Growth Factor Delivery Approaches in Hydrogels

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The controlled delivery of growth factors is a very challenging task because many different issues have to be addressed to develop the best suited system. A wide range of approaches have been employed for the controlled delivery of growth factors by hydrogels. Direct loading, electrostatic interaction, covalent binding, and the use of carriers are the main strategies presented in the literature. They are all detailed in the first part of this review. Recent work emphasizing biologically inspired strategies is also included. Also, both natural and synthetic materials are discussed. The second part comprises the methods to evaluate such delivery approaches. Both in vivo and in vitro techniques are presented. Improvements based on the discussed approaches may illustrate future paths toward the development of an ideal growth factor delivery system.

Introduction

Cells communicate with each other through direct molecular interactions or though the secretion of hormones or mediators that act locally or systemically to modulate cell functions. Growth factors, while included in the latter group, refer to an expanding class of proteins that have either pro- or antiproliferative effects, sometimes all-specific, under different circumstances. Growth factors modulate cell differentiation, migration, adhesion, and gene expression.[1−3] Purified growth factors are heat and pH unstable and highly sensitive to proteolytic degradation.[4] Therefore, techniques using delivery systems have been employed in order to protect them and control their release, thus enabling the growth factors to efficiently exert their biological effects.[5]

Natural and synthetic molecules have been employed as bulking agents to embed growth factors. In general, materials from natural sources (e.g., purified protein components such as collagens from animal tissues) are advantageous because of their inherent properties of biological recognition, including presentation of receptor-binding ligands and susceptibility to cell-triggered proteolytic degradation and remodeling. Other representative naturally derived polymers include agarose, alginate, chitosan, fibrin, gelatin, and hyaluronic acid (HA). Issues on natural products associated with purification, immunogenicity, and pathogen transmission have encouraged the development of synthetic biomaterials as cellular substrates. In contrast, synthetic materials present consistent composition and predictable manipulation of properties. Their mechanical properties can be highly tailored and they are easy to synthesize. Even though, it is important to consider that synthetic bulking agents for growth factors lack functional sites to interact with soluble or cell-surface proteins. Synthetic materials include poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), poly(acrylic acid) (PAA), poly(propylene fumarate-*co*-ethylene glycol) [P(PF-*co*-EG)], and polypeptides. In fact, researchers are now bridging the gap between natural and synthetic gels by combining well-characterized synthetic materials with biomimetic clues to support physiologically relevant cell−gel interactions,[6−9] as it will be further discussed herein.

Synthetic or natural bulking agents to embed growth factors are ideally processed in a way to mimic natural extracellular matrix (ECM). From a structural perspective, natural ECMs are gels composed of various protein fibrils and fibers interwoven within a hydrated network of glycosaminoglycan chains. In their most elementary function, ECMs thus provide a structural scaffold which, in combination with interstitial fluid, can resist tensile (via the fibrils) and compressive (via the hydrated network) stresses. In this context, it is worth mentioning how a small proportion of solid material in solvent is needed to build a mechanically quite robust structure, in many cases less than 1% (w/v).[7,8] The architecture of natural ECM components has inspired several researchers to use materials with similar structure, such as hydrogels.

Hydrogels are defined as three-dimensional polymer networks swollen by aqueous solvent, which is the major component of the gel system. These low-density materials can be classified on the basis of the mechanisms by which the cross-links within the networks are produced. Physical gels are formed by molecular self-assembly due to secondary forces, such as ionic or hydrogen bonds, while chemical gels are formed by covalent bonds. Intermediate-type of gels are produced as physical gels, but become additionally covalently cross-linked by specific chemical reactions. The cross-link density in these materials can be such that the permeability of proteins, including growth factors, through the average order 100 nm pores, is relatively rapid.[10,11]

When incorporating growth factors in such systems, some points should be considered:[10,12] (1) the loading capacity corresponds to the amount of growth factor that can be mixed or grafted into the system; (2) the distribution relates to the

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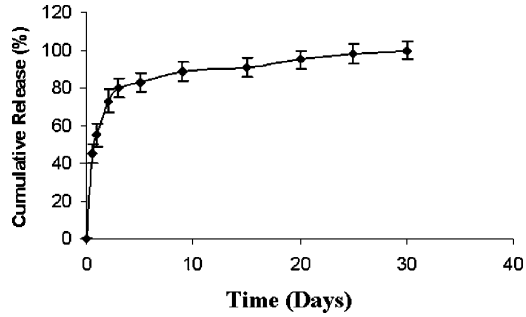

Figure 1. Illustration of a typical release profile of growth factors when direct loaded in hydrogels.

way the growth factor is dispersed, which will influence the release kinetics; (3) the binding affinity defines how tightly the growth factor binds the system; this must be sufficiently low to allow release, but high enough to prevent uncontrolled release; (4) the release kinetics: its control allows the appropriate dose of growth factor to reach the cells over a given period of time; (5) the long-term stability: the system should enable growth factors to maintain their structure and activity over a prolonged period of time; (6) the economical viability: such biomaterials must further be easy to manufacture and to handle, and be cost-competitive;

In the following four sections, different approaches concerning growth factor delivery will be highlighted.

Strategies for growth factor release

1. Direct Loading. The easiest way to add proteins and peptides to polymeric systems is their direct loading into the polymeric matrix. However, if proteins are incorporated into hydrogels without any further modification, typical release profiles show a rapid burst release during the initial swelling phase, eventually followed by the extended release of a certain amount of protein that was retarded by the gel network.[13] In fact, a controlled release of protein over a long time will not be expected from hydrogels because the rate of protein release is generally diffusion-controlled through aqueous channels within the hydrogels.[5]

For instance, Kanematsu et al.[14] developed a matrix of synthetic type-I collagen and added growth factors into it. The hydrogel was formed in aqueous solutions containing basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), platelet derived growth factor-BB (PDGF-BB), vascular endothelial growth factor (VEGF), insulin like growth factor-1 (IGF-1), or heparin binding epidermal growth factor-like growth factor (HB-EGF). It was observed that the so-called heparin-binding growth factors, bFGF, HGF, VEGF, and HB-EGF, showed distinct release profiles. This may be evidence that the interaction between collagen and growth factors have different mechanism depending on each growth factor. Although growth factor release lasted some days, the burst effect was very remarkable. Other studies concerning direct loading have shown similar results.[15,16] A typical release profile form such systems is illustrated in Figure 1.

To alter the release profiles, modifications to the cross-linking density of the polymer networks can be applied,[13] as observed by Hiemstra and colleagues.[17] Dextran hydrogels were formed in situ upon mixing aqueous solutions of dextran vinyl sulfone conjugates and tetrafunctional mercapto-poly(ethylene glycol). The hydrogel degradation time and storage modulus could be controlled by the degree of vinyl sulfone substitution and dextran molecular weight. Proteins could be easily loaded into the hydrogels by mixing protein containing aqueous polymer solutions. The bFGF was released quantitatively from such hydrogels, with a close to first order kinetics in 28 days. Importantly, the release of proteins from these dextran hydrogels did not show a burst-effect.[17]

Direct loading of recombinant human FGF-2 in a 3% hydroxypropylcellulose (HPC) vehicle was tested for a clinical trial.[18] Subjects comprised 74 patients displaying a 2- or 3-walled vertical bone defect. Patients were randomly assigned to four groups: group P, given HPC with no FGF-2; group L, given HPC containing 0.03% FGF-2; group M, given HPC containing 0.1% FGF-2; and group H, given HPC containing 0.3% FGF-2. Each patient underwent flap operation during which we administered 200 μL of the appropriate preparation. During 36 weeks following administration, patients underwent periodontal tissue inspections and standardized radiographies of the region under investigation. To confirm soft-tissue regeneration, the millimeter of clinical attachment level regained was observed as a main outcome measure. Although no statistically significant differences were noted for gains in clinical attachment level and alveolar bone gain for FGF-2 groups versus group P, the significant difference in rate of increase in alveolar bone height ($p = 0.021$) between groups P and H at 36 weeks suggests that some efficacy could be expected from FGF-2 in stimulating regeneration of periodontal tissue in patients with periodontitis.

In a similar approach, recombinant human PDGF-BB directly loaded in a gel was tested in a double-blind, placebo-controlled, multicenter study for the treatement of chronic diabetic ulcers.[19] A total of 118 patients were randomized to receive either topical recombinant human hPDGF-BB gel (2.2 μg/cm ^{2} of ulcer area) or placebo until the ulcer was completely resurfaced or for a maximum of 20 weeks. A total of 29 (48%) of 61 patients randomized to the PDGF-BB group achieved complete wound healing during the study compared with only 14 (25%) of 57 patients randomized to the placebo group ($p = 0.01$). The median reduction in wound area in the group given PDGF-BB was 98.8% compared with 82.1% in the group given placebo ($p = 0.09$). Their results demonstrated that repeated, once-daily, topical application of recombinant human PDGF-BB gel was safe and stimulated rapid healing of chronic, full-thickness neurotrophic ulcers of the lower extremity in patients with diabetes mellitus.

The presented studies basically rely on the passive diffusion of the growth factors from the matrix. Apart from natural biopolymers, for example, fibrin, collagen, and HA, hydrogel materials are biologically inert, that is, they neither provide biologically specific signals for molecular interaction with the delivered growth factor nor interact specifically with cells of the target tissue. Rather, delivery of growth factors from inert matrices is primarily either driven by passive diffusion or is coupled to the rate of matrix resorption that occurs independently and often not in tune with cellular demands and responses at the target site. This apparent need for feedback between delivery matrix and responding cells has been widely ignored by designs of growth factor delivery systems.[20] However, recent active strategies have been developed.

Such an active approach concerned, for example, commercial fibrin precursor preparations, termed “fibrin glue”. It consists in a mixture of concentrated fibrinogen and thrombin usually derived by cryoprecipitation of human plasma (e.g., Tissucol, Baxter). In the presence of cells, fibrin gradually becomes degraded by plasmin or matrix metalloproteinase (MMPs)

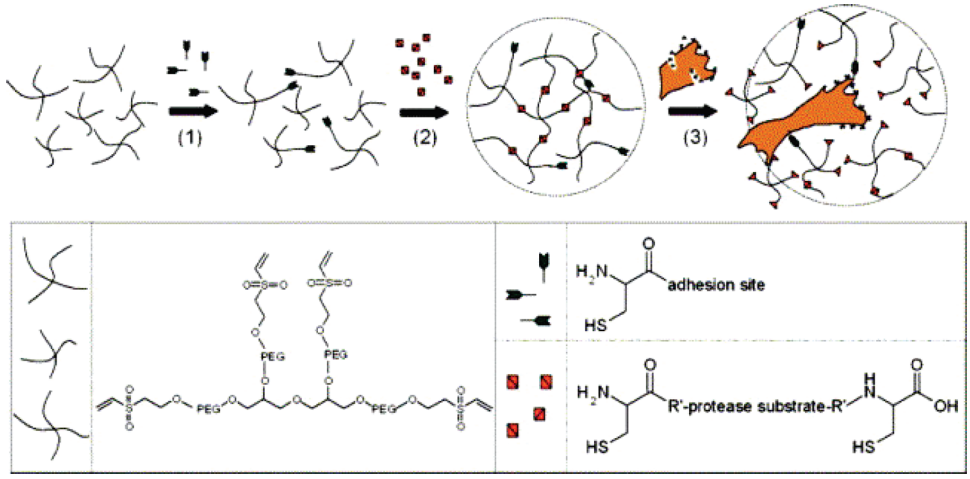

Figure 2. Stepwise synthesis of a PEG-based hydrogel by reacting multiarm vinyl sulfone-terminated PEG with a monocysteine-containing adhesion peptide (1) followed by reaction with an excess of bis-cysteine containing MMP substrate peptide (2). Incubation with a cell-secreted MMP is also shown (3). Structures of the multiarm vinyl sulfone-terminated PEG (cross lines); monocysteine-containing adhesion peptide (green wedge shaped arrows), and bis-cysteine containing MMP substrate peptide (orange divided squares) are shown in the legend. Reprinted by permission from Lutolf, M. P.; Lauer-Fields, J. L.; Schmoekel, H. G.; Metters, A. T.; Weber, F. E.; Fields, G. B.; Hubbell, J. A., "Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: Engineering cell-invasion characteristics.", *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*(9), 5413−5418.[26] Copyright (2003) National Academy of Science, U.S.A.

produced in the extracellular milieu. Because fibrin lyses slowly and locally, it has been used as a reservoir for growth factors. In spite of some positive results with simple mixtures of FGF or VEGF proteins in fibrin glue, the release kinetics of such preparations are indicative of an uncontrolled burst.[10]

Another strategy, which is not merely based on a passive diffusion, relies on mechanical-responsive system. In fact, delivery systems are mainly designed to work under static conditions. However, the environments in many tissues, such as the vasculature or musculature, are mechanically dynamic, and one may have to account for the possibility that the release of growth factors can be controlled by these mechanical signals. Mechanical compression leads to the release of unbound molecules, as a result of increased pressure within the gels. Additionally, upon gel relaxation, growth factor bound to the hydrogel dissociates, which replenishes the pool of soluble drug available for release by subsequent compression.[21,22] Lee et al. developed a biomaterial approach with VEGF-containing alginate hydrogels.[23] When these hydrogels were exposed to mechanical strain in vitro, the release rate of VEGF increased. After implantation in ischemic hind-limbs of diabetic mice, this mechanically induced release also increased collateral vessel formation.[23]

For all direct incorporation of proteins in hydrogels one has to consider the difficult step of loading the protein. If, for example, chemical cross-linking is involved in the hydrogel preparation, it is important to evaluate the chemical integrity of the added proteins. Additionally, if loading is performed after the cross-linking, the even distribution of the biomolecule within the gel has to be verified.[13]

2. Covalently Binding Growth Factors. As an alternative to the previously described method of incorporation, proteins can also be covalently attached to polymers. This covalent attachment can be achieved by reacting the different side chain functionalities of polymers with the amino acids of the growth factors (e.g., lysine, cysteine).[13]

Fan et al.[24] investigated cellular responses of mesenchymal stem cells (MSC) on a biomaterial surface covalently modified with EGF. The polymer substrate presented clusters of closely spaced ligands on PEG tethers to covalently tether EGF via the N terminus. They found that surface-tethered EGF promoted both cell spreading and survival more strongly than saturating concentrations of soluble EGF.

In an elegant strategy, Zisch et al. immobilized an adhesion peptide and VEGF within a MMP-sensitive, synthetic hydrogel networks via a mechanism that enabled cell-controlled release. The methodology proceeded in three steps. First, PEG vinyl sulfone (PEG-VS) was conjugated with RGD adhesion peptides. Afterwords, VEGF was reacted with a large excess of RGD-functionalized PEG-VS to create another covalent bond. Subsequently, a MMP-sensitive cross-linking peptide was added to induce gelation. Such molecule was a bis-cysteine peptide Ac-GCRD-*GPQG*↓*IWGQ*-DRCG (↓ indicates the MMP cleavage site; the two reactive cysteines are underlined). The first and the third steps are shown in Figure 2. The cross-linking reaction took place between reactive thiols of cysteine peptides and vinyl sulfone of PEG-VS. In the resulting system, the growth factor could be delivered on cell demand. Indeed, an active VEGF liberation was confirmed from PEG-peptide hydrogels by incubation with MMP-2 or plasmin. Besides, when implanted subcutaneously in rats, these VEGF-containing matrices were completely remodeled into native, vascularized tissue.[20] Such synthetic hydrogels prepared through Michael-type addition reaction of thiol-containing peptides onto vinylsulfone-functionalized PEG have also successfully been used to deliver other proteins, like human bone morphogenetic protein.[25]

Such bioresponsive systems have also been widely employed for drug delivery, tissue engineering and development of biosensors, as reviewed by Ulijn and co-workers.[27]

Transforming growth factor beta 1 (TGF-β1) is another growth factor that was covalently attached to PEG. The conjugation was achieved by reacting TGF-β1 with acryloyl-PEG-*N*-hydroxysuccinimide. In vivo experiments demonstrated that the growth factor remained active after binding the polymer, but the obtained effect was lower than when unmodified TGF-β1 was used.[28] In the literature, it is also reported the covalent binding of PEG to epidermal growth factor, EGF,[29] bFGF,[30] and TGF-β2.[31]

Besides bulk conjugation of growth factors to polymers, surface immobilization is feasible. Backer and colleagues

developed a strategy to immobilize recombinant proteins based on the expression of the protein of interest with a 15-amino acid long fusion tag (Cys-tag), which contains a free sulfhydryl group for site-specific conjugation. They conjugated a single-chain vascular VEGF (scVEGF) expressed with an *N*-terminal Cys-tag to fibronectin (FN) using a common thiol-directed bifunctional cross-linking agent, sulfosuccinimidyl 6-[3′(2-pyridyldithio)-propion-amido] hexanoate. They showed that their method was reliable for immobilizing growth factors on polymers.[32]

In a different approach, carbodiimide chemistry may be used to conjugate growth factors onto a polymer surface. For instance, Shen et al.[33] bound VEGF onto collagen scaffolds using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI). Immobilized VEGF, compared to soluble VEGF, successfully promoted the penetration and proliferation of endothelial cells in these collagen scaffolds.

3. Carrier Systems. If the retardation of protein release from the presented approaches is not sufficient to provide enough protein for long-term applications, carriers may be added to the hydrogels to retain the bioactive factors for an extended time.[15] To achieve this, microparticles or drug-releasing implants, can be incorporated into the gel.[13]

Such delivery systems may be capable of recreating certain qualitative and quantitative aspects of physiological growth factor delivery. More specifically, they may supply adequate local concentration [*C*] of the molecules, in the form of temporal gradients (d[*C*]/d*t*) and spatial gradients (d[*C*]/d*x*), and in combination with other biomolecules. Both physicochemical and biological characteristics of the synthetic and natural polymers used to fabricate the systems are critical parameters.[34]

In addition, a carrier can offer to its "protein cargo" adequate protection from inactivation occurring in biological environments, and guarantee bioactivity preservation. The carrier can be designed in different shapes as particles or implants. Besides, architectures may also vary from reservoirs to matrices. In the former, the molecule of interest is contained within a central core and is surrounded by a polymeric membrane that regulates the rate of release. In the latter, the protein is dispersed within a solid continuous polymer phase.[35] Such systems may be made with different biodegradable and nonbiodegradable materials offering a tunable control over the release rate.[36] In the following section, some strategies are described.

Nondegradable Systems. Diffusion-controlled systems based on nonbiodegradable polymers have been first used for the controlled release of drugs. In such systems, protein transport out of the device is driven by a concentration gradient and limited by the presence of an insoluble polymeric matrix which regulates drug diffusion. Mass transport occurring through polymer chains or pores is the only rate-limiting step of the process. Reservoir and matrix systems can be designed to respectively achieve zero- or first-order release kinetics, with different biological implications.[36]

One example of material used for such a system is ethylene-vinyl acetate (EVAc). The EVAc systems allow a high degree of control over agent release, versatility in allowing the release of a wide range of agents, and good retention of biological activity, as demonstrated by Kim et al.[37] In their work, polymer pellets were dissolved in methylene chloride to obtain a 10% solution (w/v). A mixture of PDGF and/or TGF was then added to the polymer solution, vortexed, snap frozen in liquid nitrogen, and lyophilized. The resulting EVAc-growth factor solid was extruded into continuous polymer rods. Rods were overcoated by dipping in a 10% EVAc methylene chloride solution using an automated dipping apparatus. In such a process, the main drawback was the loss of physical mass and bioactivity.[38]

Other materials[39] and other techniques such as fluid-bed, coacervation, spray-drying, spray-cooling, extrusion, and emulsification/cross-linking may also be used. A review of other useful methods, such as fiber bonding, melt molding, solvent casting/particulate leaching, and gas foaming/particulate leaching, is provided elsewhere.[40]

Degradable Systems. Current studies have also been directed toward the development of soluble/biodegradable systems. Among synthetic biodegradable polymers, thermoplastic aliphatic polyesters like polylactide (PLA) and poly(lactic-*co*-glycolic acid) (PLGA) have generated tremendous interest due to their excellent biocompatibility.[36,41]

The techniques used to produce nondegradable systems may be used to produce degradable ones. One of them is the water in oil in water (W/O/W) emulsion extraction-evaporation approach. It consists in preparing an aqueous solution of the growth factor that is emulsified in an organic solution containing the polymer. This primary emulsion is mixed with an external aqueous solution to obtain a double emulsion. Organic solvents were then extracted from the resulting double emulsion to obtain the hard particles.[42] Inside a hydrogel, particles degrade and the encapsulated protein is released. Then, it can slowly diffuse away to act on cells.[13]

PLGA bFGF-containing microparticles produced by this method were incorporated in an alginate matrix. In vitro, bFGF was released from the porous composite scaffolds in a controlled manner and it was biologically active, as assessed by its ability to induce the proliferation of cardiac fibroblasts. The controlled delivery of bFGF from the three-dimensional scaffolds accelerated the matrix vascularization after implantation on the mesenteric membrane in rat peritoneum. The released bFGF induced the formation of large and matured blood vessels, as judged by the massive layer of mural cells surrounding the endothelial cells.[43]

In the study of Ferreira et al.,[44] human embryonic stem cells (hESCs) were encapsulated in a dextran-based hydrogel containing VEGF-loaded microparticles of PLGA. The aim was to investigate if the release of such growth factor would enhance the vascular differentiation. Microparticles were prepared using a double emulsion solvent evaporation procedure. The release kinetics in VEGF-loaded microparticles showed an initial burst followed by a lower steady-state release. The bioactivity of VEGF released from microparticles was evaluated in vitro by assessing its survival effect on an endothelial cell line. Cells grown in wells containing VEGF showed a significant higher viability (as measured by the MTT viability assay) than cells grown in wells without this growth factor ($100 \pm 7.7\%$ vs $67.7 \pm 5.7\%$, respectively). After culturing hESCs in the hydrogel with VEGF-loaded microparticles, they examined the expression of a vascular marker, the VEGF receptor KDR/Flk-1. It was observed that the incorporation of VEGF-containing microparticles in the hydrogel network did not contribute significantly for the up-regulation of this marker.

A different research also concerned stem cells. Ashton et al.[45] investigated the incorporation of PLGA microspheres loaded with alginate lyase into alginate hydrogels. They demonstrated that alginate hydrogels could be enzymatically degraded in a controlled and tunable fashion. Neural progenitor cells could be cultured and expanded in vitro in this degradable alginate hydrogel system. They proposed growth factors to be introduced in the microspheres formulation in order to improve the performance of the system.

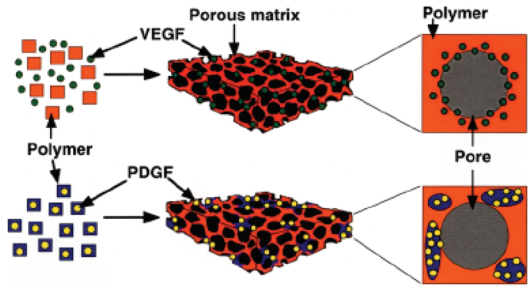

Figure 3. Schematic representation of scaffold fabrication process. Growth factors were incorporated into polymer scaffolds by either mixing with polymer particles before processing into scaffolds (VEGF), or pre-encapsulating the factor (PDGF) into polymer microspheres used to form scaffolds. Reprinted by permission from Macmillan Publishers Ltd: *Nat. Biotechnol.* **2001**, *19*(11), 1029−1034,[46] copyright 2001 (http://www.nature.com/nbt/index.html).

Biodegradable carriers offering a time-control of the delivered dose can be useful to trigger the release of a bioactive molecule and maintain a specific concentration for extended duration. Furthermore, this strategy gives the opportunity to deliver more than one protein at different preprogrammed rate according to the needs of a specific application.[36]

Richardson and colleagues described a system from biodegradable polymers that enabled the delivery of two or more growth factors with distinct kinetics. The utility of this system was investigated using a well-characterized model, the formation of new blood vessels (angiogenesis). Growth factors were incorporated into polymer scaffolds by either mixing with polymer particles before processing into scaffolds (VEGF), or pre-encapsulating the factor (PDGF) into polymer microspheres used to form scaffolds. The VEGF incorporation approach resulted in a rapid release. In contrast, the PDGF incorporation approach resulted in a more even distribution of factor throughout the polymer, with release regulated by the degradation of the polymer used to form microspheres. The two growth factors were incorporated together into the same scaffolds by mixing polymer microspheres containing pre-encapsulated PDGF with lyophilized VEGF before processing into scaffolds (Figure 3). They were implanted into the subcutaneous pockets on the dorsal side of rats. After histological analysis, it was observed that the dual delivery of growth factors, each with distinct kinetics, from a single, structural polymer scaffold resulted in the rapid formation of a mature vascular network.[46]

Recently, Holland et al.[47,48] have also worked on the development of systems for dual release of growth factors, TGF-β1 and IGF-1. Their delivery technology employed the oligo [poly (ethylene glycol) fumarate] (OPF), a water-soluble polymer, in the fabrication of biodegradable hydrogels which encapsulated gelatin microparticles. Using these OPF-gelatin microparticle composites, dual controlled release was achieved with growth factor loading in either the microparticle phase or OPF phase of gels. Parameters such as microparticle cross-linking extent and density within these gel networks, as well as the phase of growth factor loading, provided an effective means of controlling the release profiles of these growth factors.

Besides involving its cargo, the carrier may also interact with it. Controlled release is then achieved by both a mechanical and chemical mechanism. This is the case of this latter study and also of the one performed by Park et al.[49] It consisted of a hydrogel of oligo[poly(ethylene glycol) fumarate] encapsulating gelatin microparticles loaded with TGF-β1. At pH 7.4, an ionic complex of gelatin and TGF-β1 due to the negative charge of the acidic gelatin and positive charge of TGF-β1 is formed. The following section will deal with ionic interactions for the controlled release of growth factors.

4. Electrostatic Interaction. Some of the methods presented so far may result in protein denaturation and a subsequent lowering in the activity.[5] Therefore, a different strategy that relies on the electrostatic interaction between growth factors and the matrix seems to be very promising.

Despite the fact that growth factors are originally described as soluble molecules, evidence shows that the binding of growth factors to ECM is a major mechanism regulating their activity.[50] An increasing number of growth factors, including IGFs, FGFs, TGF-3′s, EGF, VEGF, PDGF, and HGF, have been found to associate with the extracellular matrix proteins or with heparin/heparan sulfate (HS). The largest group of growth factor interactions involve the binding to heparin or to HS.[4,10,50] To produce mimetics of ECM that display such a functional interplay between matrix and factors described above, it may be more appropriate to present the growth factors bound to the matrix rather than in soluble form. The desire for functional mimetics of natural ECM that protect growth factor activities and achieve more timely and spatially controlled delivery has led to employ natural and synthetic molecules as bulking agents to interact with growth factors.[10] This will be detailed in the following section.

A. Interaction with glycosaminoglycans (GAG). Heparin and HS. Heparin and HS are linear sulfated polysaccharides with molecular weights of roughly 10000∼100000. The largest part of each chain is constructed with a number of disaccharides repeating units that are composed of uronic acid and an amino sugar (glucosamine). Because of their high negative charge, the GAG chains bind to a plethora of proteins, including the members of FGF family and their receptor tyrosine kinases, TGFs, BMPs, Wnt proteins, chemokines, and interleukins, as well as to enzymes and enzyme inhibitors, lipases and apolipoproteins, ECM, and plasma proteins.[51] Studies have demonstrated that specific sulfation motifs function as molecular recognition elements for growth factors and modulate neuronal growth. It is proposed the existence of a "sulfation code", whereby glycosaminoglycans encode functional information in a sequence-specific manner analogous to that of DNA, RNA, and proteins.[52] Due to such interactions, proteins and especially growth factors become more resistant to proteolysis and thermal denaturation when bound to HS or heparin.[53]

Considering their properties, attachment of GAGs to biomaterials may result in an appropriate matrix for the binding, modulation, and sustained release of growth factors, as commented below.

In the literature, GAGs have been associated to different polymers. One of them is PLGA. Heparin-conjugated PLGA nanospheres (HCPNs) were developed for long-term, zero-order delivery of bFGF. HCPNs were prepared by using a coupling reaction between amino-terminated PLGA nanospheres and heparin in the presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDCI). The release of bFGF from HCPNs was sustained for 3 weeks with no initial burst release. The bFGF release period was increased to over than 4 weeks using a delivery system of HCPNs suspended in fibrin gel for further retention. The release was nearly of zero order. The rate of bFGF release from HCPNs in fibrin gel was inversely correlated to the fibrinogen concentration in the fibrin gel. The bioactivity of bFGF released from HCPNs in fibrin gel was assessed using human umbilical vein endothelial cell (HUVEC) culture. Controlled bFGF released from HCPNs in fibrin gel supported HUVEC growth for 15 days, similarly to cultures feeded with

soluble bFGF, suggesting that the delivery system of HCPNs in fibrin gel could release bFGF in a bioactive form for a long period. In vivo tests using a mouse limb ischemia model were also successful.[54]

Heparin can also be attached to hydrogel by using maleimide groups. For instance, a four-arm star PEG was heparin-decorated by the reaction of its terminal thiol with maleimide-functionalized low molecular weight heparin. The produced hydrogel was demonstrated to sequester and deliver bFGF in a controlled manner. The release kinetics of bFGF closely matched the matrix erosion kinetics mediated by dissociation of the constituent molecules, and lasted several days.[55]

A different approach for covalent heparin attachment consists in modifying its carboxyl groups to obtain thiol groups. Such molecules reacted with PEG diacrylate, and underwent Michael addition to form thioether linkages. The mechanical properties, gelation kinetics, and swelling ratios of the hydrogels could be controlled by modulating the precursor concentration and degree of substitution of functional groups. Fibroblast cells were encapsulated in situ and remained viable after gelation. Their proliferation was also observed inside this heparin-containing gel.[56]

In another study, bFGF was bound to cross-linked collagenous matrices with and without covalently attached HS. The location of bFGF and its release from the matrices were analyzed. Respectively, immunolocalization and radioactivity measurements using human recombinant ^{125}I-bFGF were employed. The tissue response to the matrices was evaluated by light and electron microscopy after subcutaneous implantation in rats. Attachment of HS to collagen matrices increased by a 3-fold factor the bFGF binding capacity, and resulted in a more gradual and sustained release of the growth factor in vitro. In vivo, the presence of HS without bFGF resulted in a transient vascularization, predominantly at the matrix periphery. Angiogenesis was further enhanced by combining HS with bFGF. In contrast to collagen-HS and collagen/bFGF matrices, collagen-HS/bFGF matrices remained highly vascularized throughout the matrix during the 10-week implantation period.[57]

Willerth et al.[58] used an affinity-based delivery system for mouse embryonic stem cell differentiation inside fibrin scaffolds. The affinity-based delivery system presented three components: a bidomain peptide, heparin, and growth factor. The peptide contained a Factor XIIIa substrate derived from α2-plasmin inhibitor, allowing it to be covalently cross-linked into the fibrin scaffold, and a heparin-binding domain derived from antithrombin III, which bound heparin noncovalently and retained it inside the fibrin scaffold. The heparin could in turn bind growth factors and retain them inside the scaffold. This delivery system was used to deliver different molecules, such as PDGF, at various doses. The controlled delivery of these molecules simultaneously increased the fraction of neural progenitors, neurons, and oligodendrocytes while decreasing the fraction of astrocytes obtained compared to control cultures seeded inside unmodified fibrin scaffolds with no growth factors present in the medium.

In a different study, heparin was modified with methacrylate groups, copolymerized with dimethacrylated poly(ethylene glycol), and analyzed as a delivery vehicle for bFGF and synthetic extracellular matrix for the differentiation of hMSCs.[59,60] Results demonstrated that methacrylate-modified heparin retained its ability to bind heparin-binding proteins both in solution and when copolymerized with dimethacrylated PEG in a hydrogel. In addition, the heparin functionalized gels could deliver biologically active bFGF for up to 5 weeks. The gels were examined as a potential scaffold for hMSC culture and were found to promote adhesion, proliferation, and osteogenic differentiation.

Besides the possibility of using chemical groups to functionalize and attach GAGs to hydrogels, an alternative is to create hydrogels from artificial proteins presenting heparin binding sites. For instance, Halstenberg and colleagues,[61] by recombinant DNA methods, produced an artificial protein containing repeating amino acid sequences based on fibrinogen and antithrombin III, comprising a heparin-binding site. It was found that heparin bound strongly to this region. The hydrogel of this protein was able to make cells survive, spread, and migrate. Through the design and expression of artificial genes, it was possible to prepare artificial proteins with controlled properties and with domains chosen to modulate cellular behavior. This approach avoids several important limitations encountered in the use of natural ECM proteins, including batch-to-batch (or source-to-source) variation in materials isolated from tissues, restricted flexibility in the range of accessible materials properties, and concerns about disease transmission associated with materials isolated from mammalian sources.[62]

Heparin-alginate slow-release microcapsules were tested in a clinical trial for the induction of collateral growth in patients undergoing coronary artery bypass grafting.[63,64] Heparin-sepharose beads were mixed with filter sterilized sodium alginate (1.2% w/v). The mixed slurry was dropped through a needle into a beaker containing a hardening solution of calcium chloride (1.5% w/v). Microcapsules were formed instantaneously as the mixture entered the hardening solution. bFGF was incorporated within the microcapsules, after calcium alginate matrix formation and hardening, by incubation in PBS with 0.05% gelatin and bFGF for16 h under gentle agitation at 4 °C.[65] These clinical trials demonstrated the safety and technical feasibility of therapeutic angiogenesis with basic fibroblast growth factor delivered by heparin-alginate slow-release devices.

B. Interaction by Means of Biomolecules other than GAG. Gelatin. Gelatin is another charged biopolymer able to interact with growth factors. The isoelectric point of gelatin can be modified during its extraction from collagen to yield either a negatively charged acidic gelatin, or a positively charged basic gelatin. This allows for flexibility in terms of enabling polyion complexation by a gelatin carrier with either positively or negatively charged therapeutic agents. For instance, acidic gelatin with an isoelectric point (IEP) of 5.0 should be used as a carrier for basic proteins in vivo, while basic gelatin with an IEP of 9.0 should be used for the sustained release of acidic proteins under physiological conditions.[66]

A study was reported in which bFGF was sorbed into the hydrogel of acidic gelatin with IEP of 5.0. The profile was compared with the one from basic gelatin, and PAA (anionic) hydrogels. In addition, the influence of solution temperature and ionic strength on the bFGF sorption and desorption was analyzed. It was observed that the bFGF sorption to the acidic gelatin hydrogel increased when gelatin was further carboxylated. The bFGF protein sorbed into the acidic gelatin hydrogel more slowly than into PAA hydrogel, probably because of the lower density of negative charge of gelatin. When comparing the acidic and basic gelatin hydrogels, the bFGF diffusion seemed to depend on the gelatin type. This dependence could be explained in terms of the electrostatic interaction between bFGF and gelatin. Basic bFGF must be electrostatically repelled by the basic gelatin constituting the hydrogel, leading to reduced diffusion through this hydrogel. On the contrary, bFGF sorption neutralized the negative charges of acidic gelatin, which would enable bFGF to diffuse into the hydrogel. A significant

temperature dependence of bFGF sorption to the acidic gelatin hydrogel was observed. It may be attributed to the intermolecular interaction change of gelatin accompanied with its conformational change by the reduced temperature. Probably, intermolecular aggregation of gelatin molecules by partial helical conformation induced at lower temperature reduced the number of charged groups which, otherwise, could ionically interact with bFGF. The binding molar ratio of bFGF to the acidic gelatin was around 1.0. The bFGF sorption decreased with an increase in solution ionic strength, indicating that an electrostatic interaction was the main driving force for bFGF sorption to the acidic gelatin hydrogel. The large K_d value of bFGF for the acidic gelatin hydrogel demonstrated that the electrostatic interaction of bFGF with the acidic gelatin is not as strong as that of bFGF-HS but still enough to achieve the initial bFGF sorption to the hydrogel.[67]

A related study, however, presented controversial results. Ionic, gelatin-based hydrogels were prepared by the carbodiimide-mediated amidation reaction between the carboxyl groups of gelatin or poly-L-glutamic acid (PLG, anionic gels) molecules and the amine groups of poly-L-lysine (PLL, cationic gels) or gelatin molecules, respectively. The presence of either PLL or PLG significantly affected the rate of FGF-2 release as compared to the gelatin gels without PLL or PLG. Gelatin-PLL and gelatin-PLG hydrogels demonstrated a sustained release profile of the FGF-2 in comparison to gelatin hydrogels that exhibit pronounced burst release profiles. A total of 80% of the FGF-2 was released at controlled rates from gelatin−polylysine (gelatin-PLL) and gelatin−polyglutamic acid (gelatin−PLG) hydrogel scaffolds over a period of 28 days.[68] This study showed that there was a minimal difference on the release profile of FGF-2 from gelatin−PLL and gelatin−PLG over 28 days, contrary to the fact that one may expect that positively charged scaffolds (gelatin−PLL) would tend to release FGF-2 in a burst manner.

The possibility of delivering multiple growth factors from gelatin hydrogels was also demonstrated. For instance, the immobilization of human recombinant bFGF and human recombinant granulocyte colony-stimulating factor in an ionic, gelatin-based hydrogel was reported. A controlled release extended for several days has been reported for both growth factors.[69]

A gelatin-based delivery systems for bFGF controlled-release was approved for a clinical trial in the treatment of limb ischemia.[70] A concise review on gelatin as a delivery vehicle for growth factors and other biomolecules is provided elsewhere.[71]

Collagen. Collagen is another biomolecule capable of interacting with growth factors. For instance, bFGF spontaneously interacts with type I collagen solution and sponges under in vitro and in vivo physiological conditions, which protects it from the proteolytic environment. The bFGF incorporated in a collagen sponge sheet was sustainedly released subcutaneously in mouse according to the biodegradation of the sponge matrix, and exhibited local angiogenic activity in a dose-dependent manner. Intramuscular injection of collagen microsponges incorporating bFGF induced a significant increase in the blood flow in the murine ischemic hindlimb, which could never have been attained by bolus injection of bFGF. These results suggested the feasibility of type I collagen as a reservoir of bFGF.[72] In other studies, it was demonstrated that collagen could also interact with genetically engineered EGF[73] and HGF.[74]

C. Interaction with Dextran Derivatives. Heterogeneity in the composition and in the polydispersity of GAGs has motivated the research of other molecules for potential replacement.[75,76] Modified dextrans are the main representative examples.

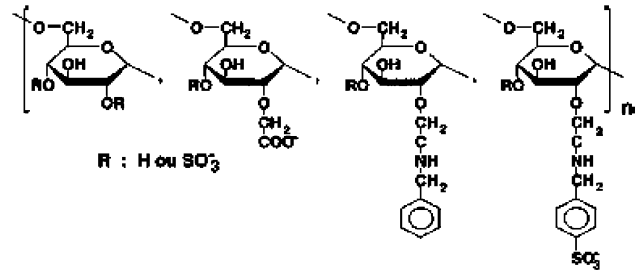

Figure 4. Structure of water-soluble derivatized dextrans: carboxymethyl benzylamide sulfonate dextran (CMDBS). Reprinted by permission from D. Logeart-Avramoglou and J. Jozefonvicz: "Carboxymethyl benzylamide sulfonate dextrans (CMDBS), a family of biospecific polymers endowed with numerous biological properties: A review", *J. Biomed. Mater. Res.* **1999**, *48*(4), 578−590.[4] Copyright 1999 John Wiley and Sons, Inc. Reproduced with permission of John Wiley and Sons, Inc.

The functionalized dextrans termed carboxymethyl benzylamide sulfonate dextran (CMDBS) represent a family comprising a wide range of polymers (Figure 4). As reported by the Jozefonvicz's group,[77] CMDBS may be prepared using four distinct reactions: carboxymethylation, benzylamidation and sulfonation of aromatic ring with concomitant sulfation of free hydroxyl groups. These soluble macromolecular compounds, which are substituted with specific chemical functional groups, are designed to interact with living systems. Their properties depend on the various anionic groups in the molecule and on their distribution along the macromolecular chain.[78] By analogy with heparin, it is postulated that CMDBS compounds also possess binding sites capable of specific interactions with biological constituents, depending on the overall composition of the polymer. These synthetic macromolecules offer the benefit of no potential risk of virus or unconventional particle contamination from donors and represent a relatively inexpensive and easily obtainable source of new bioactive molecules.[4]

In fact, the size and degree of sulfation of heparin-derived saccharides are directly related to their enhancing role on bFGF-mediated cell proliferation. High molecular weight heparins and bFGF likely form a complex involving several sites of interaction between the positively charged lysine and arginine residues of the bFGF and the negatively charged sulfated groups of heparin. In the hypothesis that CMDBS act as functional analogues of heparin, the formation of a complex between HBGF and high molecular weight dextran derivatives might involve the same kinds of interactions.[4] The fact that heparin competes with CMDB and their fragments for binding to FGF-2 strongly supports such an assumption.[79]

Several CMDBS differing in degree and positioning of the substituent groups can mimic heparin in regard to the protection, stabilization, and potentiating effects with aFGF or bFGF. Jozefonvicz group's data establish that some dextran derivatives can act as potentiating agents for FGFs, while native dextran has no effect. Dextran derivatives can also protect aFGF and bFGF from heat as well as from pH denaturation and against trypsic and chymotrypsic degradation. A dextran derivative with 82% methylcarboxylic acid, 23% benzylamide, and 13% sulfonate was studied and exhibited a greater protection for bFGF and a lesser protecting effect for aFGF than heparin. Derivatized dextrans that have very weak anticoagulant activity are of great interest as alternatives to heparin for use as stabilizers, potentiators, protectants, and slow-release matrices for FGFs in phar-

maceutical formulations.[80] On the other hand, the interaction potential of dextran derivatives is limited in comparison to GAGs. Although CMDBS can also interact with TGFβ,[81] no interaction was observed with IGF-1 nor EGF.[79]

Additional compounds have also been studied to replace GAGs. The interested reader may refer to refs 75 and 8284 for further information.

Growth Factor Delivery Systems: Characterization Methods

When delivering proteins, it is important to keep in mind that their chemical and physical integrity is important for their activity, and the delivery of necessary doses is essential. Consequently, sensitive techniques are urgently needed to fully evaluate the action of such systems.[13] These evaluation methods are the focus of the following section.

1. Incorporation and Release Studies. The incorporation of fluorescence-labeled model substances allows one to track the labeled proteins in delivery systems. Standard procedures use, for instance, covalent FITC binding. Alternatively, the incorporation and the release of proteins can be monitored using colorimetric tests, radioactive ones or enzyme linked immunosorbent assays (ELISAs) sensitive for the protein of interest.[13] They are detailed below.

As stated above, fluorescent labeling of growth factors are frequently performed for incorporation and release studies. For instance, bFGF was labeled with rhodamine isothiocyanate (RITC) and its incorporation kinetics into hydrogels was assessed using high performance liquid chromatography (HPLC) equipped with a heparin affinity column. Release studies were performed using the same technique.[67]

Such studies can also be carried out by using radioactively labeled growth factors.[85] In the article of Pieper and colleagues,[57] the amount of bFGF incorporated into the hydrogel was determined using human recombinant ^{125}I-bFGF. The radioactivity of the matrices was measured using a gamma-counter. A similar approach was used in other work. Human recombinant bFGF was labeled using ^{125}I Iodobeads. Hydrogels were loaded with bFGF either using unlabeled bFGF or ^{125}I-labeled one. For quantification, a sandwich ELISA and a gamma-counter were respectively used. The results from unlabeled bFGF and ^{125}I-labeled bFGF were in good agreement.[86] ELISA analysis have also been used in many other similar papers.[43,68,87,88]

For all assays it is important to evaluate if they are robust enough to exclusively detect the intact protein, and not its degradation products, and if modifications to the proteins, for example fluorescence labeling, have an impact on the release kinetics, for example, by altering the adsorption behavior of the proteins.[13]

2. GAG Content/Localization. The determination of glycosaminoglycan contents attached to a hydrogel may be carried out by hexosamine analysis using *p*-dimethylaminobenzaldehyde. Absorbance is measured at 525 nm using a UV−visible spectrophotometer.[57,89,90]

GAG content may also be determined using the dimethylmethylene blue dye (DMMB) assay. A red color is produced by DMMB binding to GAG, allowing for the quantification of GAG by measuring the absorbance at 520 nm. The GAG content in the hydrogels is calculated by a comparison with a curve generated from the standards of known amounts of GAG.[91−93]

Alternatively, the toluidine blue method may also be used.[54,55] For this test, the sample is added to a toluidine blue solution in 0.1% NaCl and mixed. Hexane is added and the mixture is vortexed for 30 s and left standing for 12 h. The absorbance of the aqueous layer is measured at 631 nm and the amount of immobilized GAG is calculated from a standard calibration curve. Such standard calibration curve is produced via organic extraction and absorbance measurements (631 nm) of the aqueous phase of toluidine blue solutions containing known amounts of GAG and a GAG-free hydrogel sample. The toluidine blue-heparin complex is extracted into the organic phase, and the remaining toluidine blue concentration of the aqueous phase is measured. Concerning microscopic glycosaminoglycan visualization, safranin-O (red) is proposed.[90]

3. Immunolocalization of Growth Factors. Immunological techniques may be employed for determining growth factor distribution in the matrix. For example, bFGF-containing hydrogels were incubated with rabbit antirat bFGF. After incubation with biotin-conjugated donkey antirabbit for 1 h and in avidin−biotin complex (ABC-Elite) for 2 h at 20 °C, the bFGF-antibody peroxidase complex was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB). Sections were incubated for 10 min in a chromogen solution. This resulted in a blue-black staining that enabled the localization of growth factors.[57]

4. Determination of the Physical State and Immunoreactivity. Besides growth factor quantification, ELISA tests indicate inactivation of the proteins.[13] For further determination of the physical state and immunoreactivity of the released growth factors, Western blots are a promising tool. According to the methodology proposed by Anat,[43] releasing media from a bFGF-containing hydrogel at different time intervals were subjected to this analysis. The samples were run on 15% polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes. An equal loading and transfer efficiency were verified by staining with 2% Ponceau S. The membranes were blocked overnight with PBS and 5% fat-free skim milk and then incubated for 4 h at room temperature with a 1:500 diluted monoclonal antihuman bFGF. For detection, horseradish peroxidase-linked anti human IgG antibody (1:200, 1 h at room temperature) and enhanced chemiluminescence substrate were used.

5. Bioassays. For a complete evaluation of the release systems, additional biological tests are very important, since the methods presented above do not represent an absolute evaluation of the stability and integrity of the incorporated substances. Therefore, the biological activity of the released growth factors should be determined by testing their ability to stimulate the proliferation of cells in vitro or in vivo.[15,43,68,94−97]

Conclusions

The controlled delivery of growth factors is a very challenging task since many different issues have to be addressed to develop the best suited system. First of all, its design must take many facts into consideration, as stated in the introduction section of this paper, ranging from the loading capacity to the economical viability. Then, to produce the growth factor hydrogel delivery systems, the choice should be based not only on the characteristics of the hydrogel, but also on the purpose for which the system is designed. After producing the delivery system, its feasibility must be tested. As described in the section "Growth Factor Delivery Systems: Characterization Methods", studies such as growth factor content, release, stability, and biological activity are the basis for determining the efficacy of system. Altogether, the appropriate development of growth factor

delivery systems for hydrogel lies on the suited carrying-out of all these steps.

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