGF-β1 (32) delivery to stimulate new bone formation in animal models. Liposomes have also been used as carriers for HGF (33,34), and NGF (35) for effective delivery to liver and brain. The lipid-based NPs seem to be advantageous in delivering therapeutics to the brain, due to the facilitated penetration of such NPs across the lipophilic brain–blood barrier (BBB).

A limitation of lipid-based NPs is the relatively low loading efficiency (typically <35%), given the incompatibility of proteins with lipids. The stability of lipid-based NPs is also an issue, since unsaturated lipids are susceptible to enzymatic degradation, undergo ready dissociation of self-assembled lipids and form particle aggregates due to their thermodynamically unstable structure under aqueous conditions. All these factors may result in uncontrolled or rapid release and destabilization of growth factors, which is the main obstacle for widespread use of liposome as drug delivery system. A core/shell NP aimed to overcome the stability issue was designed and examined for VEGF delivery *in vitro* (36). Lecithin was used to form the anionic nanolipid core, and VEGF was loaded into the core by electrostatic interaction. The shell was formed by Pluronic F-127 (poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock polymer) through freeze-drying with trehalose, which was used as a cryoprotectant to preserve the core/shell structure and VEGF bioactivity. The *in vitro* VEGF release was sustained for more than 30 days. The release was highly dependent on the lecithin composition, presumably due to the increased interaction between VEGF and anionic lecithin. During a 2-week study period, the nanolipids without a shell underwent significant aggregation while the core/shell structure remained stable. This core/shell system appears to be promising for sustained VEGF delivery to ischemic areas. Another core/shell NP system was composed of cholesterol/dimethyldioctadecyl-ammonium bromide (DDAB) cationic liposome, which was then coated layer-by-layer with sodium

alginate and chitosan for BMP-7 delivery (37). By monitoring by changes in NP size, beneficial effect of layer-by-layer coating was clearly demonstrated, especially in the presence of serum. The BMP-7 encapsulation efficiency was also augmented as the number of layers on liposome increased. Several SLN systems have been used for peptide and protein delivery, which can be easily adapted for the delivery of growth factors (38,39).

Polymer-Based Systems

Polymer-based NPs have been extensively studied for growth factor delivery in the form of nanospheres, nanocapsules, micelles and dendritic particles. The nanospheres represent a matrix system with the bioactive agent dispersed throughout the particle, while nanocapsules represent vesicular system with bioactive agents confined to a cavity surrounded by a polymeric membrane. Micelles arise from self-assembled polymers that form quasi-stable structures under aqueous conditions, while dendritic particles are ordered molecular systems with nano-scale dimensions and high density of repeating and/or functional units. Building blocks for nanoparticulate systems are derived from synthetic and natural sources and can be formulated from pre-formed polymers or by polymerization of a monomer (28). The minimal requirements for polymers used for therapeutic delivery should be biocompatibility, biodegradability, non-toxicity, non-immunogenicity and sterilizability (40).

Polymeric Nanospheres and Nanocapsules

The synthetic polymers used for NP fabrication include polyesters, polyanhydrides, polyphosphazenes, poly(alkyl cyanoacrylate) and hemiesters of copolymers of maleic anhydride and alkyl vinyl ethers, as well as the block copolymers (41). Among them, polyesters, such as polylactides, poly(ε-caprolactone), poly (phosphoesters), and poly(ortho esters), have been widely utilized in clinics due to their accepted safety profile and biodegradability. Polylactide (PLA), polyglycolide (PGA) and poly(*DL*-lactide-*co*-glycolide) (PLGA) copolymers have been the most extensively used materials (Fig. 3). The well-understood degradation pattern, long history of clinical applications, and well-established manufacturing techniques make PLGA the "gold standard" of biodegradable polymers (14). Emulsion-solvent evaporation/extraction (single and double emulsion), phase separation, solvent displacement (nanoprecipitation, coprecipitation, and dialysis method), and self-assembly are representative techniques for polymeric NP fabrication (see Ref. (41) for detailed explanation of these techniques). Polymeric NPs have been tailored for growth factors with a delivery rate dependent on environment stimuli as well as the chemical composition of the polymeric material.

IGF-I was encapsulated in PLGA nanospheres by two methods, salting-out and solvent evaporation/double emulsion processes (42). Though the former method typically produced smaller particles, particles from solvent evaporation/double emulsion method provided sustained release for up to 40 days with ~70% of IGF-I release *in vitro*, while the IGF-I was not significantly released over a 30 day period (~15% release) for former NPs. The salting-out process was suggested to lead to strong IGF-I adsorption to the polymer, or simply deactivated IGF-I during the fabrication. VEGF was also entrapped in heparin-functionalized PLGA nanospheres for sustained release (43). The PLGA formed the hydrophobic core, while Pluronic F-127 formed a hydrophilic surface layer. The heparin-PLGA nanospheres were prepared by a solvent diffusion method with no chemical modifications; heparin was dispersed in the nanospheres, with majority being located on the NP hydrophilic surface layer. Due to the specific interaction between VEGF and heparin, VEGF was entrapped in the nanospheres and the VEGF release depended on heparin amount in the nanospheres, with higher heparin amount providing prolonged release. The highest heparin percentage in the nanospheres (4.7 wt.%) yielded VEGF release for up to 40 days without an initial burst. PLGA nanospheres were also used to deliver BMP-7 (44) and FGF-2 (45) with heparin-free particles, as well as BMP-2 (46) delivery after heparin conjugation to NPs for specific interaction with BMP-2.

However, there are several issues associated with PLGA use for growth factor delivery: (1) the possible adverse effect of the use of organic solvent during fabrication on growth factor bioactivity (47); (2) the increased acidity in the local microenvironment due to the formation of lactic/glycolic acid when the polymer degrades; this can lead to irritation at the target site or denaturation of growth factors (48); (3) the poor clearance because of its synthetic origin, specially for high molecular weight polymers; and (4) chronic inflammatory response (49). For these reasons, other synthetics materials have been investigated, such as poly(ε-caprolactone) (PCL), polyanhydrides, and poly(alkylcyanoacrylate). PCL-PEG-PCL copolymer was used to prepared anionic NPs by emulsion solvent evaporation method to incorporate bFGF by electrostatic interaction (50,51). A relatively high encapsulation efficiency (61%) was obtained in this approach, and the release was sustained for up to ~10 days with an initial burst release of ~60%. The same group also reported functionalized PCL-PEG-PCL NPs with mannan-coating for targeting to dendritic cells to improve humoral immunity (52). Poly(butylcyanoacrylate) (PBCA) NPs were prepared by emulsion polymerization from n-butylcyanoacrylate, and then coated with polysorbate-80 to prolong the circulation time of particles in the blood and increase their concentration in cerebral vessels (53). This system demonstrated enhanced uptake by human and bovine primary brain capillary endothelial cells, and then used for NGF delivery for Parkinson's therapy in mice (54). NGF was adsorbed on the surface of PBCA NPs, and then the particles were incubated with polysorbate-80 for coating. The intraperitoneal injection of NGF-loaded NPs decreased the Parkinsonian symptoms in this animal model. Another polymeric nanoparticulate system, composed of non-degradable polystyrene core (hydrophobic) and poly(methacrylic acid) shell (hydrophilic), was prepared by dispersion copolymerization for EGF delivery (55). EGF was entrapped in the NPs by electrostatic interaction with a high loading efficiency (~100 wt.%). The release of EGF from the NPs showed a pseudo-zero order kinetics over a 7-day period after an initial burst release of ~40%. The proliferation of A431 cells was superior for NP-delivered EGF (compared to free EGF), indicating a beneficial effect of sustained delivery.

As an alternative, natural polymers are attractive for NP fabrication because of their good compatibility in physiolog-

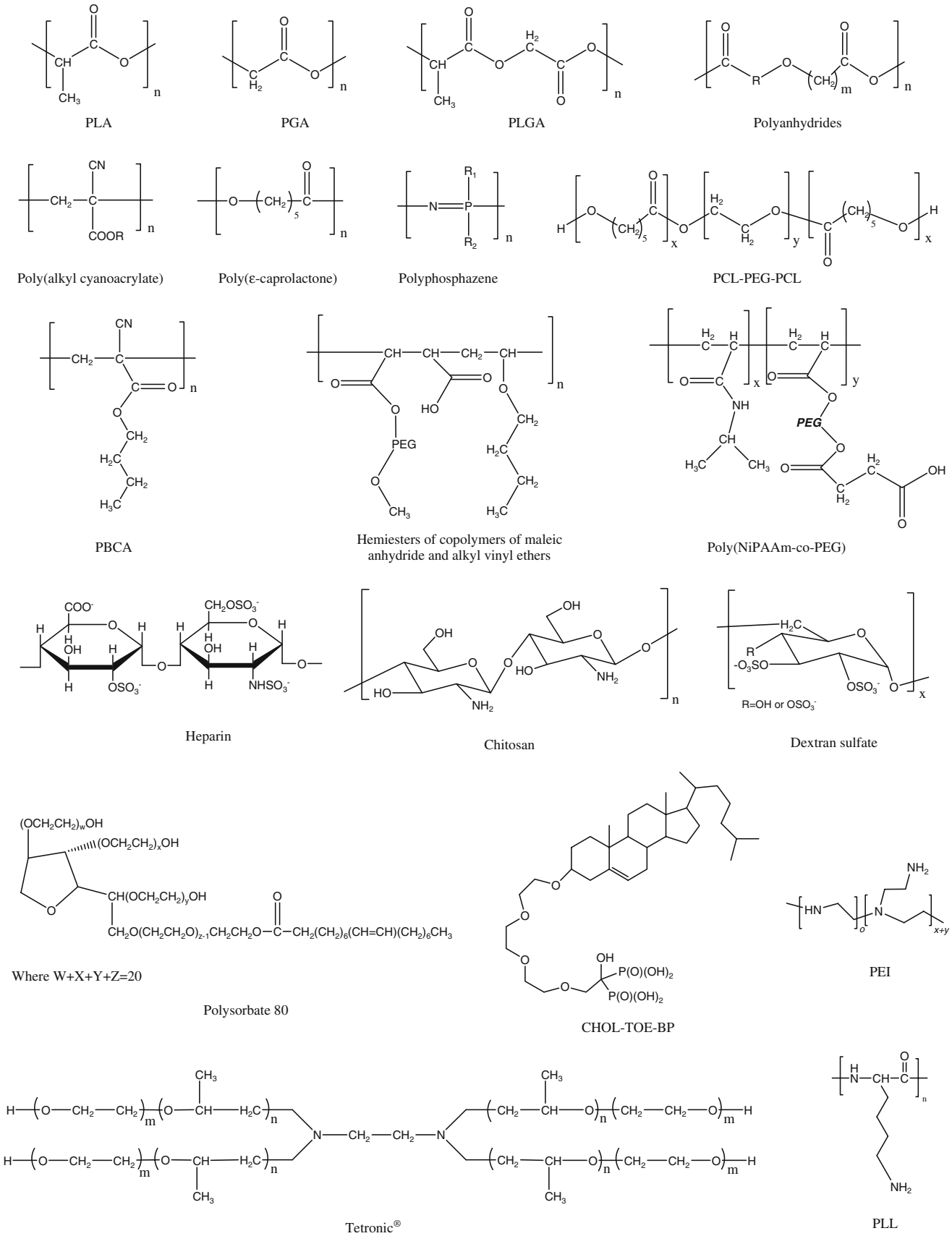
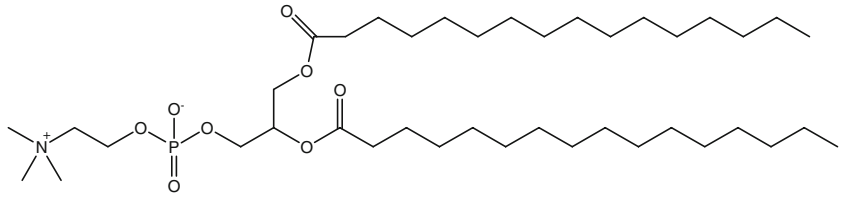
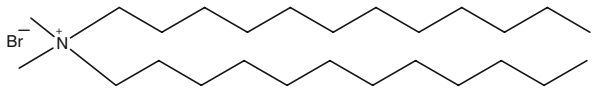


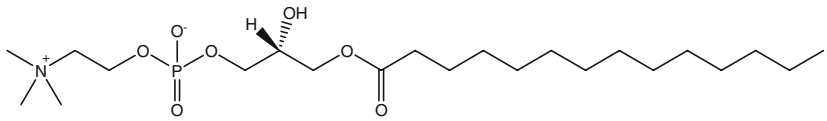
Fig. 3. Chemical structure of various building blocks used for the fabrication of NP delivery systems. The list is not intended to be exhaustive, but rather to provide a summary of representative entities used to construct NPs.



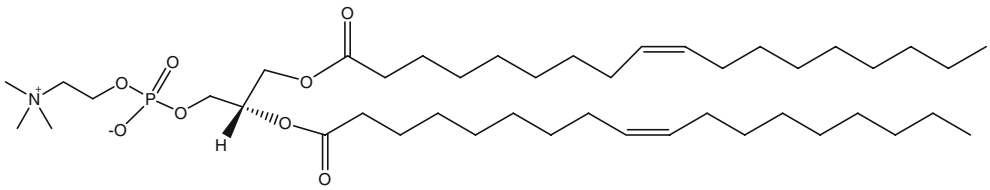
Dipalmitoylphosphatidylcholine (DPPC)



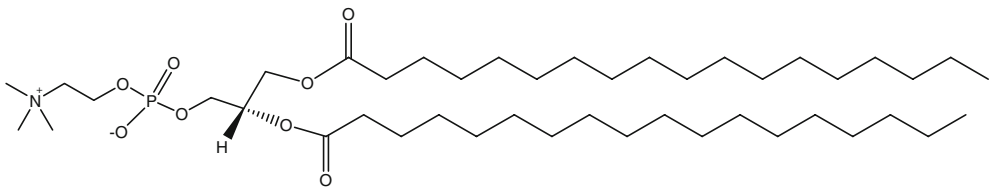
Dimethyldioctadecyl ammonium bromide (DDAB)



lysophosphatidylcholine (LPC)



1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE)



1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)

Fig. 3. (continued).

ical systems and their suitability for chemical modifications (41). Proteins and polysaccharides are the main natural polymers for this purpose. The former typically include albumin, collagen and gelatin, while the latter contains chitosan, cellulose, alginate, hyaluronan, pullulan and dextran (14,41). In particular, the abundant multifunctional moieties, such as amines, carboxylic groups and thiol groups, provide exciting opportunities for covalent or non-covalent interactions with therapeutic agents and tailoring the carrier for tissue-specific targeting.

Albumin has been extensively studied for NP fabrication by using emulsion/solvent extraction, simple and complex coacervation processes (18). Simple coacervation method under mild conditions has been considered the preferable means for encapsulation of growth factors. Conventional albumin NPs prepared by this method often use glutaraldehyde (GA) as a cross-linker to stabilize the particles (56–58); however, GA may react with encapsulated drugs during NP cross-linking (59,60) and it must be completely removed before clinical use owing to its known carcinogenicity. One alternative approach to stabilize the NPs is to use a non-covalent coating on NPs surfaces; polycations such as polyethylenimine (PEI) and poly-*L*-lysine (PLL) were used to coat the albumin NPs in aqueous medium. Such a coating not only stabilized the particles, but also controlled the release of a growth factor (BMP-2) from the system (61,62). In this system, albumin NPs achieved high BMP-2 encapsulation efficiency (~90%) and BMP-2 release could be controlled by the amount of PEI coated on the NPs (Fig. 4). Toxicity of cationic polymers is a concern, but the bioactivity of the released BMP-2 was retained based on an established *in vitro* bioassay. Chitosan NPs are also promising carriers for growth factor delivery. bFGF was encapsulated in chitosan NPs prepared by an ionotropic gelation process based on the interaction between cationic chitosan and anionic tripolyphosphate (63). The encapsulation efficiency was relatively

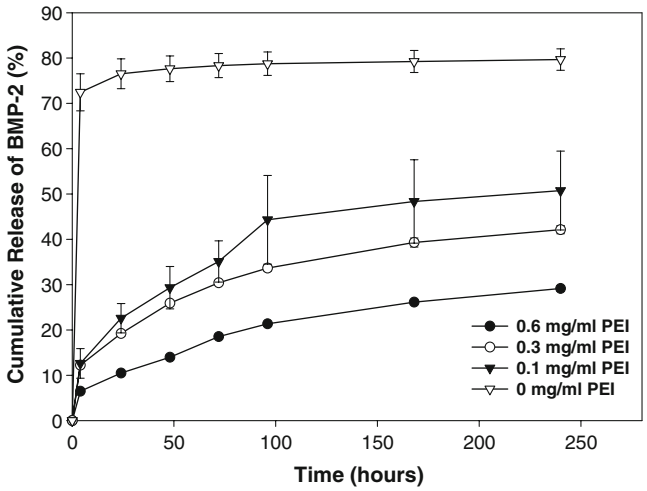


Fig. 4. The release profile of BMP-2 from the PEI-coated BSA NPs with different PEI coating concentrations. The release was performed after encapsulating ^{125}I-labeled BMP-2 in BSA NPs and using a release medium of DMEM containing 1% penicillin/streptomycin at 37±1°C. There was a burst release of BMP-2 for uncoated BSA NPs within hours of incubation in the release medium. The burst release was effectively suppressed by the PEI coating. Note that the gradual reduction of BMP-2 release as the PEI coating concentration was increased from 0.1 to 0.6 mg/mL. In case where SDs are not seen, they are smaller than the symbols (n=2; Ref. (62)).

low (~27%), probably due to cationic nature of both bFGF and chitosan, and ~70% of bFGF was released intact after 24 h. Injectable NPs for VEGF delivery were developed by coacervation of VEGF-bound dextran sulfate with chitosan (64). The encapsulation efficiency of VEGF was 50–80%, and VEGF release into a phosphate buffer persisted for >10 days with ~75% of the encapsulated VEGF released. Similar to NP-based delivery of EGF (55), the NP-delivered VEGF significantly stimulated endothelial cell proliferation in comparison to VEGF in solution. The same strategy was employed to construct NP system for another dextran sulfate-bound growth factor, FGF-10 (keratinocyte growth factor-2, or KGF-2), and the proliferation of human umbilical cord vascular endothelial cells was found to be significantly enhanced as compared to the free protein (65). Chitosan, dextran sulfate and PLL were also used for layer-by-layer fabrication of nanocapsules for bFGF encapsulation (66,67). One concern with chitosan is its poor solubility or insolubility in water, which will complicate its metabolism *in vivo* (14). Dextran was also used to prepare NPs for BMP-2 delivery to stimulate proliferation and differentiation of bone marrow stem cells *in vitro* (68).

A significant issue associated with natural polymers is widely different chemical compositions and physical properties depending on the variations in sourcing. This makes the characteristics and especially *in vivo* performance of NPs relatively less predictable. This is unlike highly precise synthetic polymers whose known chemical composition provides more predictable performance. Natural polymers are often mildly immunogenic (23), and the use of non-recombinant proteins are concerning for diseases transmission (18). Proteins produced from recombinant technology or plant origin may be preferable (69).

Polymeric Micelles

Amphiphilic polymers typically self-assemble in aqueous milieu to form colloidal NPs called micelles (70). Such polymeric micelles have a unique core-shell structure, with an inner hydrophobic core and an outer hydrophilic shell. Owing to smaller size (10–100 nm) and a narrow size distribution, polymeric micelles have several advantages, such as high extravasating and tissue penetrating ability, and reduced toxicity. The structure and physiochemical properties of the micelles can be modulated by the nature of polymer constituent. Micellar formulations have been used primarily for antitumor drug delivery in clinical or preclinical trials (71), but they are beginning to be explored for growth factor delivery. Lee *et al.* reported bFGF entrapment in heparin-conjugated Tetronic®-PCL (Fig. 3) polymeric micelles as an injectable vehicle for bFGF delivery (72). The block copolymer Tetronic®-PCL was synthesized by bulk ring-opening polymerization, and heparin was subsequently conjugated to this copolymer. The polymeric micelles, prepared by single emulsion/solvent evaporation method, demonstrated high bFGF loading efficiency (~70%) due to the specific interaction between bFGF and heparin, which was located mainly on the outer shell of the micelles. The bFGF release from the micelles was almost complete over a 2 month-period without an initial burst release. Interestingly, the same group later reported a dual release of bFGF and indomethacin from this micellar system, after indomethacin was encapsulated in the hydrophobic core and bFGF entrapped in the outer shell by heparin (73).

Polymeric Dendrimers

Dendrimers are highly branched, globular macromolecules with many arms emanating from a central core (74). The stepwise synthesis of dendrimers provides precise control over the branching pattern, molecular weight, density, polarity, solubility and surface functional groups. Due to the well-organized synthesis strategy, dendrimers have uniform nano-scale dimensions of ~20 nm (75). For drug delivery purposes, dendrimers should be water soluble and biocompatible with tissues (76). One of the most widely used dendrimers is polyamidoamine (PAMAM). Generation 5 PAMAM dendrimers were conjugated with EGF (77), and the dendrimer-conjugated EGF effectively stimulated cell growth to a greater extent than the free EGF. The conjugate was suggested to favor cross-linking of the receptor, resulting in a sustained EGFR-mediated signal transduction and increased cell growth. This system was also reported as a targeting system for EGF receptor over-expressing tumors (78,79), in addition to VEGF-conjugated dendrimers in the case of VEGF receptor over-expression (80). The dendrimers, however, displays significant cytotoxicities and liver accumulation due to high density of polycationic (amine) surfaces, which need to be charge-neutralized (e.g. acetylation) or chemically modified with anionic moieties (e.g. carboxylation) (74) for improved biocompatibility.

Other Nanoparticulate Systems

Although the delivery configurations summarized above are thought as the most versatile, a few other 'nano' materials

have also been utilized for growth factor delivery. For example, ceramic materials, exemplified by tricalcium phosphate, hydroxyapatite (HA) and carbonate apatite, are often used in hard tissue applications to provide the needed mechanical strength. Nano-sized particles of calcium sulfate were synthesized and used for PDGF delivery for enhancement of bone tissue regeneration (81). Histological analysis in an animal model revealed a better quality of newly-formed bone, in terms of tissue type and space maintenance for the guided bone regeneration, by the NP system than the PDGF solution.

Since growth factors are expected to readily absorb on and desorb from inorganic NP surfaces, they may need to be incorporated into other 'protective' environments to prevent burst release or enzymatic degradation. Calcium phosphate NPs were employed to absorb BMP-2 and then encapsulated in PLGA microspheres for tunable release of BMP-2 as the acidic degradation product of PLGA manipulated the dissolution of the basic inorganic substrate (82). A controllable release of BMP-2 was obtained for over 7 weeks in a reasonably linear profile, and the released BMP-2 was able to induce much higher osteocalcin expression than those released from the collagen sponge *in vitro*. A nanocomposite formed by cross-linking of HA nanocrystals with collagen was also used for BMP-2 delivery after protein adsorption on HA. An animal study showed that the HA/collagen composite was effective in controlling both the mechanical strength and the bioresorbability, and the new bone formed in and around the implant (83).

Quantum dots (QDs) were conjugated to β-NGF by a streptavidin-biotin immobilization method and used for NGF delivery (84). The β-NGF activity was retained on β-NGF-QD conjugate, but lower than the free β-NGF and β-NGF-biotin, probably due to the steric hindrance of the presence of the bulky QD. Despite this, the activity of β-NGF in the β-NGF-QD conjugate was significant and initiated neuronal differentiation in PC12 cells.

TARGETED NANOPARTICULATE SYSTEMS

One of the most promising attributes of NPs is their potential for targeting to specific tissues. While offering great opportunities, designing tissue-seeking NPs is a challenging task that requires overcoming physiological and biochemical barriers (85). For intravenously administered NPs, *in vivo* fate of NPs is predominately determined by their size and surface charge. To avoid opsonization and subsequent phagocytosis, and to remain in circulation for sufficient time to reach the targeted site, NPs are generally surface modified with PEG and their size is restricted to <200 nm. Different tissues possess unique barriers for delivery systems. For instance, the BBB restricts transportation of even small molecular weight drugs from circulation to the brain. Bone possesses a membrane consisting of lining cells, which function as a marrow-blood barrier with pores of approximately 80–100 nm (86). These strict barriers which complicate the microenvironment *in vivo* could account for unsatisfactory performance of '*in vitro* engineered' systems. Identifying the targeting ligand and designing the appropriate NPs are keys for successful delivery of growth factors to the site of action at therapeutic levels for a desired period of time. Table II summarizes the targeting achieved for several growth factors.

Receptor-Mediated Targeted Delivery

Unique cell surface receptors on specific cells or elevated receptor levels on diseased cells provide possible targets for active delivery of growth factors. With the appropriate ligand, the NPs can be induced to undergo enhanced and/or specific cellular uptake. The BBB imposes one of the strictest barriers for therapeutics delivery of drugs, especially for transport of hydrophilic compounds, small proteins, and charged molecules into central nervous system (87). This barrier is formed at the level of the endothelial cells of cerebral capillaries; tight continuous circumferential junctions between cells restrict any aqueous paracellular transport (88). Given the near impossibility of growth factors to cross BBB, NPs can be harnessed to transport growth factors across the BBB. Coating NPs with polysorbates is known to result in greater transport of NPs across the BBB (88). The polysorbate-80 on the surface of the NPs seemed to facilitate adsorption of apolipoprotein E in plasma, turning the NPs into low density lipoprotein (LDL) particle 'mimics' and interacting with LDL receptors on BBB endothelial cells (88). It was also suggested that apolipoprotein A-I adsorbed on polysorbate-80 coated NPs interacted with the scavenger receptor SR-BI at the BBB (89), since a significant correlation between the adsorption of apolipoprotein A-I on NP surfaces and drug delivery across the BBB was observed. Similarly, poloxamer-188 (Pluronic® F68) was also utilized to coat NPs for facilitated brain delivery of NPs (90). While this may increase brain targeting, it remains to be seen if such a strategy also increases tissue distribution of NPs non-specifically.

While tight junctions at BBB can be ~100 times less permeable than the other capillary endothelium (91), the BBB still displays lipophilic properties of a continuous cell membrane. Liposomes, therefore, may be preferred for brain delivery. Xie *et al*. studied the targeting ability of a liposome-based NGF delivery system (35). In this system, Cereport (RMP-7, receptor mediated permeabilizer-7), a bradykinin analogue with a longer half-life and greater selectivity for B2 receptor on brain microvascular endothelial cells, was combined with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*n*-(poly (ethylene-glycol))-hydroxyl succinimide (DOPE-PEG-NHS) to obtain DOPE-PEG-RMP-7. The DOPE-PEG-RMP-7 was incorporated onto surfaces of liposomes, which were prepared from soybean phospholipids and cholesterol to form sterically-stabilized liposomes. The encapsulation efficiency of NGF was 34%, with the size <100 nm. Using an *in vitro* BBB model as well as a rat intravenous injection model, liposomes were shown to enhance the transport of the NGF across the BBB as compared to the free NGF, and the highest transport rate was obtained with the liposomes containing RMP-7 as the targeting ligand. The average concentration of NGF in the brain was almost 10 times more in the case of targeted delivery as compared to free NGF. Compared to non-targeted liposomes, targeting efficiency of RMP-7 guided liposomes was ~2.1 times higher. A physical mixture of liposomes and RMP-7 was not that effective, and this was attributed to different arrival time of RMP-7 and liposomes at BBB, which results in non-synchronous "opening" of BBB

Table II. Summary of Studies on Targeted NP Systems for Growth Factor Delivery

Growth factor	NP system	Route	Study outcome	Reference
bFGF (FGF-2)	Mannan modified PCL-PEG-PCL	SC	bFGF-specific autoantibody titer in mice was significantly higher when bFGF was delivered with mannan-bearing NPs.	(52)
	PLGA	IV	>80% enhancement in diameter of the posterior collateral arterial vessel and an ~11-fold increase in flow capacity of this vessel as compared to BSA NPs-treated control.	(45)
NGF	PBCA coated with polysorbate-80	IP	Injection of PS-80 coated NGF-NPs showed 1.8~2.9-fold higher capacity in the restoration of motor activity than the control (MPTP injected, but no NPs) 7 days after injection. The motor activity was completely restored till day 21 in the NPs treated group, but not in the control.	(54)
	DOPE-PEG-RMP-7 liposome	IV	The targeting efficiency of RMP-7 guided liposome was ~2.1 times higher than the non-targeted liposomes.	(35)
HGF	DOPE-PEG-RGD liposome	IP	HGF encapsulated DOPE-PEG-RGD liposomes stimulated the remission of liver cirrhosis to a significantly higher extent than HGF in liposome without RGD or HGF alone.	(34)
BMP-2	Magnetic EPC liposomes	Topical injection	Magnetic liposomes with BMP-2 showed 1.5~1.7-fold higher radiographic scores and bone formation areas at the defect site than BMP-2 liposomes without magnetite 9 weeks post-operation.	(31)
TGF-β1	Magnetic EPC liposomes	Topical injection	Abundant chondrocyte-like cells by histological analysis and clear positive immunohistological staining around the chondroncyte-like cells at the defect site 8 weeks after treatment with magnetic liposomes containing TGF-β1, but not in other groups.	(32)

SC subcutaneous injection; *IV* intravascular injection; *IP* intraperitoneal injection; *PCL* poly(ε-caprolactone); *PEG* polyethylene glycol; *PLGA* poly(DL-lactide-*co*-glycolide); *PBCA* poly(butylcyanoacrylate); *MPTP* a neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; *DOPE* 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; *RMP-7* receptor mediated permeabilizer-7; *RGD* arginine-glycine-aspartate peptide; *EPC* egg phosphatidyl-choline

by RMP-7 (unlike RMP-7 physically inserted into liposomes) (35). Other ligands to facilitate drug delivery across the BBB include transferrin (92) and apolipoprotein E (93, 94). Peptidomimetic monoclonal antibodies may be alternative ligands, since they can cross the BBB via receptor-mediated transport and act as molecular "Trojan horses" to assist the proteins and other drugs across the BBB (see (91) for a review on this subject).

The sterically stabilized liposomes (SSLs) were employed for targeted delivery of HGF to liver for remission of liver cirrhosis (34). The liver cirrhosis originates from an excessive deposition of extracellular matrix by the activated hepatic stellate cells. A desirable therapeutic strategy is to interfere with the activation process of the stellate cells. The cyclic RGD peptide C*GRGDSPC* is known to target the collagen type VI receptors on stellate cells (95), and specifically inhibit the attachment of collagen type VI. Cyclic RGD peptide was used as the targeting ligand on SSLs, which were prepared with EPC/cholesterol/DOPE-PEG/DOPE-PEG-MAL, with the maleimide (MAL) group for cyclic RGD coupling. The obtained HGF-SSL-RGD had a size of ~90 nm, and encapsulation efficiency of ~30% with HGF (34). In a rat model, the HGF-SSL-RGD was shown to improve the efficiency of HGF to a significant extent in reducing liver cirrhosis in rats, which is higher than the SSL-HGF without the RGD, as well as HGF alone. However, there was no quantitative targeting efficiency reported in this study. It is worthwhile to mention that hepatic neoplasms were induced in rats treated with the non-targeted SSL-HGF and HGF alone, but not in the targeted group, which may indicate a reduced frequency of adverse events due to targeted delivery of the growth factor. Although the reason for this phenomenon was not clear, it is possible that non-specific distribution of HGF and uptake by a different population of hepatic cells might have been the reason for this unexpected result. The RGD density on SSL surfaces was not reported in this study, which is important parameter for design of tissue-specific NPs.

Utilizing bFGF to improve the humoral immunity was investigated by Gou *et al.* (52) with the hypothesis that epitope mimicry (i.e., similarity between infectious agents and self-proteins) might induce autoimmune response against self-molecules. Overcoming tolerance against self-molecules, such as bFGF and VEGF, might be a useful approach for therapy in certain cancerous growth. To test this, bFGF was loaded in mannan-guided PCL-PEG-PCL NPs, and subcutaneously injected into C57BL/6 mice for 3 weeks (52). Mannan was considered to be a useful ligand that can target to dendritic cells capable of initiating and amplifying immune responses. The results showed that bFGF-specific autoantibody titer was significantly higher when bFGF was delivered with mannan-bearing PCL-PEG-PCL NPs, which seemed to specially interact with lectin-like

surface receptors on dendritic cells. The enhanced uptake of mannan-modified NPs increased the bFGF delivery, breaking the innate tolerance against bFGF.

Adsorption-Mediated Targeted Delivery

Bone is distinguished from other tissues by the presence of the inorganic HA, which is a unique template for selective drug delivery (96). BPs are a class of chemical compounds that have "bone-seeking" properties. A series of functional BP derivatives have been synthesized and used for protein delivery to bone by chemical conjugation, such as bovine serum albumin, lysozyme, bovine fetuin and bovine non-specific IgG, as well as BMP-2 derived from *E. coli* (96). The highest *in vivo* targeting efficiency determined in rats was ~7.5-fold higher than the free proteins. Such BP compounds also have great potential for targeting NPs to bone after covalent or non-covalent conjugation to particles. Alendronate, a BP-drug used for osteoporosis treatment, was employed to fabricate drug-incorporating NPs for bone delivery (97). NPs (40–60 nm) were prepared from PLGA-alendronate conjugate and PLGA-PEG copolymer by dialysis method. The HA affinity of the NPs was enhanced after alendronate modification. Although this system has not been tested *in vivo*, it is a promising system for growth factor delivery to bone as: (1) the PEG segment is beneficial for avoiding the opsonization and clearance by the RES; (2) the bone affinity of alendronate as the targeting ligand is well-established; and (3) the NP size was <80 nm, the expected pore size on bone-marrow barrier required for bone delivery. The growth factor entrapment in these NPs has not been reported, but the past history of PLGA for entrapment of growth factors in microparticles is likely to facilitate these efforts.

A liposomal system was also constructed for bone delivery by using BPs (98). The carrier was prepared from a BP modified lipid, cholesteryl-trisoxyethylene-bisphosphonic acid (CHOL-TOE-BP), cholesterol, EPC, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE)-PEG by lipid film extrusion method. The size of the prepared liposome was 100–135 nm (i.e., larger than the 'expected' pore sizes at bone-marrow interface), and *in vitro* HA affinity increased remarkably after BP attachment on liposomes. The *in vivo* bone targeting and drug release of this liposomal system has not been reported. Since the liposomes were generally regarded as "soft" NPs, transport through bone vasculature membrane should be easier than the "solid" NPs, such as polymeric or ceramic NPs (13), as long as integrity of the liposomal particles is maintained.

There are several reports on NP formulations of growth factors for bone delivery, but these systems are intended for local delivery (i.e., administration by implantation or local injection). Local delivery of NPs (incorporating growth factors) with scaffolds is a more popular choice for inducing new bone formation with mechanically resilient scaffolds. Moreover, the expression of biomolecules with biological affinity suitable for targeting may be low in mineralized tissues, which implies that utilizing biological affinity for bone-specific drug targeting other than HA is difficult (99). The pharmacologic effects of BP, i.e., apoptosis of osteoclasts, could be additionally utilized to achieve a synergetic effect with bone regeneration activities of growth factors.

Induction-Mediated Delivery

NPs responsive to physical induction has been utilized for targeted delivery of growth factors. Low-frequency ultrasound, for example, can destruct circulating microbubbles and selectively increase the microvessel permeabilization so that the circulating materials can extravasate into target tissue. A microbubble contrast agent-based system was used to facilitate FGF-2 release from PLGA NPs deposited at the skeletal muscle in a mouse model of hind-limb ischemia (45). The FGF-2 loaded PLGA NPs (~100 nm) was prepared from double emulsion method and co-administered with microbubbles through a catheter in an ischemic region exposed to the ultrasound. The results showed that the ultrasound facilitated NPs deposition at the ischemia site, and the sustained FGF-2 release elicited arteriogenic remodeling more significantly than the control groups (i.e., BSA loaded NPs), with >80% enhancement in diameter of the posterior collateral arterial vessel and a ~11-fold increase in flow capacity of this vessel as compared to BSA NPs-treated control. However, FGF-2 loaded NPs without ultrasound application were not examined in the study, which is necessary to demonstrate the efficacy of the ultrasound application. Nevertheless, by controlling the ultrasound and microbubble parameters, this method can be widely applied to enhance the delivery of NPs to other organs.

Liposomes have been designed for bone targeting based on magnetic guidance. BMP-2 was encapsulated in magnetic liposomes, prepared from EPC, cholesterol and magnetite particles for topical injection into bone defect in rats (31). The NPs had a size of ~100 nm, and encapsulated BMP-2 with 22% efficiency and guided to a critical-sized bone defect with permanent magnet attachment. The magnetic liposomes showed complete bone reunion in 9 weeks, while the group non-magnetically delivered BMP-2 showed approximately half of the bone formation based on micro-CT assessed bone volume. The magnetic liposomes seemed to be accumulated at the target site for prolonged BMP-2 residence time. Magnetically-guided liposomes could be adopted for other tissues with the appropriate design of the magnetic field. Although the long-term consequences of magnetic treatment remains to be explored (especially the fate of magnetic particles), the same group also developed TGF-β1 encapsulated magnetic liposomes for treatment of articular defects in a rabbit model (32).

GROWTH FACTOR DELIVERY FOR TISSUE REGENERATION

Approaches to regain tissue function by inducing the intrinsic repair process, rather than replacing the tissue with unnatural materials, are increasingly desired. Since growth factors are expressed during different phases of tissue healing and control tissue regeneration, they can be employed as 'stimulants' in therapeutic repair. Growth factors were incorporated in various NP delivery systems based upon the unique requirement of specific target tissues. A wide range of

materials were used for such NPs, which have been delivered by local or systemic administration (Table III).

Bone and Cartilage Regeneration

To obtain mechanically-resilient engineered bone that is permissive for neo-vascularization, osteoinductive and osteoconductive matrices need to be developed. Several growth factors, especially the members of transforming growth factor-β (TGF-β) superfamily such as TGF-β1, TGF-β3, BMP-2, and BMP-7, have been used to accelerate and enhance bone formation. These osteogenic growth factors were intended to induce migration, proliferation, and differentiation of bone-forming and bone remodeling cells. Osteoconductive materials include porous coralline ceramics, exemplified by HA and tricalcium phosphate, collagen and synthetic materials such as PLGA and porous metal implants (100). The osteoconductive scaffold provides an appropriate matrix for osteoprogenitor cell attachment and a porous structure for newly formed bone tissue and vascular vessels.

The growth factors for bone tissue engineering were primarily delivered locally, whereby a bioactive growth factor is maintained at high concentration locally without systemic build-up. The current clinical devices incorporate the growth factor by the impregnation of protein solutions into biomaterial scaffolds (101). This passive adsorption may not afford precise control over the protein release (102). A more attractive approach is to incorporate the growth factor containing-NPs in matrices or scaffolds (103, 104), so that the release of growth factor can be controlled in a more desirable way, since the NPs can be engineered appropriately for the preferred release pattern (105, 106). Wei *et al.* investigated BMP-7 encapsulated PLGA NPs which were then post-seeded into PLA scaffolds (44). The *in vitro* release kinetics indicated that the delivery system provided a sustained BMP-7 release for up to 6 weeks, and the release profile was controlled by PLGA molecular weight and composition. This scaffold induced significant bone formation while passive adsorption of BMP-7 alone into the scaffold failed to generate bone at 6 weeks. The difference was attributed to better localization of BMP-7 with NP-impregnated scaffold.

Several growth factors, such as bFGF, BMP-2, and TGF-β, were known to bind heparin avidly. Heparin was accordingly used in the fabrication of NPs to sustain growth factor release (107). Park *et al.* investigated the controlled release of growth factor from TGF-β3-containtaing heparin/poly(L-lysine) NPs immobilized on PLGA matrix (108). The NPs and matrix combination provided sustained release of TGF-β3, which were able to promote neocartilage formation from mesenchymal stem cells *in vitro*. Chung *et al.* studied the BMP-2-loaded, heparin-functionalized PLGA NPs in fibrin hydrogel in a rat calvarial defect model (46). This NP system was composed of a PLGA core and an F-127 shell, where heparin was entrapped on the surface for BMP-2 complexation. The NPs were then incorporated into a fibrin gel which served as a matrix for new bone formation. Significantly higher bone formation was found in the

Table III. *In vivo* Investigations of NP Systems for Growth Factor Delivery for Tissue Induction or Regeneration

Growth factor	NP system	Regeneration	Targeted	Targeted tissue	Ligand/tissue interaction	Reference
bFGF (FGF-2)	PLGA	Arteriogenesis	Y (systemic)	Skeletal muscles	Ultrasound facilitated NP deposition	(45)
	Gelatin NPs	Nerve	N			(120)
	Peptide amphiphile	Angiogenesis	N			(127)
	Heparin-conjugated PLGA	Angiogenesis	N			(126)
	Mannan modified PCL-PEG-PCL	Anti-cancer effect	Y (systemic)	Dendritic cells	Mannan and lectin-like receptors on DCs	(52)
NGF	PBCA NPs coated with polysorbate-80	Nerve	N			(54)
	Streptavidin (strep-QDs)	Nerve	N			(118)
	DOPE-PEG-RMP-7 liposome	Nerve	Y (systemic)	Brain	RMP-7 and B2 receptor on BBB	(35)
	P80 coated PBCA	Nerve	Y (systemic)	Brain	P80-apolipoprotein E and BBB	(54)
HGF	DOPE-PEG-RGD liposome	anti-fibrotic effect	Y (systemic)	Liver	Cyclic RGD and hepatocytes	(34)
PDGF	Calcium sulfate	Bone	N			(81)
BMP-2	Heparin conjugated PLGA NPs	Bone	N			(46, 110)
	PLGA/F-127 /heparin NPs	Bone	N			(108)
	Magnetic EPC liposomes	Bone	Y (local)	Bone	Magnetic induction	(31)
	HA/collagen nanocomposite	Bone	N			(83)
	PLGA/HA NPs composite	Bone	N			(102)
	Peptide amphiphile	Bone	N			(103)
EGF	DPPC and LPC liposome	Teeth	N			(30)
	PEG coated liposome	Gastric ulcer healing	N			(29)
BMP-7	PLGA NPs	Bone	N			(44)
TGF-β1	Heparin/PEI NPs	Cartilage	N			(109)
	Magnetic EPC liposome	Cartilage	Y (local)	Bone	Magnetic induction	(32)

Y Yes, *N* No

BMP-2-loaded NP-fibrin gel complex 4 weeks post surgery, suggesting higher localization of BMP-2 *in situ*. Another NP system for BMP-2 delivery involved chemical conjugation of heparin to amino-terminated PLGA NPs (109). The NPs were then suspended in a fibrin gel and osteogenic efficacy of this system was evaluated in a rat ectopic implant model. BMP-2 delivered by heparin-conjugated PLGA NPs in fibrin gel was able to induce bone formation to a much greater extent than fibrin gel containing normal PLGA NPs, with 2.0-fold greater bone area and 3.5-fold greater calcium content after 8 weeks of implantation. The successful results obtained with these approaches should be applicable to other heparin-binding growth factors and, more generally, any ligand-growth factor combination.

Polycation-coated BSA NPs were recently developed by our group (61, 62). Polycations such as PEI and PLL were used to coat the NPs, and control growth factor (BMP-2) release profiles. This approach can be widely applied to any bioactive molecules since it does not require specific interactions with a ligand such as heparin. The implantation of such NPs in a collagen scaffold prolonged the BMP-2 release in rat ectopic model and, by using PEGylated PEI for improved biocompatibility of the NPs, significant bone formation was obtained in this model (Zhang and Uludağ, unpublished).

Since new tissue formation requires cellular participation, co-delivery of growth factor with therapeutic cells is expected to lead to more extensive repair. On this front, TGF-β1-loaded PEI/heparin NPs were incorporated into injectable hydrogel constructs prepared from the thermoreversible polymer, poly (*N*-isopropylacrylamide-*co*-vinylimidazole), along with rabbit chondrocytes for cartilage regeneration (110). Another study investigated bone formation by BMP-2 using heparin-conjugated PLGA NPs and bone marrow-derived mesenchymal stem cells (BMMSC) (111). Undifferentiated BMMSCs delivered with BMP-2 loaded, heparin-conjugated PLGA NPs induced more extensive bone formation than either implantation of BMP-2 loaded NPs or osteogenically differentiated BMMSCs.

Skin Regeneration

In skin and wound healing, growth factors have the potential to accelerate the healing process by attracting cells into the wound site, promoting cell migration, stimulating the proliferation of epithelial cells and fibroblasts, as well as initiating the formation of new blood vessels and participating in the remodeling of the scar (112). The growth factors involved in these processes include PDGF, EGF, FGF, IGF, TGF-β and VEGF. Delivery of growth factors in NPs is attempted to reduce their side-effects, to provide an extended availability at the local environment, as well as to protect them against degradation by proteolytic enzymes.

Particulate systems used for wound healing are mostly at the 'micro' scale, such as TGF-β1 in gelatin microparticles (113), EGF in PLA microspheres (114), and bFGF in gelatin microspheres (115). Li *et al*. reported a PEG-coated liposomal NPs for oral delivery of EGF in treatment of gastric ulcer healing (29). The size of the liposomal NPs was 250–310 nm, prepared from DPPC and PC. The liposomal formulation was beneficial for protecting EGF against degradation by Caco-2 cell homogenate and facilitated EGF transport depending on the liposomal formulation. After oral delivery into rats, the bioavailability of EGF increased 1.7 and 2.5-fold for PC and DPPC liposomes, respectively. The healing of ulcers was significantly increased by DPPC liposomal formulation of EGF as compared to PC liposome formulation or free EGF. Resistance to enzymatic degradation, increased permeability and prolonged *in vivo* circulation contributed to improved performance of PEG-coated DPPC liposomes in oral EGF delivery. A series of bioadhesive liposomes was also reported for topical delivery of EGF, which was fabricated from gelatin, collagen and hyaluronic acid modified systems (116). The NP system constructed with dextran sulfate/chitosan for FGF-10 delivery also provided a promising system for systemic (intravenous) or local injection formulation for the wound healing therapy (65).

Nerve Regeneration

Diseases arising from nerve degeneration, such as Parkinson's, Alzheimer's and Huntington's, were extensively investigated in the last decade. As new therapeutics are identified for treatment of neurodegenerative diseases, effective delivery of these drugs to brain remains a challenge. High molecular weight, hydrophilic protein and peptide drugs cannot be transported across the BBB for a satisfactory therapeutic effect. Although several specialized approaches have been taken to overcome this delivery challenge (117), NPs is emerging as one of the most promising strategies for effective delivery to brain. The NPs surface can be modified with substrates for transporters expressed on BBB (e.g., lipoprotein transporters) so as to facilitate NPs endocytosis by the endothelial cells. The drugs may subsequently be released in these cells and diffuse into the brain interior or the particles may be transcytosed (88). The small size of the NPs is ideal for this transport. NPs may also be modified by bradykinin analogs, such as RMP-7, enabling the opening of the tight junctions for paracellular transport (118). The efflux transporters such as P-glycoprotein, on the other hand, tend to prevent drugs from entering the brain and the inhibition of such transporters might be necessary for effective delivery. However, these approaches might allow brain entry of unwanted molecules or toxins (87). Once the NPs are located in the brain, a slow release of the drugs should occur, exerting therapeutic effects without affecting other organs, which reduces the peripheral or systemic toxicities. Several mechanisms related to NP transport through the BBB were proposed and discussed in detail (88).

NGF is a neurotrophin responsible for the regulation of survival, differentiation, and maintenance of the responsive neurons. Other neurotrophins structurally related to NGF include brain-derived and glial-derived neurotrophic factors. Owing to the limitation of systemic administration, NPs were explored for NGF delivery by using NPs fabricated from PCL and PBCA with the polysorbate 80 coating. The latter probably mimics the LDL protein, apolipoprotein E, to interact with BBB for enhanced cellular uptake and promote active transport of NGF across the BBB. Polysorbate-80 coated NPs were employed for targeted delivery of NGF as an anti-Parkinsonian therapy in C57B1/6 mice (54). Upon systemic treatment with the NPs, a therapeutic effect was readily obtained and the NGF effect was shown to persist for 7 to 21 days. Liposomes have been also widely studied for NGF delivery to brain, such as sterically stabilized liposomes modified with PEG and BBB-targeting ligands (35). The

main concern with liposome delivery system is the *in vivo* instability (117). Quantum dots (QDs) were utilized as a new imaging technology, as one of its unique properties is the particular photostability. Coupling of NGF to QD was proposed and served as nanocarrier for NGF delivery, which also provided the convenient tracking and real-time analysis of individual QD-NGF receptor complexes within neuritis. Such a NP system was shown to be active in the differentiation, binding, internalization and transport of NGF (119).

The bFGF is capable of stimulating proliferation of a wide range of cells. It displays a protective neurotrophic activity in regional brain ischemia after covalent conjugation to an monoclonal antibody for BBB delivery (120). Gelatin NPs incorporating bFGF were intravitreally injected into rats for a protective effect against photoreceptor degeneration (121). The intraocular kinetics showed the injected bFGF-gelatin NPs can be delivered to the photoreceptor continuously with a sustained release in the vitreous for over 30 days. The bFGF-gelatin NPs had a significant protective effect on photoreceptor degeneration, morphologically and functionally, by local targeted delivery and sustained release of bFGF.

One study investigated different physicochemical characteristics, charge and lipid coating, on the ability of polysaccharide NPs to cross an *in vitro* BBB model consisting of bovine brain capillary endothelial cells and rat astrocytes (122). Without lipid coating, the neutral NPs showed 3-fold higher transport than the charged NPs; however, the lipid coating caused a marked increase in the transport of charged NPs, with a 3-fold increase for anionic NPs and 4-fold for cationic NPs. The lipid-coated cationic NPs were tested to transport albumin, which has a very low transport across the BBB. A 27-fold increase in albumin transport was obtained by such a system, which is very encouraging for delivery of growth factors by transcytosis through the endothelial cells in BBB.

The metallic NPs based on silver and iron were reported as imaging agents for central nervous system. However, several studies showed the toxic effect of such NP systems after exposure for a period of time or above a certain concentration (123, 124). To avoid the toxicity concerns, an approach that combined peptide technology with nanotechnology was summarized by Teixidó *et al*. for BBB delivery (125). The NP system in this approach was made from all-peptide components, including peptidic drug, peptidic NP and peptide coating as targeting ligand.

Angiogenesis

In vascular diseases such as coronary artery disease, formation of new vessels sprouting from the preexisting vessels is a promising solution. Angiogenesis is a complex process (126), involving endothelial cell activation, recruitment and migration to sprout the neovessels to finally the mural cells (pericytes or smooth muscle cells) making up the surrounding vessel wall for stabilization. Several growth factors, such as FGF, VEGF, PDGF-B, and TGF-β, exert influence at different stages of this process. The formation of an extensive network of blood vessels is generally required for the regeneration of tissues. Though the direct administration of growth factor in solution form is the simplest way to induce angiogenesis, the utilization of growth factors in such format is clinically limited due to low potency and systemic side-effects.

A heparin-conjugated PLGA NP system was developed for sustained bFGF delivery and potentiating the angiogenic efficacy of bFGF (127). The specific interaction between bFGF and heparin enabled the NP system to provide a controllable release of bFGF over 3 weeks with no initial burst release. Since these NPs embedded in a fibrin gel exhibited an increase in the duration of bFGF release, the angiogenic potential of bFGF was evaluated in a mouse limb ischemia model by subcutaneous injection. The results revealed that a single injection of bFGF in NP-fibrin system induced ~3.2-fold higher density of growing capillaries than daily injection of the same total amount of free bFGF for 4 weeks. The heparin amount conjugated to PLGA NPs was assumed to affect the bFGF loading efficiency, release pattern and subsequent angiogenesis; in this study, however, this issue was not explored, though it was demonstrated that the low molecular weight, or star-shaped PLGA exhibited up to 29-fold higher heparin conjugated amount than the high molecular weight, or linear PLGA. Another PLGA NP system for FGF-2 delivery was studied by using ultrasound for targeted arteriogenesis at the ischemic site (45) (See "Induction-Mediated Delivery").

A self-assembly system prepared from a peptide amphiphile ($CH_3(CH_2)_{14}CO$(AAAAGG GERGD)) was employed for sustained release of bFGF to enhance angiogenesis (128). The pharmacokinetics studies showed a 20-day retention of the bFGF at the injection site with the PA nanoscaffold, compared to a 2-day retention by injecting bFGF alone. This prolonged retention of bFGF offered a significant increase in the number of new vessels formed at the injection site: ~8 times higher for the nanostructured bFGF delivery system as compared to free bFGF after 7 days of subcutaneous injection. The tissue hemoglobin levels were about ~5 times higher for the nanostructured bFGF delivery system than the bFGF alone for over 28 days.

VEGF is an important angiogenic growth factor that plays a significant role in tissue engineering, and numerous systems were designed for effective VEGF delivery. However, the current matrix/scaffold/particulate systems are at the 'micro' scale. As the burst release is always an issue with such systems, NP systems may provide a more defined release pattern, prolonged growth factor presence at the defect site, and enhanced therapeutic effect. Several *in vitro* NP systems have been developed and characterized for VEGF delivery (see "TYPES OF NANOPARTICULATE SYSTEMS FOR GROWTH FACTOR DELIVERY") and their *in vivo* performances will be worthwhile to explore for an angiogenic effect.

CONCLUSIONS AND FUTURE DIRECTIONS

Efforts have been devoted to development of NP systems for growth factor delivery in recent years, and a significant expansion of this activity is anticipated in the near future. Growth factors possess enormous potentials in tissue engineering, and NP-based delivery systems for growth factors will be highly beneficial to augment their therapeutic powers, since NPs have the potential to overcome traditional limitations related to release kinetics and targeting capability. The success of growth factor delivery by NPs can be attributed to the targeting ability of the NP systems, which can improve the therapeutic index of the proteins while reducing their toxicity. By incorporating a targeting ligand into NPs, as well as taking advantage of physical induction at a local site, more site-specific targeting approaches are likely

to be designed. The choice of NP material has been relatively limited so far in growth factor delivery, but given the possibility of fabricating NPs from a wide array of biomaterials (18), there will be great flexibility in tailoring NPs for particular applications. This is especially critical for tissue regeneration efforts where the biomaterial properties could augment a reparative process, or hinder the process due to undesirable attributes of the material. Despite its recognized importance, there have not been systemic studies that probe the targeting efficiency of NPs as a function of particle size. This will greatly aid the field. Significant challenges exist in NP targeting efforts due to the complicated *in vivo* environment. Accommodation of one or more means of targeting in a single NP system might be a promising approach for enhanced targeting efficiency. For example, a NP system with double-targeting vectors was reported for liver-specific tumor delivery, which employed magnetic NPs for physical induction and an antibody for receptor-mediated targeting (129).

The exploration of mechanisms of growth factor release from NPs to control *in situ* presence of the proteins will be highly beneficial. The disassembly/dissolution and degradation of NPs are likely going to be the main mechanisms that control the release. However, these mechanisms could also be responsible for the instability of NPs when applied *in vivo*. The balance of NP resiliency to reach site of action while providing sustained release is a desirable goal to achieve. In this regard, core-envelope structure is a worthwhile approach to attempt. The envelope section can be designed with the purpose of protecting inner core, prolonging circulation time *in vivo*, targeting to site of action, and also controlling the release rate of growth factors from inner core. An engineered "nanocell" composed of a nuclear PLGA nanoparticle within an extranuclear PEG-phospholipid envelope was reported as a tumor therapy (130). As an alternative to growth factor release by diffusion, NP can be designed for disintegration by chemical cleavage with compounds present in the physiological system. The disulfide-linked NPs, in this regard, is a good example for environment-responsive system (131). Despite good understanding of *in vitro* degradation of NPs, little effort has been exerted to probe *in vivo* degradation of NPs. The impact of such studies on growth factor availability at the repair site is obvious. Such studies should also discriminate between growth factor release into a microenvironment (i.e., usually resulting in a pharmacological effect) *versus* NP loss (i.e., leading to non-pharmacological effect), which was not explored in the literature.

The "integrative" approach of co-delivery of synergistic growth factors to target multiple signaling pathways will be the likely trend in future growth factor delivery, given the potential to achieve elevated therapeutic efficacy by such a delivery approach. Key growth factors needs to be identified based on the understanding of their biological roles in a particular application and the precisely timing of growth factor release to achieve a sequential effect. However, the inappropriate combination of growth factors or improper release sequence of growth factors may produce little enhanced effect, even stimulate multiple signal pathways that produce undesirable effects (132,133). A series of dual delivery of growth factors have been reported, such as VEGF and PDGF for stimulation of, respectively, vessel growth and maturation/stabilization of the nascent vessels (134–136), and BMP-2 and IGF-I for elevated mineralization in bone repair (137). The sequential release was achieved by affinity differences of growth factors to system components, or spatially restricting the growth factors to different sections in delivery systems (Fig. 5). However,

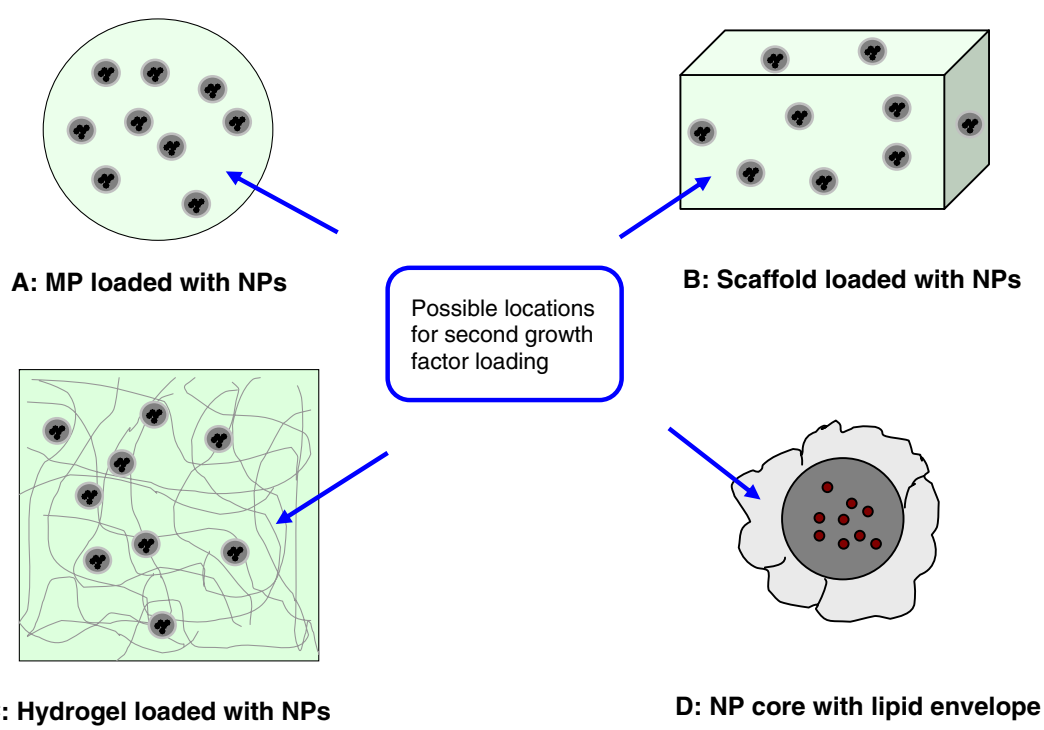


Fig. 5. Illustration of the combinational design of NPs with incorporation into a microsphere (*A*), a tissue-engineering scaffold (*B*), a hydrogel (*C*), and NP core with lipid envelope (*D*) for the sequential release of multiple growth factors. These systems also have the potential to offer a more sustained release of growth factor as compared to simple NP formulation of a growth factor.

none of these co-delivery systems was fabricated at the nanoscale and uniquely efficacious agents should arise from such an approach.

Engineering different sections in the NP delivery system, such as co-delivery of growth factors, diverse types of materials as NP components, as well as various means of targeting ligands for a combinatorial effect, is probably the future for growth factor delivered by NP system. The ultimate goal for such combination is to integrate the advantages of different elements and minimize the disadvantage of each section for desired release kinetics, elevated targeting specificity, and the enhanced therapeutic effect that would not be achieved by either of the singular means. Continued exploration of novel NP system strategies, which can be used to provide spatiotemporal control of growth factors, may offer a gateway to the next generation of NP systems for growth factor delivery.

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