PART VI

31

DELIVERY SYSTEMS MADE OF NATURAL-ORIGIN POLYMERS FOR TISSUE ENGINEERING AND REGENERATIVE MEDICINE APPLICATIONS

ALBINO MARTINS,[1,2] HELENA FERREIRA,[1,2] RUI L. REIS,[1,2] AND NUNO M. NEVES[1,2]

[1] *3B's Research Group – Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal*
[2] *ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal*

31.1 INTRODUCTION

The administration of a bioactive factor aims to obtain a specific therapeutic response at the anatomical target, without leading to the appearance of undesirable side-effects. In conventional therapies, drugs are administered freely, and consequently may be distributed arbitrarily through the body, reaching tissues where a pharmacological response is not observed, but could be responsible by the appearance of toxic effects. In fact, some drugs administered via conventional pharmaceutical formulations have been withdrawn from the market, due to their unexpected toxic effects in nontarget tissues [1, 2]. This can be overcome, or at least reduced, by the entrapment of the therapeutic agent in delivery systems. With delivery systems, the pharmacokinetics and pharmacodynamics of a drug can be enhanced, increasing its therapeutic index [3] Additionally, delivery systems offer other advantages such as protection of the

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entrapped material against degradation by, for example, light or enzymes, incorporation of both hydrophilic and hydrophobic compounds, and they make possible drug targeting with controlled release [3–5]. Indeed, delivery systems can be used to carry not only synthetic molecules, but also peptides, proteins and nucleic acids whose protection from degradation is crucial.

Besides the conduct of the pharmaceutical compound at the site of action, other essential functions that must be provided by these carriers is to release them at concentrations lying within the therapeutic range during a certain period of time (Fig. 31.1) [6]. The possibility of drug targeting can thus decrease the dose required to observe the therapeutic response and avoid side-effects. From Figure 31.1 it is possible to observe that drug blood concentrations higher than the minimum toxic concentration (MTC) present a toxic risk for patients. On the other hand, concentrations below the minimum effective concentration (MEC) lead to drug quantities insufficient to treat the disease. In this sense, the devices must be designed to achieve therapeutically relevant concentrations through controlled release. This leads to reduced dosing frequency, culminating with a greater patient acceptance and compliance, thereby improving human health [7]. Taking into account that the main aim of a delivery system is to release a defined therapeutic agent at a controlled rate, the mechanisms that contribute to this will be addressed within this chapter.

As derivatives of extracellular matrix components, natural-origin polymers can function not only as bioactive factor delivery systems and DNA complexing agents, but also as structural scaffolds for tissue engineering applications [8]. Typically, the bioactive factors are incorporated within the internal structure of biomaterials during the processing steps or are otherwise bonded or adsorbed at the surfaces of the

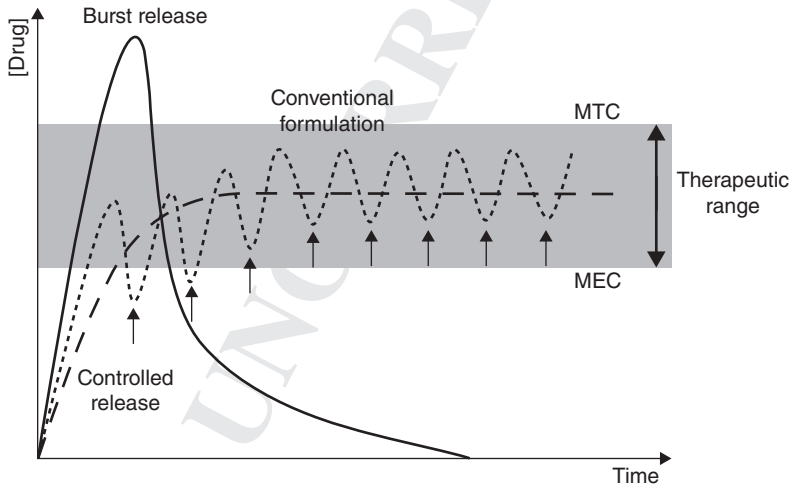

FIGURE 31.1 Drug concentration along time for a conventional formulation administered at pre-defined time intervals (↑) and for delivery devices with or without controlled release, showing the therapeutic range and the minimum toxic concentration (MTC) and the minimum effective concentration (MEC). Adapted from Liechty et al. [6].

structured biomaterials, depending on the actions of the therapeutics and the target cells/tissues. While the former is more relevant to gain long-term therapeutic effects in a more sustainable and time-dependent manner, the latter mainly targets direct actions with the contacting cells. Depending on the paths of action and roles of the bioactive factors, the design of delivery matrices and vehicles should be carefully considered, either targeting binding to cell membrane receptors (growth factors), penetration into cell membrane (drugs or genes) or even transport to nuclear pores for direct genetic modification (genes). Therefore, this chapter will discuss the current status on the use of natural polymers for drug, protein and gene delivery, with a special focus on research with applications in tissue engineering.

31.2 ADVANTAGES AND DISADVANTAGES OF NATURAL POLYMERS-BASED DELIVERY SYSTEMS

Although the majority of the delivery systems are based on synthetic polymers, natural-origin polymers have the advantage of having the intrinsic property of environmental responsiveness via degradation and remodeling by hydrolysis or cell-secreted enzymes (Table 31.1). They are generally nontoxic, mucoadhesive, biocompatible and biodegradable, and therefore can readily be incorporated into oral delivery or bulk matrix delivery systems. Additionally, the typical biodegradability of the natural polymers present an advantage in terms of originating nontoxic compounds that can be eliminated by normal clearance processes of the body. The crosslinking of natural polymers in nontoxic ways, such as using agents with reduced toxicity (e.g. N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), dehydrothermal treatment), and forming interpenetrating networks has, thus, been pursued for achieving structural integrity of the host scaffolds and the sustainable release of incorporated bioactive factors [9–12].

TABLE 31.1 Advantages and disadvantages of natural polymers-based delivery systems

Advantages	Disadvantages
• Readily available from natural sources • Biodegradability • Biocompatibility • Hydrophilicity • Availability of functional groups for drug immobilization • Presence of biologically recognizable moieties that support cellular activities	• Faster drug release • Shorter biodegradability • Necessity of crosslinking for stability in physiological conditions • May evoke immune/inflammatory responses • Difficult to isolate natural polymers with high purity and at industrial scale amounts • Batch to batch variation • May contain pathogens from other species • Regulatory issues for commercial approval

The most common scaffolds used to incorporate bioactive factors are polymers, and the bioactive factors have been incorporated either during the process of the scaffolds or after their fabrication. The incorporation of bioactive factors within scaffolds needs careful consideration to maintain the bioactivity of the factors and consequently to potentiate their therapeutic effects. Although synthetic biopolymers have shown better mechanical properties than natural ones when processed by solvent-based techniques, the organic solvents used to dissolve the synthetic polymers are not readily available for the use of bioactive factors. Because most bioactive factors require water-based solutions, natural biopolymers are preferred over synthetic ones. Therefore, the sequestering of bioactive factors within natural polymers formulated in nanostructures helps avoid the harsh processing conditions of most synthetic polymers. Furthermore, in synthetic-origin delivery systems the hydrophilic bioactive factors are segregated and not homogenously distributed within the synthetic matrices. Many natural polymers including collagen, gelatin and chitosan, have charged functional groups and present a more or less ionic affinity to therapeutic biomolecules such as growth factors [13].

From the current research, there appear to be hints that natural polymeric carriers have a different mechanism for intracellular escape and transfection than their synthetic polymer counterparts. These differences can be at the cell surface level, the endosome release level, nuclear transport level, or any other potential rate-limiting steps [14]. Polymer molecular weight, charge density, as well as the possibility to change the overall drug or gene/polymer surface charge, surface ligands, and enhancement of availability near the cell surface are some of the factors that can be optimized to improve transfection efficiency of any given carrier [15]. Systematic studies varying these degrees of freedom should provide significant insight into development of natural polymers for use in gene therapy and tissue engineering [16].

31.3 FUNDAMENTALS OF DRUG DELIVERY

The entrapment or chemical conjugation of a therapeutic agent with a polymer for the treatment of a wide variety of diseases have received much attention over the past decades. Due to the advantages presented by natural polymers (Table 31.1), they have been continuously investigated to develop innovative and more effective and specialized release dosage forms. For example, insulin and pectin can be used to achieve a colon-specific drug delivery and using alginate is possible to obtain a gastro-retentive formulation [17]. Therefore, the selection of the most adequate biodegradable polymer is crucial to obtain systems with the desired properties of drug release. The lower or higher rate of polymer biodegradation in the biological fluids will result in a slower or faster release of the drugs dissolved or dispersed in the selected delivery system. At the same time, the drug release profile can also be influenced by the delivery system preparation method whose choice depends, for example, on the nature of the therapeutic agent and on the selected delivery route [18].

Numerous efforts have been made to design delivery systems that allow releasing of the bioactive agents in a predictable and controlled manner, when administered

by oral, topical, rectal, intravenous or other route. Independently of the delivery system geometry, the most usually required release profile is a uniform drug concentration within a therapeutic range over a considerable period of time (Fig. 31.1). However, in recent years, efforts have been made to achieve a release of the therapeutic agent in a pulsatile fashion, triggered by changes in the surrounding environment or by an external stimulus [6, 19]. Due to the relevance of the various mechanisms behind the drug release profile, mathematical models have been developed to describe it [20–28]. In fact, the existence of a mathematical model that can predict the exact drug release mechanism and, consequently, the concentrations that are obtained *in vivo* during the delivery system 'half-life' would be an asset in the development of new pharmaceutical formulations. Nevertheless, *in vitro* release studies are always useful to predict and to provide a basis for the *in vivo* release profile, despite the fact that a direct correlation between *in vitro* and *in vivo* behavior cannot always be achieved [29].

Typically, the polymeric pharmaceutical delivery systems can be divided into five categories according to the mechanism that control the release of the therapeutic agent: diffusion controlled systems, chemically controlled systems, solvent-activated systems, externally triggered systems and self-regulated delivery systems (Fig. 31.2) [6, 19, 30, 31]. Notwithstanding, a delivery system can present not only one of these mechanisms, but can actually involve two or more mechanisms. For instance, a study in which bovine serum albumin (BSA) and human serum albumin (HAS) microspheres were used to carry piroxicam showed that the nonsteroidal anti-inflammatory release was conducted by drug diffusion and polymer chain degradation [32].

31.3.1 Diffusion Controlled Systems

The diffusion controlled systems can be monolithic (matrix) or reservoir systems (Fig. 31.2a and 2b) [6, 33]. In monolithic systems, the drug dissolved or dispersed in the matrix is released by diffusion. Therefore, these systems maintain their structure and do not suffer alterations by swelling, degradation or erosion. The drug can be released through pores present in the particles structure [27] or can be conducted simply by the drug passage through the polymer chains [33]. In the monolithic systems, the distribution of the drug must be, ideally, uniform through the polymer matrix [33]. Usually, faster releases occur for molecules closer to the particle surface and, as they become further embedded in the particle, the release velocity decreases. On the other hand, it is possible to obtain a desired release rate by including the therapeutic agents in the system core surrounded by a uniform polymeric layer that controls the diffusion rate [24, 33]. As for monolithic systems, the diffusion of the drug through the polymer in the reservoir diffusion controlled systems is the rate-limiting step [33]. The release rate in this type of system is time-independent (zero order) for planar, cylindrical or spherical systems in geometry [24]. In fact, the geometry of the system constitutes a factor that can control the drug release rate, as well as the membrane thickness, the drug concentration across the membrane, the system thermodynamic properties via the partition coefficient and the polymer structure through the solute diffusion coefficient.

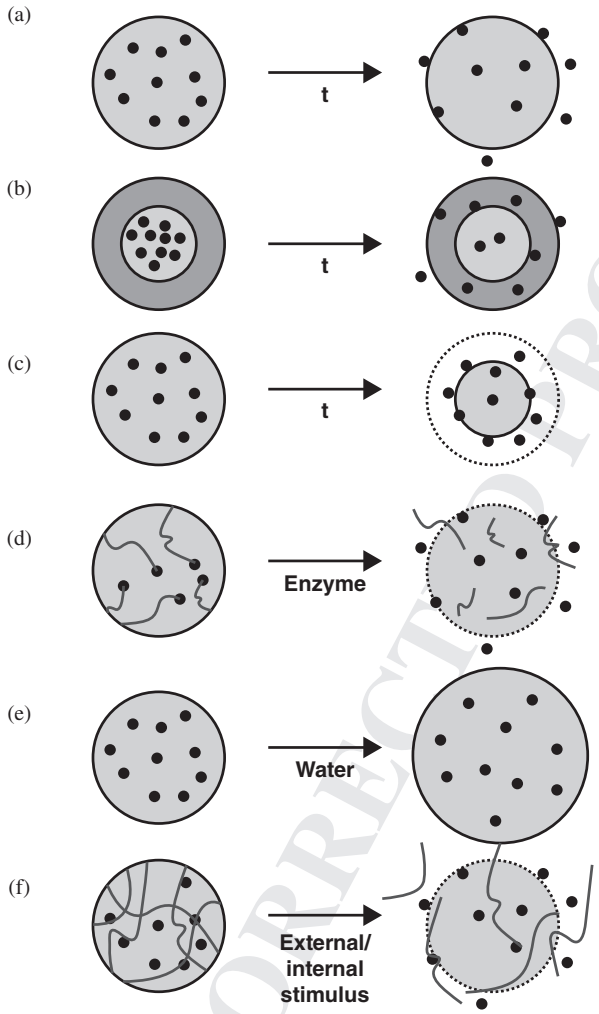

FIGURE 31.2 Illustration of diffusion controlled systems (a; b), chemically controlled systems (c; d), solvent activated systems (e) and externally-triggered/self-regulated systems (f).

31.3.2 Chemically Controlled Systems

The chemically controlled systems encompass biodegradable and bioerodible carriers, as well as the systems in which the drug is linked to the polymer (Figure 31.2c and d) [6, 24]. Biodegradable polymers suffer degradation to smaller compounds when incorporated into physiological conditions and, contrariwise, with the erodible polymers did not experience a chemical modification of their structure, but their dissolution. Consequently, in these systems the higher or lesser degradation or dissolution of the polymer chains will influence the bioactive factors release rate [24].

For systems having the drug linked to the polymer, the drug release will be controlled by the velocity at which the drug–polymer bonds are cleaved, either enzymatically or hydrolytically [24]. The link of the drug to the polymer may or may not be performed via a spacer group, which can also affect the drug release rate and the hydrophilicity of the system [33].

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31.3.3 Solvent-Activated Systems

Solvent-activated systems are vehicles that swell when in contact with physiological fluids, increasing their volume and allowing drug diffusion to the surrounding media (Fig. 31.2e) [6, 25, 33, 34]. Therefore, the drug release depends on the ability of the biological fluids to penetrate the polymeric delivery system [22, 25, 35]. The release profile of these systems and of bioerodible carriers are similar, if the surface erosion in the bioerodible is the unique factor responsible for the release of a drug [33]. These systems are based in hydrogels, which can absorb a considerable quantity of water or physiological fluids. In systems not chemically crosslinked, the dissolution creates an erosion front, besides diffusion and swelling [6]. During swelling, the solvent-activated systems go from the glassy state to the rubbery state, this last state being the one that allows the drug release [24]. Consequently the systems present two phases until all systems stay in the rubbery state. The velocity and position of the glassy–rubbery interface will determine the drug release rate [24].

31.3.4 Externally Triggered Systems

The externally triggered systems are devices in which the drug is released due to an external stimulus, as magnetic, ultrasonic, thermal, electric and irradiation (Fig. 31.2f) [19]. In magnetically triggered systems, the polymer matrix is enriched with magnetic beads together with the therapeutic agents [36, 37]. For example, a study performed with alginate spheres containing insulin and magnetic particles showed that the release rate of the protein was much higher (around 50 times higher) when the magnetic field was applied [37]. The same study also demonstrated that when alginate spheres were crosslinked, the released of insulin was also higher than in the absence of the magnetic field. However, this effect did not occur immediately, but it happened only after applying the oscillating magnetic field. In this perspective, it is possible to conclude that the release rate in these systems also depends on mechanical properties of the polymeric device. Additionally, this event depends on the position, orientation and magnetic strength of the magnets and on the amplitude and frequency of the magnetic field applied [19].

31.3.5 Self-Regulated Delivery Systems

In self-regulated delivery systems the release rate is adjusted in response to the evolution of the illness or the physiological need (Fig. 31.2f) [19, 31]. Several mechanisms can be used to modulated the feed-back action of drug delivery systems: pH-sensitive

polymers, enzymes, illness markers, pH-dependent drug solubility, competitive binding and metal concentration-dependent hydrolysis [19]. For example, a design of this closed-loop controlled system consists of immobilizing urease to the polymeric vehicle [38]. This enzyme converts urea to ammonium bicarbonate and ammonium hydroxide, thereby increasing the pH, that can leads to a higher erosion or swelling of the polymer that enhances the drug release [39]. The solvent activated systems mentioned before become even more attractive when the swelling is controlled by an internal stimulus that can be a change in pH, temperature, solvent composition or ionic strength in the environmental surroundings [34, 40]. In this approach, these systems will only release the entrapped material at specific sites, where and when the environmental aspect for which they are sensitive is present. For instance, pH-sensitive polymers that swell at low pH, as observed in inflammatory processes, will only release the entrapped material in anatomic/cell locations characterized by this physiological condition. The swelling at low pH is also attractive, for example, when it is necessary to obtain a localized delivery at the stomach to treat an infection caused by *Helicobacter pylori* [41]. The polymers used to swell at low pH are polybasic in nature, such as chitosan [42], unlike the polyacid polymers which will shrunk at low pH, but swells with the increasing pH, like carboxymethylcellulose [19,39]. pH stimulus is generally associated with a change in body conditions such as at the site of tissue and organ or intracellular compartments. Common examples are found in the fields of gastrointestinal tract, changes in blood stream pH, or tissues in a pathological situation such as clot or cancer. Therefore, biomaterials for such fields have mainly been developed as nano/microparticulate carriers that allow oral uptake or intravenous injection. The responsiveness of candidate biomaterials to pH is accompanied mainly by a change in size/shape (swell/shrink or collapse), thereby allowing the release of certain drugs from the interior [43]. Some natural polymers like chitosan, alginate, and gelatin and many synthetic polymers have been developed for this purpose. Protein release from alginate, chitosan, and pectin microparticles analyzed at different pH simulating gastric (pH 1.2 and 5.0), intestinal (pH 7.4) and colonic (pH 6.0 and 6.8) activity is highly pH dependent; release is sustained at gastric pH but increased at intestinal and colonic pHs [44].

pH-sensitive polymers can also be used linked to an enzyme whose reaction product alters the pH of the surrounding media, as the urease mentioned before [38], and consequently the release rate is adjusted by the presence of a specific compound in the system. Another approach that can be used is to proceed to the immobilization of glucose oxidase to the devices for controlled release of insulin [28, 45]. This enzyme converts glucose into gluconic acid, which leads to a decrease of the pH within the system and as a result gel swelling occurs. In this way, as required, insulin delivery is controlled by glucose concentrations. In fact, pH-sensitive polymers are usually loaded with enzymes whose product of reaction leads to an alteration of the pH in the local microenvironment, being glucose oxidase commonly used for this purpose [40]. Another delivery system composed of 6-mercaptopurine-carboxymethyl chitosan showed a release rate pH-sensitive and dependent on the glutathione presence [46]. As this is a reducing agent at a higher concentration in the cells, these devices were developed to provide an intracellular controlled release.

In another work, poly(ethylene glycol) (PEG)-modified thiolated gelatin nanoparticles were used to take advantage of intracellular glutathione concentrations for DNA delivery [47]. The authors showed that the delivery systems developed were able to transfect cells *in vitro* to a greater extent than Lipofectin-plasmid DNA complexes.

31.4 *IN VITRO* AND *IN VIVO* APPLICATIONS OF NATURAL-BASED DELIVERY SYSTEMS

Natural polymer-based delivery systems such as micro- or nano-particles, gels, membranes, sponges and scaffolds have been broadly proposed for drug administration, targeting and delivery. Besides targetting and delivery in a certain tissue or cell, efforts have been made to intracellularly carry and/or to release in response to physiological needs. Natural polymers can be mixed with other compounds or chemically modified to improve their properties for the development of innovative drug delivery systems. Additionally, functionalized delivery systems of hybrid, composites and grafted polymers can strengthen the responsiveness to a wide range of external or internal stimulii, as previously mentioned.

A biodegradable, porous carrier system is convenient for the clinician, as it limits and protects the release of drugs, proteins or genes in a predictable and time-controlled manner. Simultaneously, these delivery systems should allow cell growth and act transiently as an extracellular matrix until sufficient cells are present to build a new substratum.

31.4.1 Drug Delivery Systems

There are numerous works in the literature that evidence the value of natural polymers in the formulation of spatiotemporal controlled drug delivery systems. Chitosan, for instance, is a polymer usually used as drug delivery carrier, since it shows antibacterial activity [48,49] and the ability to accelerate wound closure and healing [50], thereby being an attractive wound dressing device. The use of chitosan sponges incorporating the antibiotic norfloxacin as wound or burn dressings [51] is an example which uses the intrinsic therapeutic properties of the device. In another study, starch-conjugated chitosan microparticles incorporating gentamicin presented a sustained release of the antibiotic in effective concentrations that exert its antibacterial activity and consequently has potential to treat bone infections, as suggested by the authors [52]. In another attempt, carboxymethyl-chitosan grafted onto low generation poly(amidoamine) (PAMAM) dendrimers and carrying dexamethasone demonstrated the ability to induce osteogenic differentiation of rat bone marrow stem cells *in vitro* [53]. This glucocorticoid and mouse lung fibroblastic cells were also encapsulated into chitosan-based spherical particles as a two-in-one tissue engineering construct [54]. These systems showed pH-sensitive behavior and the ability of drug sustained release, preserving encapsulated cells viability.

The pH-responsive delivery was also demonstrated in devices of carboxylmethyl-chitosan grafted with phosphatidylethanolamine incorporating the hydrophobic drug

ketoprofen [55]. This study demonstrated that, in the presence of an acidic medium, a higher release of the drug occurred. A pH-sensitive hydrogel made of a blend of chitosan with polyvinyl pyrrolidone (PVP) was developed to obtain a selective controlled release of the antibiotic amoxicillin in the acidic pH of the stomach [56]. In fact, chitosan has a limited utilization for oral formulations, since it solubilizes at the stomach, leading to a fast release of the incorporated drugs. On the other hand, it is also possible to obtain particles that did not disintegrate at the stomach by the surrounding environment, by developing a system composed by a chitosan core with a hydrophobic cellulosic shell [57]. This system presents the ability to modulate the *in vitro* release profile of sodium diclofenac and fluorescein isothiocyanate-labeled BSA. It was also described in the literature that chitosan shows tumor growth inhibitory activity [58]. An example relates to the local administration of a photo crosslinkable chitosan hydrogel (with azide and lactose moieties) containing paclitaxel [58]. This study showed that the drug delivery system presented a higher inhibitory activity over angiogenesis and tumor growth in mice, than the chitosan hydrogel or the paclitaxel alone. Pectin-coated chitosan hydrogel used to incorporate 5-fluorouracil also demonstrated a pH-dependent release, which was slower at acidic pH and higher at neutral pH, supporting the anticancer therapeutic action of the drug and therefore can be used, as suggested, for the treatment of colon tumors [59]. The mucoadhesive property of chitosan was also explored in bladder cancer treatment to obtain the desired attachment of the mytomycin-C delivery system to the bladder wall [60]. Tissue engineering scaffolds can also be made of chitosan by the use of green processing technology (i.e. supercritical fluids), which show the ability for the sustainable release of dexamethasone [61].

Besides chitosan, other natural polymers showed a great potential in drug controlled release. Hyaluronic acid-based hydrogels demonstrated the potential for slow and sustained release of hydrophobic anti-inflammatory steroids [62]. A mixture of hyaluronic acid and chitosan allows the production of a temperature sensitive hydrogel, first prepared by Fang et al. [63]. This study demonstrated, *in vivo*, that the developed system showed the ability of nalbuphine-controlled release, leading simultaneously to an increase duration of its analgesic effect.

Gellatin nanoparticles were also used to incorporate and to sustain release of the anticancer drug 5-fluorouracil [64]. Starch-based porous materials also demonstrated the ability for the controlled release of meclofenamic sodium salt, a nonsteroidal anti-inflammatory drug [65]. Gellatin and HSA nanoparticles functionalized with HER-2 (human epidermal growth factor receptor-2) specific antibody trastuzumab (Herceptin®) were used to drug target tumor cells [66]. This study demonstrated the effectiveness and specificity of the protein nanoparticles to bind and be internalized in HER-2-overexpressing cancer cells.

Crosslinked oxidized alginate hydrogels were considered to locally deliver three anticancer drugs, namely methotrexate, doxorubicin and mitoxantrone [67]. While methotrexate was localized into the hydrogel pores, the doxorubicin and mitoxantrone were covalently bound and ionically complexed, respectively, to the polymer. These hydrogels can, therefore, be used to deliver one or more therapeutic agents, by different controlled-release mechanisms. Under a magnetic field, curcumin, an

anticancer drug, was released in a higher rate when administered in a magnetic hydrogel of gum arabic, chitosan or maltodextrin [49]. A pH- and redox-responsive alginate-based microcapsule for intracellular degradability and docetaxel delivery was developed [68]. This delivery system was produced through dynamic covalent assembly of a Schiff base and disulfide, taking advantage of the redox potential presented in the intracellular environment.

31.4.2 Protein Delivery Systems

The use of polymeric vehicles to locally deliver growth factors (GFs) in various formats provides a method of controlled, localized delivery for the desired time frame [35]. Various encapsulation processes utilizing harsh solvents, crosslinking agents and high temperatures have been used, which could result in the denaturing and deactivation of the incorporated proteins. A variety of processing techniques have been developed to bypass these issues including soaking the polymeric scaffold in a solution of a defined growth factor after processing, the use of hydrogel delivery systems where growth factor incorporation can be achieved at low temperatures [69], and supercritical carbon dioxide processing [70]. Chemical/genetic modifications of GFs were also conducted to improve their stability and bioactivity with proved success.

Collagen, the industry's favored natural-origin polymer, has been used to create various delivery vehicles: collagen sponges, strips, gels, membranes, and others [71]. Bone morphogenetic protein (BMP) 2, an osteogenic growth factor, FDA approved and routinely used in orthotropic sites for bone generation, was absorbed to collagen sponge and the feasibility of their use in local alveolar ridge preservation/augmentation [72], in maxillary sinus augmentation [73] and in femoral rat defects [74] demonstrated. Further on, it has been reported that recombinant human rhBMP-2 and rhBMP-6 are retained better than rhBMP-4 [75]. In another work, collagen sponges carrying cartilage derived morphogenetic protein 2 (CDMP-2) were implanted subcutaneously, intramuscularly or inside a freshly created defect in the achilles tendon of rats [76]. Large amounts of bone were induced subcutaneously, smaller amounts intramuscularly and, in the tendons, only small amounts of bone or cartilage were seen in a few animals. Thus, the amount of bone appeared inversely related to the degree of mechanical environment. A native type I collagen gel augmented with insulin-like growth factor 1 and 2 (IGF-1 and -2) significantly enhance the osteoconductive repair of nasal critical-size defects in a rodent model [77]. The results indicate that rat nasal defects treated with IGF-2-augmented collagen gels showed healing that was significantly greater than the IGF-1 augmentation, the combination of IGF-1 and IGF-2 augmentation, and that of collagen gels-only treatment. In a similar approach, a porous collagen–glycosaminoglycan scaffold was loaded with a range of IGF-1 concentrations to evaluate its potential as a controlled delivery system [11]. The bioactivity of released IGF-1 was confirmed by seeding of the systems (pre-adsorbed with IGF-1) with human osteoarthritic chondrocytes, demonstrating an increased proteoglycan production *in vitro*. The effect of exogenous platelet-derived growth factor (PDGF) BB on bone healing was also demonstrated using a

collagen [78] or a composite of chitosan/tricalcium phosphate (TCP) sponge [79] as carriers, in a tibia defect of rabbits or in a calvarial defect of rats, respectively.

The BMP-2 was also directly immobilized on silk fibroin films, which were able to induce an enhanced osteogenic differentiation of human bone marrow stromal cells (hBMSCs) when compared to the ones cultured in the presence of soluble osteogenic supplementation [80]. Indeed, this permanent immobilization of biomolecules leads to long-term presentation of bioactive molecules to the seeded cells from the polymeric surface. Chondroitin sulphate, which consists of a network of highly negative-charged groups and is categorized as GAG-like heparin, can improve the binding ability to BMP-2 and, subsequently, prolonged release when combined into collagen scaffolds [9]. A more recent study highlights the spatiotemporal control of the regenerative process by utilizing a hybrid growth factor delivery system that consists of an electrospun nanofiber mesh tube for guiding bone regeneration combined with peptide-modified alginate hydrogel injected inside the tube which was developed for the sustained delivery of rhBMP-2 [81]. This system resulted in complete bony bridging of challenging 8 mm femoral bone defects in a rat model, when compared to the current clinical standard of collagen delivery.

Fibrin matrices functionalized with heparin were also developed as GFs delivery systems, consisting of a heparin-binding peptide (HBP) derived from antithrombin III (ATIII) and a Gln acceptor substrate [82]. The covalent tethering of this linker-peptide to fibrin matrices during crosslinking confers heparin affinity to the fibrin matrix. Thus, heparin and GFs with a natural affinity for heparin are preferentially retained within this modified fibrin matrix leading to a slow and continuous delivery modality. This strategy was employed for the delivery of a variety of GFs such as beta-nerve growth factor (β-NGF) [83], neurotrophin-3 (NT-3) [84, 85], FGF-2 [86] or PDGF-BB [87]. *In vitro* and *in vivo* data demonstrated the ability of such heparin-binding GFs systems to promote nerve regeneration, angiogenesis and tendon healing. A fibrin gel incorporating transforming growth factor beta (TGFβ) 1 displayed a slow release profile and was, consequently, effective in chondrogenic differentiation while suppressing osteogenic differentiation [88]. For skin regeneration, epidermal growth factor (EGF) fused with the fibrin-binding domain of fibronectin has reportedly shown higher affinity than the EGF alone to the fibrin matrix, with the EGF-loaded fibrin promoting the growth of fibroblasts and keratinocytes, and subsequent wound repair [89]. Since heparin is a widely used anticoagulant, immobilization of heparin on collagen matrices reduces the thrombogenic activity of collagen and may therefore prevent platelet adhesion and blood coagulation. Therefore, heparin-modified collagen matrices were employed as vascularization scaffolds able to deliver vascular endothelial growth factor (VEGF) [90] or fibroblast growth factor (FGF) 2 [91], as well as an *in vivo* recruitment scaffold of hematopoietic cells using the stromal cell-derived factor-1 alpha (SDF-1α) [92] or its involvement on the wound contraction inhibition and re-epithelialization stimulation in a mouse full-thickness excision skin wound model [93].

Along similar lines, the development of an injectable hyaluronic acid (HA)-based hydrogel crosslinked with PEG-diacrylate and consisting of thiol-modified heparin (heparin-DTPH) was reported [94, 95]. This hydrogel network was further modified

with chondroitin sulphate (CS-DTPH) or thiol-modified gelatin, and has been used to deliver several proteins, including FGF-2 [96, 97], hepatocyte growth factor (HGF) for the concomitant recruitment of hBMSCs [98], and combinations of VEGF with bFGF [94], VEGF with angiopoietin-1 (Ang-1) [95], and VEGF with keratinocyte growth factor (KGF) or PDGF [99].

To prolong the release profile of the bioactive factors from the polymeric delivery systems, the candidate molecules are often encapsulated first within microspheres that release them more slowly, and then embedded within the scaffolds or hydrogels. In order to achieve spatiotemporal control over GFs delivery, an anisotropic double-layered collagen membrane was developed, comprising a dense layer and a loose layer, which incorporated basic FGF-loaded chitosan-heparin nanoparticles [10]. The nanoparticles were prepared by a polyelectrolyte gelation process and, then were sandwiched between the two layers of the collagen membrane. Different release amounts of bFGF from the different layers of the membrane induced a significant difference in cell proliferation, when fibroblastic cells were seeded on the different layers of membrane. Another system combining protein-loading poly(lactic-co-glycolic acid) (PLGA) microspheres within collagen and hyaluronic acid gel-like scaffolds was developed to allow tunable and sustainable protein release kinetics [100]. For the support of neural stem cell maintenance and proliferation, a composite system made of hyaluronic acid hydrogel that incorporates PLGA microsphere loaded with brain-derived neurotrophic factor (BDNF) and VEGF was developed [101]. The composite appears to be a promising scaffold that provides an ECM mimicking niche for stem cells and creates a permissive microenvironment for angiogenesis and neural regeneration. In another attempt, a composite delivery system made of alginate-poly(L-lysine)-alginate microencapsulated myoblasts incorporating dexamethasone-loaded PLGA microspheres has proven to be an effective composite release system [102]. The dexamethosone released from the PLGA generates a potential immune-privileged local environment to the cells that are microencapsuled and ensheathed.

While biopolymers are versatile in incorporating bioactive factors, bioactive inorganics such as calcium phosphates and glasses have significant limitations in delivering bioactive factors, because they primarily require high thermal processes in the shape formulation. In this manner, the bioactive inorganics are generally made into composites with natural-origin to allow shape formability [13]. However, some of the valuable physicochemical properties of bioinorganic nanoparticles (mainly calcium phosphates), such as high electrostatic charge, surface area and roughness, improve the interaction with and affinity to bioactive factors, allowing suitable matrices for drug delivering scaffolds [103]. Among the bioactive inorganics, calcium phosphate cements (CPCs) are among the most attractive group of inorganic biomaterials to be used as bioactive factor delivery systems. α-tricalcium phosphate-based CPCs can self-harden and be formulated into microspheres with the help of collagen to deliver biomolecules. Bovine serum albumin (BSA), used as a model protein, was safely loaded within the microspheres and then released sustainably over a month [104]. In order to stimulate osteoinduction, BMP-2 was also incorporated within tetracalcium phosphate/dicalcium phosphate anhydrous-based CPCs composite with chitosan, which showed significant improvement of osteoblastic cell functions [105].

The addition of alginate into CPCs-based on calcium carbonate/monocalcium phosphate monohydrate prolonged the release of gentamicin, providing a reservoir system for antibiotic delivery with bone regeneration capability [106].

Currently, one interesting and attractive form of biomaterial scaffolds is the nanofiber, which is mainly produced by an electrospinning process. A number of target tissues including skin, nerve, muscle, blood vessel, cartilage and bone, have utilized the nanofibrous meshes as support for cell culture, for the implementation of tissue-engineered constructs [107]. Therefore, the development of nanofibrous scaffolds as drug delivery systems has become an attractive research area. For the loading of GFs, some common biological proteins such as BSA were used to hold and stabilize those bioactive factors. For example, NGF was mixed with BSA and, subsequently, dispersed in the co-solvent of the synthetic copolymer ε-caprolactone-ethyl ethylene phosphate and, then electrospun into nanofibers [108]. The use of BSA significantly stabilized the NGF, showing a sustainable release profile over 90 days. Instead of using BSA, collagen was used with a synthetic polymer, showing similar effects on epidermal growth factor (EGF) release from the electrospun nanofibers [109]. Heparin has also been highly effective in stabilizing GFs like EGF and bFGF within PLA nanofibers [110]. However, these mixture systems are considered rather case-specific and have limitations in controlling the drug release profiles. An elegant and general strategy to gain sustainable and controlled release pattern of bioactive factors from the electrospun nanofibers is the core-shell (or dual-concentric) design. Some recent studies have highlighted the effectiveness of this core-shell design for prolonged delivery of GFs. For example, silk fibroin/PCL core-shell nanofibers were proposed as a potential tissue engineering and drug release system [111].

Tissue regeneration may be enhanced by the delivery of combinations or sequences of bioactive factors, as single GF delivery has a number of limitations. Challenges with this combinatorial or sequential delivery of multiple GFs approach include the selection of proper GF cocktails, understanding their synergies, and rigorously controlling their concentrations, gradients and releasing timing (Fig. 31.3) [13, 112, 113]. Each GF has a specific physiological mechanism of action, and this drives the selection of a specific release profile. Indeed, if not appropriately chosen, the delivery of a combination of GFs could lead to inhibitory, as well as stimulatory responses in bone formation [114–116]. Often, though, the most effective dosage and release profile is not known, and must be empirically explored.

A work developed by Ripamonti et al. [117] showed, for the first time, that rhTGF-β1 induces endochondral bone formation in extraskeletal sites of adult baboons. Furthermore, it was also shown that TGF-β1 and recombinant human osteogenic protein-1 (OP-1, bone morphogenetic protein-7) synergize in inducing large ossicles in extraskeletal sites of the primate, as early as 15 days after implantation. A single application of OP-1, in conjunction with an insoluble collagenous matrix as carrier (5, 25, and 125 μg/100 mg of carrier matrix) induced bone differentiation in the rectus abdominis of the baboon. This level of tissue induction was raised several-fold by the simultaneous addition of comparatively low doses of TGF-β1 (0.5, 1.5, and 5 μg), which by itself induces bone formation in the rectus abdominis at doses of 5 μg/100 mg of carrier matrix. A composite gelatin/β-TCP sponge loaded with BMP-2 and

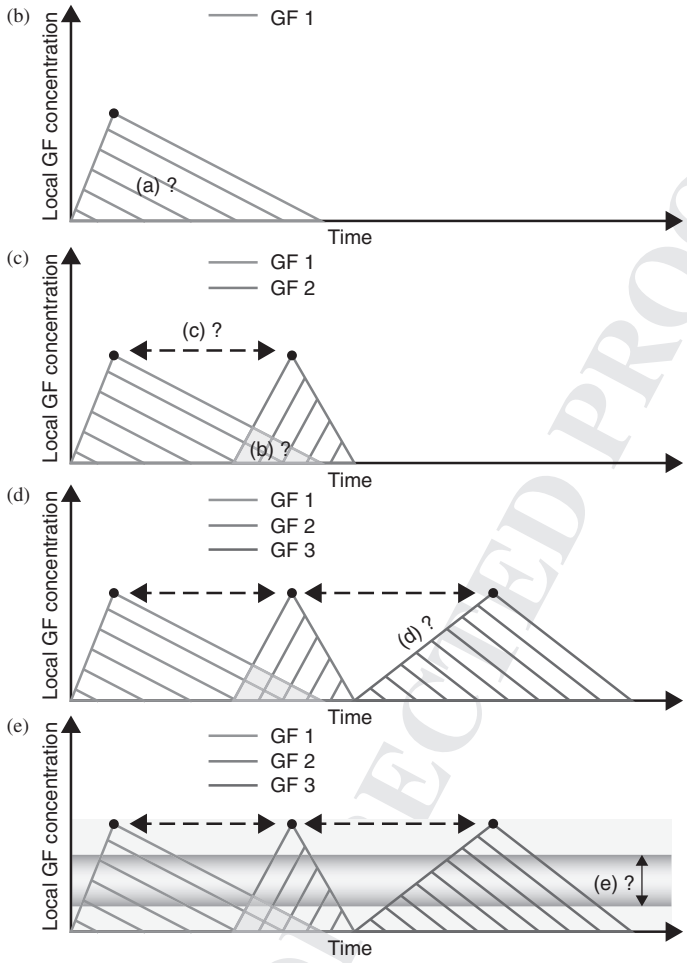

FIGURE 31.3 Multiple morphogens can be delivered in sequence or in combination to gain control of the different phases in healing. The time interval between each growth factor delivery, the total amount and concentrations, and synergies between factors need to be considered for the design of these delivery systems. (b) The release of a single growth factor – GF 1. The area under the curve (a), the peak concentration, and duration of exposure can be used to control cell behavior. (c) Release of two growth factors (GF 1 and GF 2). The factors can be delivered with some overlap (region b) or purely in sequence, and the timing of their peak concentration may also be regulated (c). The choice of overlap depends on their synergistic or inhibitory effects on osteogenic behavior. Multiple growth factors can also be released using controlled delivery from biomaterials (GF 3). (d) Rate of release (parameter d) can be defined based on *in vitro* experiments that demonstrate cellular response to a desired concentration and time of exposure for a certain behavioral response. (e) The effective dosage that elicits a maximum cellular response may have a lower and upper limit defined by a range (parameter e). A range outside this may cause a lowered or a different response in cellular behaviour. All these parameters may be similar or different for varying GFs (1, 2, 3, or more). Adapted from M. Mehta et al. Advanced Drug Delivery Reviews 64 (2012) 1257–1276 [112]. For a color version of this figure, see the color plate section.

Wnt1 inducible signaling pathway protein-1 (WISP-1) showed a synergistic ectopic bone formation in middle-aged mice, suggesting that a scaffold incorporating multiple osteoinductive agents could be effective in age-related bone disease by inducing new bone formation [118].

Initial approaches to chemically conjugate GFs to collagen matrices/hydrogels were based on the use of homobiofunctional poly(ethylene glycol) (PEG)-based crosslinkers containing terminal and primary amine selective succinimidyl groups [119, 120]. The use of such linkers then expanded to simultaneous crosslinking of collagen matrices and covalent tethering of GFs. The sustained delivery of tethered TGF-β2 and VEGF resulted in an enhanced and prolonged response *in vivo*, compared to the unmodified GFs. In another attempt, longitudinally oriented poly(L-lactide-co-D,L-lactide) scaffolds were functionalized with alginate hydrogels incorporating rrhBMP-2 and rhTGF-β3, these molecules being co-delivered at relatively low doses (i.e. 200 ng and 20 ng, respectively) and able to promote the repair of a challenging rat femoral defect [121]. In a similar approach, a combinatorial delivery of BMP-2 with TGF-β3 within alginate hydrogels has been reported for the transplantation of stem cells [122]. Previously, the hydrogel was covalently modified with RGD-containing peptides to stimulate the attachment of rat bone marrow stromal cells (rBMSCs). When implanted ectopically in mice, the delivery of BMP-2 or TGF-β3 individually from the alginate hydrogels containing transplanted rBMSCs resulted in negligible ectopic bone formation. On the other hand, when these GFs were delivered together from the alginate hydrogels, there was significant bone formation by the transplanted rBMSCs, suggesting the synergistic role of multiple-delivered GFs.

Based on the anatomophysiology of the bone tissue, the sequential delivery of angiogenic and osteogenic GFs is a promising strategy for bone regeneration. In a recent study, a composite biomaterial scaffold made of PLA matrix with alginate fibers was developed, where VEGF was loaded into the alginate and the BMP-2 was incorporated into the PLA matrix, aiming at initial VEGF release and, then, BMP-2 release at a much later stage [123]. When the delivery systems were implanted in mouse segmental femoral defects with hBMSCs, significantly higher bone regeneration was observed with respect to the composite scaffolds without GFs. When collagen scaffolds combined with heparin were subcutaneously implanted in rats, the presence of both FGF2 and VEGF displayed the highest density of blood vessels and more mature vessels than the cases delivering either of the individual GFs, suggesting the synergistic roles of both GFs in the series of events involved with blood vessel formation [203]. A sustained and synergistic effect of a composite system made of fibrin hydrogel and ionic-albumin microspheres loaded with FGF-2 and granulocyte colony-stimulating factor (G-CSF) was also demonstrated in a murine critical limb ischemia model [124]. Another study comprises a cocktail of GFs made of VEGF, Ang-1, IGF and SDF-1, which were incorporated within composite hydrogels of dextran and PEG diacrylate. The subcutaneous implantation of hydrogels comprising multiple angiogenic factors dramatically increases the size and number blood vessels compared with any of the GFs used individually [125]. An interesting approach to stimulate angiogenesis by a sequential delivery of GFs that have different time dependent roles was recently proposed. A combined system made of micelles in

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Ca-alginate microparticles in PVA hydrogel was developed, where paclitaxel (PTX) was incorporated into the micelles and VEGF was loaded into the alginate microparticles [126]. *In vitro* results demonstrated a short-term release for VEGF and a long-term release for PTX, aimed at stimulating the proliferation of endothelial cells in early stages and inhibiting the later proliferation of smooth muscle cells to prevent the vascular intimal hyperplasia.

The combinatorial delivery of rhBMP-2 and rhIGF-I functionally encapsulated in either PLGA or silk microspheres, and further incorporated in alginate and silk scaffolds to form concentration gradients, can significantly affect the osteogenic and chondrogenic differentiation of hBMSCs, suggesting their usefulness in osteochondral tissue regeneration [127]. Two bone GFs, i.e. BMP-2 and BMP-7, were encapsulated in PLGA and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanocapsules which were then incorporated into chitosan-PEO fibrous scaffolds produced by wet spinning [128]. The sequential release of both BMPs performed better in inducing rBMSC proliferation and osteogenic differentiation (ALP activity) than individual nanocapsule populations or the populations designed to provide simultaneous release of the BMPs.

The co-delivery of glial cell line-derived neurotrophic factor (GDNF) and NGF within collagen nerve conduits was also shown to be effective in early axonal regeneration in the peripheral nerve system [129]. Drug release studies point out that crosslinked collagen tubes sustained the initial release of both neurotrophic factors more effectively than the non-crosslinked ones (2% vs. 12–16% during 3 days). The nerve regeneration in a 10 mm rat sciatic nerve gap model, evident as axonal outgrowth and Schwann cell migration, was significantly improved by the sustainable release and co-delivery of both factors.

Another elaborated system for multiple bioactive factor delivery was reported to initially suppress inflammatory reactions and, subsequently, to improve reparative and regenerative ability of skin tissue. A system of co-delivery of two anti-inflammatory drugs, i.e. spantide II and ketoprofen, was designed from nanogels composed of hydroxypropyl methyl cellulose (HPMC) and bilayered nanoparticles of PLGA and chitosan [130]. The increase in skin permeation of spantide II and ketoprofen was further responsible for improved response in allergic contact dermatitis and a psoriatic plaque-like model, suggesting a promising gel for the treatment of percutaneous delivery of combine drugs into the deeper skin layers for treating skin inflammatory disorders.

Biomaterials responsive to applied stimuli are another fascinating design of smart matrices for tissue engineering and carriers for drug delivery. Stimuli can be given externally, such as light, electrical or magnetic force, or by internal changes in temperature, pH, and enzymatic reaction. The response of these biomaterials to stimuli accompanies changes in diverse properties, including surface charge, shape, and temperature.

Along with pH-responsiveness (described above), thermo-sensitivity is a widely accepted action of smart biomaterials with stimuli-responsiveness. One of the most well-known thermo-responsive biopolymers is poly(N-isopropyl acrylamide) (pNIPAAm), which presents a typical sol–gel transition at approximately 32°C [131].

However, its poor biocompatibility and nondegradability generally require a composite approach with other biocompatible materials to produce stimuli-responsive and biologically active composite materials. Moreover, its composites with natural polymers or hydrophilic synthetic polymers generally modulate the transition point near body temperature as well as allowing the delivery of hydrophilic drugs, enabling better applicability in tissue engineering and drug delivery. For example, alginate composite with pNIPAAm was developed as a protein delivery system [132]. The release of a model protein (i.e. BSA) was highly dependent on the temperature, showing a higher release rate with temperature decrease. A composite of hyaluronic acid with pNIPAAm was also exploited to produce thermo-reversible hydrogels for cartilage tissue engineering [133]. Rabbit chondrocytes were encapsulated into the composite gel, which also contained TGFβ-3. The thermo-reversible hydrogel construct could be injected subcutaneously in mice, enhancing the production of cartilage-specific ECM in the cell-growth factor delivering condition better than those without the GF. A hyaluronic acid/pluronic thermo-sensitive composite was also developed for the delivery of cells and GFs in cartilage tissue engineering [134]. Human adipose derived stem cells and TGF-β1 could be loaded within the composite gel via sol–gel transition at body temperature allowing *in vivo* injection. The growth factor release was moderate, and the *in vivo* result of the construct loading into a full-thickness defect of rabbit knee articular cartilage demonstrated the formation of cartilaginous matrix by the tissue-engineered construct.

31.4.3 Gene Delivery Systems

Originally, the therapeutic application of genes was proposed for the correction of genetic defects, such as single mutations. Recently, gene therapy has been used to induce the expression of molecules that are normally involved in the regenerative response in the tissue of interest [135]. Most of the gene therapy models use vectors to enhance DNA entry into target cell nuclei and expression of the desired genes. An ideal vector would possess the following characteristics: avoidance of an immunological host response, preferential binding to specific target cells, transduction of dividing and nondividing cells, integration of genes into host cell DNA without disruption of the normal cell function, expression of genes at an appropriate therapeutic level, ability to allow external control of protein expression, and easy of production at a reasonable cost [136]. However, the perfect vector has yet to be developed, because many of the currently used vectors partially fulfill the above criteria.

The choice of vector for gene therapy depends on the desired duration of protein function, anatomical location, condition to be treated, and whether an *in vivo* or *ex vivo* approach is favored [137]. The vector systems can be classified into nonviral and viral vectors. The major advantage of viral vectors is their high frequency of transduction due to the natural tropism of viruses for living cells. The main disadvantages of viral vectors are their immunogenic potential and, in the case of retroviruses and certain adeno-associated viruses, the threat of disturbing normal gene function [138]. Nonviral vectors, such as DNA plasmids, lipoplexes, or polyplexes mimic functions of viral cell entry, but avoid many problems associated with viral vectors, though generally possess a lower rate of transfection [139]. In addition, physical methods,

such as electroporation, sonoporation, magnetofection, hydrodynamic methods, and ballistic methods (the so-called gene gun) have been developed that support nonviral nucleic acid delivery to cells.

A pioneering study employing natural-derived matrices demonstrated significant bone regeneration [135, 140]. Natural polymers have been applied to a wide range of gene therapeutics, from nanoparticulates to three-dimensional scaffolds. Nano- and micro-particles have been applied to oral and intramuscular delivery successfully as nonviral gene therapy systems. These particulates can be modified with proteins, such as KNOB or transferrin, or antibodies/antigens to allow for cell-specific targeting and enhanced gene transfer. Other studies have demonstrated that nonviral vectors delivered from natural scaffolds promote tissue formation in a variety of injury models, such as bone, and the key role that microenvironmental cues play in regulating the extent of plasmid uptake and expression [141, 142].

Chitosan, a naturally derived polymer from shellfish, has been successfully used in oral and nasal gene delivery for vaccination [143, 144]. In these cases, the mucoadhesive property of chitosan is crucial to the delivery of these particles. For the periodontal tissue regeneration, a similar system has been used, where chitosan/DNA nanoparticles encoding PDGF were incorporated into chitosan/collagen scaffolds [145]. The plasmid showed a sustained release over 6 weeks and an effective protection by the chitosan. The *in vitro* results showed that periodontal ligament cells achieved high proliferation and were able to form periodontal connective tissue like structure. To prevent restenosis, stents were coated with chitosan-plasmid DNA nanoparticles encoding the enhanced green fluorescent protein (GFP), by a spray coating method [146]. The expression of gene exhibited high level of GFP and an *in vivo* study confirmed gene activity in the region in contact with the stents.

Appropriate modifications made to the natural forms of these polymers can yield targeted gene delivery to specific cell types, improvement in transfection efficiency as well as prolonging the residence time once delivered *in vivo* [147]. Although collagen gene carrier systems show limited gene transfer success *in vivo* [148], modified forms of collagen have shown the capacity for extended release of genes [149]. Ultimately, the coordinated delivery of multiple genes can be used to aid in multi-cellular tissue development, with each gene affecting different aspects and stages of tissue growth and development. Therapeutic genes can also be utilized to enhance incorporation of a tissue construct once implanted *in vivo* and enhance growth and assimilation with neighboring tissues.

31.5 CONCLUDING REMARKS

There is an emergent need in the development of more specific and effective therapeutic agent carriers to help in the regeneration of a plethora of tissues. The ultimate aim of bioactive factor delivery system development is to improve human health with the fewest possible adverse reactions. While there have been many polymeric scaffolds and matrices with different forms and compositions developed to load and deliver bioactive factors, the delivery strategy should be established based on the type of molecules to deliver and mechanisms to control their release. As most bioactive

factors such as proteins and genes are water-soluble, natural polymers are more favored than synthetic ones for this purpose. A core-shell structuring of biomaterials (in the cases of particles or fibers) where water-based polymers are placed in the inner core, may be the most common design principle to secure bioactive factors during the processing of synthetic drug delivery scaffolds.

While the physical entrapment of bioactive factors within the cargo materials is one way to gain sustainable and controllable release by the diffusion or erosion process, introduction of chemical bonds (such as ionic/covalent bonds or affinity binding) between the drug molecules with the carrier network can facilitate more stable and prolonged release. Providing stronger bonds between the scaffolds and the bioactive factors, while preserving the activity of the bioactive factors has, thus, been extensively researched. Developing affinity-driven bonds, such as utilizing heparin-binding domains for GF immobilization is considered an effective way of preserving the biological activity of the factors. The inorganic phase of bone mineral (i.e. hydroxyapatite) is also a good example that can be implemented on the surface of polymers to allow strong and even specific ionic bonds with some bone ECM proteins.

Although the therapeutic actions of the delivered molecules have some impact on cell behavior, the potential efficacy associated with complex events exerted by the multiple bioactive factors *in vivo* in the regenerative processes cannot be simulated well. The simultaneous or sequential release profile of bioactive factors at proper time periods and dosages has been achieved by placing or combining different compositions and formulations of carriers and scaffolds. Multilayered scaffolds with each layer carrying different bioactive factors are one of the methods to gain sequential release, which primarily showed its effects on the *in vitro* cellular responses, including proliferation and differentiation functions, as well as *in vivo* tissue repair such as bone formation, where a concomitant blood vessel formation is achieved by the release of angiogenic factors.

Development of biomaterials with responsiveness to stimuli including temperature, pH and ionic strength is another promising strategy to achieve more smart and multifunctional actions of delivery systems and scaffolds. The pH-responsive polymers like chitosan, alginate, gelatin, polyelectrolytes, and their possible combinations, have revealed applications in the switchable delivery of proteins under pH-dependent physiological conditions, such as ischemic myocardium (pH 6.8 to 7.4) and gastric to intestinal change (pH 5.0 to 7.4). Thermo-sensitivity has long been pursued primarily utilizing pNIPAAm, because of its dramatic sol/gel transition and swelling/shrinking property near body temperature. Significant studies have proven the utility of pNIPAAm-based copolymers and composites in temperature-responsive delivering matrices of proteins and drugs.

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