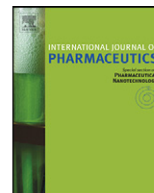
International Journal of Pharmaceutics 364 (2008) 281–297

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm



Review

Bioinspired tissue engineering: The great promise of protein delivery technologies

Fabiana Quaglia[a,b,*]

[a] *Department of Pharmaceutical and Toxicological Chemistry, University of Naples Federico II, Via D. Montesano 49, 80131 Naples, Italy*
[b] *Interdisciplinary Research Centre on Biomedical Materials, University of Naples Federico II, Italy*

ARTICLE INFO

Article history:
Received 31 January 2008
Received in revised form 11 April 2008
Accepted 15 April 2008
Available online 26 April 2008

Keywords:
Microparticles
Tissue engineering
Growth factors
Scaffolds

ABSTRACT

The concept of developing a tissue either *in vitro* or *in vivo* taking inspiration from physiological events has prompted toward the integration of molecular signals such as growth factors (GFs) in tissue engineering strategies with the aim to guide cell proliferation, differentiation and migration. After the first studies, the awareness emerged that a fine tuning of GF levels in the scaffold, when present, and at boundary with healthy tissue was needed to give successful results. Thus, the modality of GF presentation to cells has been recognized as a key fundamental in many tissue engineering applications and applied through different approaches. In this scenario the potential of particulate systems for GF delivery was promptly perceived as a mean to protect GFs during tissue regrowth and to offer adequate control over release rate. The use of tissue engineering constructs based on GF-loaded particles integrated in different scaffold types has impressively grown in recent years and led to significant advances in the field. Release of more than one GF at rates mimicking *in vivo* situation has become possible as well as to exert a fine control over GF spatial concentration by developing constructs with specific areas of bioactivities. However, if we consider the strategies for protein delivery currently applied in tissue engineering, it is soon realized that much more can be done. Thus, the aim here is to review some tissue engineering approaches involving the use of GFs by the point of view of delivery issues trying to highlight the remarkable impact that particulate systems can have in the next future.

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Contents

1. Introduction 282
2. Tissue engineering: some basic aspects 282
   2.1. Tissue engineering strategies 282
   2.2. Composition and processing of tissue engineering scaffolds 282
   2.3. Polymer scaffolds bioactivated with growth factors 284
3. Drug delivery in tissue engineering 285
   3.1. Scaffold as growth factor delivery platform 285
      3.1.1. Free growth factors incorporated in "*in situ* forming" matrices 285
      3.1.2. Free growth factors incorporated in shaped scaffolds 286
   3.2. Growth factor-loaded delivery systems in tissue engineering 289

Abbreviations: BAM, bladder acellular matrix; BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic protein; BSA, bovine serum albumin; CNTF, ciliary neurotrophic factor; DS, delivery systems; CPC, calcium phosphate cement; ECGF, endothelial cell growth factor; ECM, extracellular matrix; EGF, epidermal growth factor; EVAc, ethylene-*co*-vinyl acetate copolymer; FGF, fibroblast growth factor; GDNF, glial-derived neurotrophic factor; GF, growth factors; GGF, glial growth factor; IGF, insulin-like growth factor; NGF, nerve growth factor; NT, neurotrophin; OPF, oligo(poly(ethyleneglycol) fumarate; PDGF, platelet derived growth factor; PEG, poly(ethyleneglycol); PCL, polycaprolactone; PGA, poly(glycolic acid); PLA, poly(lactic acid); PLGA, poly(lactide-*co*-glycolide); $scCO_2$, supercritical CO_2; TCP, tricalcium phosphate; TGF, transforming growth factor; VEGF, vascular endothelial growth factor (isoform 165 unless otherwise specified).

* Correspondence address: Department of Pharmaceutical and Toxicological Chemistry, Via D. Montesano 49, 80131 Naples, Italy. Tel.: +39 081678707; fax: +39 081678707.
E-mail address: quaglia@unina.it.

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doi:10.1016/j.ijpharm.2008.04.030

3.3. Scaffolds bioactivated with growth factor-loaded delivery systems 291
3.4. Spatiotemporal control of growth factor distribution in tissue engineering scaffolds 293
4. Closing remarks 293
References 294

1. Introduction

Tissue engineering and regenerative medicine hold great promise to restore tissue and organ functions with the ambitious goal to avoid organ transplantation. Tissue engineering research includes specific areas such as biomaterial science (novel smart biomaterials able to provide both physical and chemical cues), biology (cell proliferation and differentiation, biomolecules acting as molecular signals), engineering design aspects (2D cell expansion, 3D tissue growth, bioreactors, vascularization, cell and tissue storage and shipping–biological packaging), biomechanical aspects of design (properties of native tissues, identification of minimum properties required of engineered tissues, mechanical signals regulating engineered tissues, and efficacy and safety of engineered tissues), informatics (gene and protein sequencing, gene expression analysis, protein expression and interaction analysis, quantitative cellular image analysis and tissue analysis, *in silico* tissue and cell modelling, digital tissue manufacturing, automated quality assurance systems, data mining tools, and clinical informatics interfaces).

In vitro and *in vivo* tissue engineering strategies generally include signalling molecules, which should try to reproduce the natural sequence of signals guiding spontaneous tissue repair, cells, in case tissue is not able to regenerate by itself, and if necessary a scaffold, which provides a mechanical support to the development of neotissue. Signalling molecules are mainly growth factors (GFs), a wide variety of proteins with distinct properties which are able to act on cell migration, differentiation, proliferation and organization in a functional tissue.

In the early tissue engineering approaches, GFs were simply interspersed in the polymer scaffolds which acted as delivery platforms. Poor modulation of release characteristics as well as no protection toward detrimental conditions occurring in biological microenvironments were the main drawbacks experienced. From the beginning of the century, a new trend emerged based on the attempt to realize a bioinspired approach through which naturally occurring behaviours could be reproduced. Along this direction, research was focused on the development of 3D constructs better mimicking biological complexity and able to reproduce signal cues controlling cell behaviour. Scaffolds presenting specific cell recognition area, as well as functionalized with immobilized GFs which could be released in response to cell-mediated stimuli, could create a highly regulated network of signals able to orchestrate cell migration, organization and proliferation finally giving a functional tissue.

In this context, it was soon realized that delivery systems (DS) encorporating GFs, and mainly biodegradable particles, could offer distinctive advantages such as ability to regulate release rate while protecting their protein cargo during all the stages of tissue regrowth, offer great versatility in their application and prompt to new tissue engineering strategies.

Very recently, the need to exactly control the time- and space-dependent levels of morphogen cues inside 3D constructs has emerged as a novel concept in tissue engineering area. This necessity, together with the development of scaffold processing technologies based on “bottom-up” approaches, has strongly oriented the scientists toward the use of particulate DS. It is true that some work has been done in the past but much more can be still done if one considers the tremendous efforts in protein formulation pharmaceutical technologists have provided.

Thus, the aim of this review is to trace an outline of the state of the art in the application of delivery strategies in tissue engineering focusing on GF delivery. The final intent is to focus mainly on delivery aspects of the systems developed until now and highlight the most relevant applications where preformed DS are involved. Releasing DNA encoding the GF has also been suggested as an alternative approach to bypass limitations of protein delivery and will not be treated here.

2. Tissue engineering: some basic aspects

2.1. Tissue engineering strategies

Certain tissues in the body are able to initiate regeneration or repair after injury whereas some others lack this capability (heart muscle, central nerves). In both cases cells, either present in the injured tissue or externally provided, need a specific microenvironmental context to proliferate and differentiate with the ultimate goal of repairing the defect. Depending on where tissue regeneration/organ substitution is performed, *in vitro* or *in vivo* tissue engineering is recognized. *In vitro* tissue engineering consists in the development of tissue-like constructs where cells grow and organize on a 3D support (scaffolds). If prepared in appropriate amounts, the construct can be supplied to the patient. This strategy suffers from serious limitations because it is very hard to reproduce *in vitro* all the biological events occurring *in vivo* in a functional tissue. Nevertheless, this strategy has been employed in tissue engineering of bone, articular cartilage and artery, while only the constructs of artificial skin dermis is being clinically used. Another application of *in vitro* tissue engineering relies upon the replacement of organ functions by use of allo- or xenogenic cells combined with biomaterials to protect the cells from host immune response while maintaining their biological functions (bioartificial hybrid organs). Besides having a therapeutic application, where the tissue is either grown in a patient or outside the patient and transplanted, tissue engineering can have diagnostic applications where the tissue is made *in vitro* and used for testing drug metabolism and uptake, toxicity, and pathogenicity. On the other hand, *in vivo* tissue engineering aims to recreate a biological environment suitable for tissue regeneration, provided that biological elements are mainly supplied by the living body. Thus, the basic elements of a tissue, that is ECM, cells and molecular signals, should be guided under a tissue engineering approach to repair the defect. In this context, numerous tissue engineering strategies are based on the use of polymeric scaffolds which mimics ECM and act as a temporary template for cell proliferation and differentiation. Scaffolds should provide not only mechanical support to cells but also offer a microenvironment of molecular signals able to guide cells toward the regeneration of a functional tissue.

2.2. Composition and processing of tissue engineering scaffolds

An ideal scaffold for tissue engineering should provide: (i) a 3D architecture with well-defined shape and mechanical strength; (ii) a highly interconnected porosity with an “open pore” structure to allow the seeding of a high number of cells as well as efficient

transport of nutrients and metabolites needed for cell survival; (iii) guidance for biological response through the promotion of the dynamic interaction with surrounding tissues; (iv) adequate biocompatibility, in terms of both tissue and systemic response, and biodegradability, with degradation kinetics suitable to provide support until the full-growth of neo-tissue.

A wide variety of materials, both synthetic and natural, have been used in this area, taking into account the need of avoiding any adverse foreign host response. In the case of *in vivo* application, ECM-mimicking scaffold properties must be tailored according to the body site because the template must temporarily bear mechanical stresses after implantation, eliminating potential stress discontinuities at the tissue-implant interface until the surrounding tissue is fully regenerated. The selection of the material for a scaffold still remains a key fundamental in the design and development of tissue engineering constructs, especially if it is considered that the biomaterial employed must produce controlled and predictable interactions with cells. Several options are available as recently reported in a number of reviews (Drury and Mooney, 2003; Hutmacher et al., 2004; Yarlagadda et al., 2005; Sokolsky-Papkov et al., 2007; Chung and Park, 2007; Malafaya et al., 2007). Naturally derived materials are more versatile in providing several biological functions and are generally processed in aqueous solution. Natural polymers widely used for tissue engineering applications include fibrin, collagen, gelatin, chitosan, alginate and hyaluronic acid. A wide number of synthetic biodegradable polymers has been used to produce scaffolds and chemically modified to serve a specific tissue engineering application. Biodegradable synthetic polymers processed as tissue engineering matrices include polyesters (PLA, PGA and PLGA), PEG and its derivatives, poly(fumarate)s and their copolymers with PEG (OPF), polyhydroxyalkanoates, poly(vinyl alcohol, poly(amido-amines)s, poly(urethane)s and some others. Inorganic materials such as calcium phosphate ceramics and cements, bioactive glass and ceramic/polymer composites have been especially tested in bone tissue engineering (Habraken et al., 2007).

Scaffold morphology is determined by the fabrication method and, in the case of methods involving the use of elements such as microspheres, fibers, or flat bodies (e.g. films), by the properties and arrangement of the single element. The methods of manufacturing polymeric 3D scaffold for tissue engineering applications have been widely detailed in the literature (Hutmacher, 2001; Yarlagadda et al., 2005; Weigel et al., 2006). An outline of the main processing technologies is provided in the following to facilitate reading of the next sections.

The most widely employed method to produce porous scaffolds is solvent casting-particulate leaching. A polymer solution with dispersed particles (e.g. water-soluble salts) is placed in a mould and the solvent evaporated. The solid is then poured in a suitable solvent to dissolve particles, thus generating porosity. Freeze-drying a W/O emulsion to obtain scaffolds with high porosity and interconnectivities is possible too.

Starting from a polymer solution, nonsolvent-induced phase separation (NIPS) and the thermally induced phase separation (TIPS) can be used. In the NIPS technique, a polymer solution is dripped onto a solid surface and immersed into a polymer nonsolvent. When added, the nonsolvent diffuses into the polymer solution, creating new solubility conditions in which the polymer precipitates forming the scaffold structure. Using a nozzle to cast the polymer solution into the nonsolvent, it is possible to achieve a tubular structure. Pore size from nanometers to few micrometers unsuitable for tissue engineering applications are obtained, unless combination with other techniques is carried out. TIPS technique involves a thermal induction of phase separation. A homogeneous polymer solution is prepared at elevated temperatures and the obtained solution is introduced in the desired mold and cooled to reduce the heat energy and to promote material quenching. Solvent is then removed by extraction or sublimation giving a microporous structure. One of the most attractive characteristics of TIPS is the formation of not only an intrinsically interconnected polymer network, but also an interconnected porous space in one simple process that is scalable, fast and controllable. TIPS is thus a very convenient technique for fabricating porous scaffolds as many architectures can be formed easily by manipulation of various processing parameters and system properties.

Foaming techniques consist in foaming polymers by using different types of agents (nitrogen, CO_2, fluoroforms, effervescent salts) and processing procedures. Expanding substances can be generated by chemical reaction, thermal decomposition or initiated by physically acting agents, which change their physical state during the process. A common method of scaffold preparation involves the use of $scCO_2$ which dissolves solid polymer or polymer melt at temperatures which depend on material properties (for example, PLGA requires 35–40 °C and 10–20 MPa). Thermodynamic instability is induced by reducing gas pressure to ambient conditions. Since low interconnectivity is generally obtained, foaming is coupled with particulate leaching techniques. Interest in this method mainly arises from the very mild conditions employed which allow processing labile molecules.

Corresponding to the ambition of mimicking structure and biological functions of ECM, 3D fiber networks of biodegradable polymers based on nanoscale multifibrils hold great promise. Fibers prepared by electrospinning have micro- or nanometric resolved size and can be prepared by applying a high voltage to electrically charge a polymer solution. The obtained fiber diameters and morphology strongly depend on processing parameters, such as nature of polymer, polymer solvent, viscosity, surface tension, charge density, and from environmental conditions, such as temperature, humidity and air velocity. The electrospinning process results in a 2D weave which can evolve in a 3D structure if the process time is increased. The scaffolds obtained by this technique are characterized by a wide range of pore size distribution, high porosity and a high surface area:volume ratio, which are essential parameters to promote cell attachment, growth and proliferation.

The challenge to control scaffold architecture at multiple size levels has driven the research toward the development of more sophisticated techniques to process engineered scaffolds as well as strategies to control GF levels within the scaffold in time and space. During the past decade, a dramatic increase in the resolution over scaffold architecture has been achieved through microfabrication technologies. In comparison to traditional polymer processing methods, micro- and nano-fabrication may allow for design resolution ranging from the nanometer to the supramillimeter length scale. The fine control of the internal architecture may facilitate the patterning of cells and molecular signals. Thus, efforts are underway to create high throughput technologies suitable for bulk fabrication of a wide range of materials that can guarantee spatial resolution. New scaffold processing techniques, based on computer-aid-design (CAD), permit to obtain scaffolds with more complex structures and exactly predetermined shape. Under the term rapid prototyping (RP) or solid-free form (SFF), techniques that can generate a physical model directly from computer-aided design data are grouped. Each part of the scaffold is constructed layer-by-layer in an additive process with precise control of morphological characteristics as well as chemical composition and mechanical properties. RP technologies have the potential to design a construct with any desired 3D morphology and shape. An interesting approach consists in the assemblage of microparticles made of thermoplastic polymers by laser sintering as well as compression moulding followed by exposure to vapour of a polymer

Table 1
Some characteristics of growth factors commonly employed in tissue engineering

Growth factor superfamilies	Growth factor and relevant isoforms	Molecular weight (kDa)[a]	Isoelectric point	Activity
Epidermal growth factor (EGF)	EGF	6	5.3–5.5	Keratinocyte mytogen
	Heparin-binding EGF-like growth factor (HB-EGF)	22	7.2–7.8	
Fibroblast growth factor (FGF)	Acid-FGF (aFGF or FGF-1)	18	6.5	Migration, proliferation and survival of endothelial cells
	Basic-FGF (bFGF or FGF-2)	17	9.6	Osteoblast proliferation
Platelet derived growth factor (PDGF)[b]	PDGF-AB	25	10.2	Wound healing enhancement, vessel maturation by recruitment of smooth muscle cells, osteoblast proliferation
	PDGF-BB	25	10.5	
	Vascular endothelial growth factor-121 (VEGF-121)	30	Acid	Migration, proliferation and survival of endothelial cells
	Vascular endothelial growth factor-165 (VEGF-165)	45	8.5	
Insuline-like growth factor (IGF)	IGF-1	8	8.3	Stimulation of osteoblast differentiation, synthesis of bone matrix
	IGF-2	8	5.1	
Transforming growth factor-β(TGF-β)	TGF-β1 TGF-β2 TGF-β3	25	8.9[c]	Bone proliferation and differentiation, promotion of keratinocyte migration, ECM synthesis and remodeling, differentiation of epithelial cells
	BMP-2	15	8.8	Differentiation of bone-forming cells
	BMP-7	16	7.9	
Neurotrophins and neurokines	Nerve growth factor (NGF)[d]	30	–	Axonal growth and cholinergic cell survival
	Ciliary neurotrophic factor (CNTF)	23	4.8	Trophic and differentiating effects on different types of peripheral and central neurons, glia and cells outside the nervous system

[a] Referred to quaternary structure.
[b] Dimeric glycoprotein composed of A or B chains.
[c] Referred to TGF-β1.
[d] Referred to β-NGF.

solvent to produce a scaffold with the desired shape and porosities.

2.3. Polymer scaffolds bioactivated with growth factors

In addition to acting as a bare physical support, scaffolds can be engineered to provide biological functions and actively induce tissue regeneration. The surface of a 3D scaffold, for example, can be functionalized to promote cell adhesion through specific cell–matrix interactions. In fact, the formation of complex tissues from single cells and tissue maintenance needs large amounts of information which must be transported from cell to cell and from cells to ECM.

GFs are protein molecules specific for intercellular and cell–ECM signalling which are involved in ECM dynamics through specific surface receptors regulating their activity. GFs are released by many cell types for immediate signalling and activate specific pathways controlling cell migration, differentiation and proliferation. GFs are normally synthesized as membrane-bound or high molecular weight precursors that must be modified to be released in the active form. GFs are often bound to ECM molecules, such as glycosaminoglycans (e.g. heparins). The interaction with these molecules alters GF action, by retaining the active/latent forms near cells and modifying GF transport properties. The sequestration of GFs within ECM in inert form is necessary for rapid signal transduction, allowing extracellular signal processing to take place in time frames similar to those inside cells. In addition, GF storage in ECM is crucial to maintain homeostasis through continuous GF activation upon ECM degradation. During tissue morphogenesis the presence of soluble GFs guides cell behaviours, thus governing neo-tissue formation and organization. ECM, therefore, serves as a naturally occurring sustained release reservoir for GFs, which is fundamental for molecules that are released over a short period, but which stimulate processes involved in tissue regeneration, such as angiogenesis, that take extended periods to be carried out. Some physico-chemical properties of GFs widely employed in tissue engineering along with specific activity are reported in Table 1.

Although signalling molecules can be simply added to culture medium for *in vitro* tissue formation, the translation of this concept *in vivo* is generally unsuccessful. For example, intracoronary infusion of VEGF and bFGF as well as intravenous infusion of VEGF has not yielded the expected results (Cao and Mooney, 2007). Furthermore, GF localization at the action site can be useful to confine their activity to a distinct location in the proximity of the defect avoiding side effects and exposure of non-target sites to these potent molecules. Thus, the firm conviction that a biologically inspired tissue regeneration could be obtained only reproducing naturally occurring GF presentation to cells emerged. How the signalling molecules are delivered in time and space to cells inside scaffold, thereby, has become a key issue in tissue engineering and has led to an explosion of work to develop GF delivery strategies (Biondi et al., 2008).

As underlined by Salvay and Shea (2006), the main aspects which should be taken into account in GF delivery are: (i) concentration–duration relationship, since the concentration of signalling molecules must fall within a therapeutic range and has to be maintained for periods that depends on the specific timing of repair; (ii) stable concentration gradients, since controlling the location of morphogen release can generate concentration gradients by diffusion of the factor from the release site directly inside the scaffold providing a control over direct cell migration, differentiation and proliferation; (iii) multiple factor delivery, since

multiple factor presentation to cells can be more effective than the delivery of a single factor in different situations; (iv) spatial patterning, since a control of where and when a GF acts can strongly contribute to provide spatially complex arrangements of cells in length scales ranging from micrometers to centimetres.

3. Drug delivery in tissue engineering

Different strategies have been carried out to control GF delivery from tissue engineering scaffolds. In fact, scaffolds can work as a delivery platform which incorporate GFs and release them at controlled rates. Body of literature demonstrates that this approach is feasible and results in constructs with a biological activity. Nevertheless, once optimization of the scaffold is reached in terms of composition, porosity and mechanical properties, and bioactivation with GFs is envisaged, GF loading efficiency, release rate, physico-chemical stability are all consequent properties which can be tailored only going back and changing scaffold properties. Thus, an *ab initio* design of bioactivated constructs which takes into account both scaffold and delivery requirements is very difficult to attain. This is a reason why scaffold bioactivation through a preformed DS, especially polymeric particles, has been identified as an alternative strategy (Fig. 1). Once incorporated in the scaffold, DS can provide GF protection from *in vivo* degradation thus extending the duration of scaffold bioactivation and allowing GF release at preprogrammed rates. Two or more DS types loaded with different GFs and designed to give different release rates can be introduced in the scaffold. Finally, a patterning of DS could be in principle carried out in a "bottom-up" approach thanks to the latest techniques for smart scaffold design. DS for tissue inductive factors must consider both the specific application and the requirements for efficacy, demanding for differentiated design criteria. It is clear that this approach has to face several technological issues, the main being the development of technologically optimized DS formulations (size, loading, bioactivity, release properties) for a specific tissue engineering application and the mode of integration of the DS inside the scaffolds. The aspect of GF stability strongly depends on the type of protein and has, therefore, to be assessed individually for each combination of protein and delivery device. The strategy of immobilizing GFs in the scaffold through covalent bonding will not be considered in this review.

In the following, relevant strategies to bioactivate polymeric scaffolds with GFs are reported in the attempt to highlight the main technical difficulties to face. The systems here discussed are: (i) scaffolds acting as a delivery platform for GFs; (ii) specific DS loaded with GFs useful when a 3D support is not needed for tissue regrowth; (iii) tissue engineering scaffolds integrated with GF-loaded delivery systems. In each section, organic platforms (hydrophilic natural and synthetic, hydrophobic) and inorganic supports will be treated in the order.

3.1. Scaffold as growth factor delivery platform

3.1.1. Free growth factors incorporated in "in situ forming" matrices

In situ forming injectable matrices for tissue engineering have been recently reviewed (Drury and Mooney, 2003; Kretlow et al., 2007). Interest in injectable scaffolds is mainly related to the fact that they eliminate the need for surgical implantation, increasing patient compliance. They are used as space fillers and

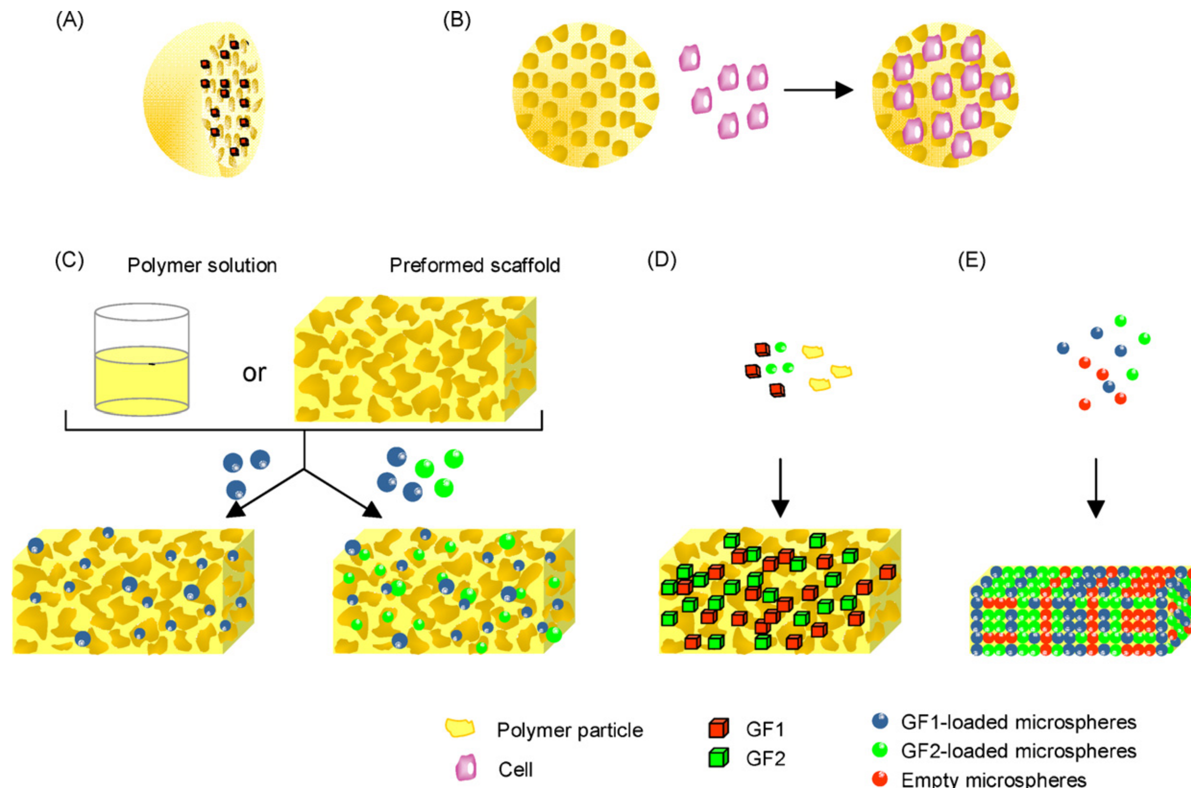

Fig. 1. Schematic illustration of the possible use of particles in tissue engineering and mode of particle integration in 3D scaffolds. (A) Microparticles loaded with GFs for direct injection at injured site; (B) empty microspheres acting as an *in vitro* scaffold for cell growth and injectable *in vivo*; (C) scaffold integrated with single or multiple types of GF-loaded microspheres. GF-loaded particles can be incorporated in scaffolds before or after scaffold processing. Shaped scaffolds can be assembled to give multilayer scaffolds with different compositions; (D) scaffold obtained from different combinations of polymer particles, free GF and GF-loaded particles resulting in GFs incorporated at different depth; (E) scaffolds made of GF-loaded particles assembled randomly. A layer-by-layer assembly can be envisaged too.

for cell and therapeutic agent delivery (small drugs as well as GFs). Since they take the shape of the cavity where they are placed, also irregular defects can be repaired avoiding the need for patient specific scaffold prefabrication. As for all 3D scaffolds, injectable matrices should have adequate porosity, pore size and interconnectivity, ensure adequate mechanical support as a function of tissue type as well as be bioactive to drive cell differentiation–migration–proliferation and tissue growth. *In situ* solidification should occur in mild conditions and be fast enough to avoid rapid extravasation in surrounding tissue and, in case the mixture contains a GF, initial *burst* effect. Solidification can be either spontaneous or achieved by a chemical reaction (chemically activated or photoinitiated crosslinking, thermogelation, as well as ionic crosslinking). The injected mixtures – including prepolymers or materials to solidify, initiators and crosslinkers – must be non-toxic to tissue. Furthermore, exothermic reactions should not reach temperatures capable of inducing tissue necrosis.

The incorporation of GFs in injectable scaffolds made of hydrophilic polymers (gel-like scaffolds) is generally achieved by adding GFs in the prepolymer-containing aqueous medium. The mixture is directly injected in the body and allowed to solidify *in vivo*, provided that GFs do not interfere with scaffolding process and do not affect its overall properties. Solidification of the gel should occur very rapidly upon injection and in the mildest conditions as possible to avoid any GF inactivation. Reports on GF-loaded *in situ* forming matrices of potential interest or tested in tissue engineering are available in the literature and, in some of them, relevant delivery issues emerged. In the following an overview is provided.

Chitosan has been used as base material for GF-loaded injectable matrices. It has been demonstrated that it is possible to prepare injectable hydrogels by photocrosslinking water-soluble chitosan obtaining a flexible insoluble hydrogel within 10 s (Obara et al., 2003; Ishihara et al., 2003). FGF-1, FGF-2, VEGF and heparin-binding EGF (HB-EGF) were incorporated inside the gel before crosslinking (Ishihara et al., 2003). About 10–25% of the GF was released into a phosphate buffer used as release medium at a rate independent on GF type within the first day, and afterwards no further substantial release took place. Incomplete release was due to GF interaction with chitosan since bioactive GFs were recovered from the hydrogel after addition of chitinase and chitosanase which partly degraded the polymer (this occurred for FGF-1, FGF-2 and VEGF165, but not for HB-EGF). Similar results were obtained with hydrogels of water-soluble chitosan crosslinked with non-coagulant heparinoids and incorporating FGF-2 (Fujita et al., 2004). When subcutaneously implanted into the back of a mouse FGF-1- and FGF-2-incorporated chitosan hydrogels induced a significant neovascularization near the implanted site (Obara et al., 2003; Ishihara et al., 2003) while no significant vascularization by FGF-1 and FGF-2 in a photocrosslinkable water-soluble chitosan aqueous solutions (without UV irradiations) was observed. The authors interpreted these results suggesting that the controlled release of biologically active FGF-2, caused by hydrogel biodegradation, allowed induction of the vascularization. FGF-2-incorporated chitosan hydrogels were also tested for therapeutic angiogenesis in a rabbit models of chronic myocardial infarction (Fujita et al., 2005). Again, the controlled release of biologically active FGF-2 from the chitosan hydrogel induced angiogenesis and possibly collateral circulation in ischemic myocardium. FGF-2 releasing chitosan hydrogel significantly accelerated wound closure in healing-impaired diabetic mice as compared to normal mice (Obara et al., 2003).

Recently, Holland et al. (2003) have developed injectable hydrogels based on OPF which can be crosslinked in physiological conditions within a clinically relevant time period and tested the hydrogels for TGF-β1 release. Experiments revealed that OPF formulation and crosslinking time may be adjusted to influence the equilibrium swelling ratio, elastic modulus, strain at fracture and mesh size of the final hydrogels and, as a consequence, also TGF-β1 release rate. An evolution of this system involves the incorporation of TGF-β1-loaded gelatin microspheres to better control release kinetics which will be treated in Section 3.3.

Thermosensitive injectable hydrogels have been proposed too. Chenite et al. (2000) developed thermally sensitive neutral solutions based on chitosan/polyol salt combinations. This platform was used successfully to deliver bone proteins derived from bovine bone (osteogenic mixture of TGF-β family members including several BMPs). Na et al. (2006) developed injectable drug delivery vehicle based on poly(*N*-isopropylacrylamide-*co*-acrylic acid) hydrogels incorporating dexamethasone, ascorbate and TGF-β3 which were used as supporting matrix for neo-cartilage formation. Thermosensitive hydrogels composed of poly(*N*-isopropylacrylamide-*co*-*n*-butyl methacrylate)/PEG loaded with IGF1 and TGF-β2 were tested in the generation of tissue-engineered cartilage *in vitro* (Yasuda et al., 2006).

Hydrophobic materials can be injected in a defect area after solubilization in a suitable organic solvent and solidified taking advantage of phase separation. This strategy has been applied for PLGA by using *N*-methyl-2-pyrrolidone as solvent. Although such a systems is available on the market for peptide delivery, no relevant tissue engineering application has been proposed yet.

Injectable pastes comprised of amylopectin and β-tricalcium phosphate (β-TCP) granules preadsorbed with TGF-β1 were prepared by Ongpipattanakul et al. (1997). *In vitro* results showed a release of 80% after 24 h incubation in serum whereas a half life of the TGF-β at the implant site of 4–6 days was found after implantation in a rabbit unilateral segmental defect model. Next to that the GF also remained intact for more than 21 days as was observed by SDS-PAGE. In other studies, TGF-β1 and BMP was incorporated into and coated on CPC during the setting, respectively (Blom et al., 2002; Szivek et al., 2004; Haddad et al., 2006). *In vitro*/*in vivo* results demonstrated stimulation of the differentiation of preosteoblastic cells isolated from rat, accelerated bone-implant interface, and promotion of complete closure of critical-sized calvarial defects in New Zealand White rabbits at 12 weeks. In addition to osteoconductivity, the slow release of GFs from CPC matrices was related to the high affinity of hydroxyapatite to certain proteins, which may contribute to the improved binding to bone tissue. As reported by Blom et al. (2002), bioactivation of CPC with TGF-β1 could be achieved without seriously affecting important physicochemical properties like hardening, compressive strength and the formation of apatite.

3.1.2. Free growth factors incorporated in shaped scaffolds

Hydrophilic and hydrophobic materials can be shaped in the desired form and processed through different technologies to obtain desired scaffold properties, as well as bioactivated through incorporation of GFs. Two main strategies are available to load GFs in tissue engineering scaffolds: (i) prepare the scaffold and then load GF(s) or (ii) incorporate GF(s) before scaffolding. Both strategies experience distinct issues and advantages which are summarized in the following. It is worth of note that many reports are focused on *in vivo* activities of GF-loaded scaffolds rather than face with delivery issues such as amount loaded, release rate and stability profile.

The incorporation of GFs in a preformed scaffold has the advantage that optimized scaffold properties are not substantially affected by the presence of protein. GF loading can be accomplished by either diffusion, impregnation of the preformed scaffold with very small volumes of GF solutions (Lee et al., 2002) or immersion of the scaffold in a GF solution (Karageorgiou et al., 2006). All the methods are followed by a dehydration step. The first technique presents the risk that non-homogeneous distribution of GF in the

scaffolds is obtained. To this respect, Kanematsu et al. (2004) incorporated radioactive bFGF in a BAM and analysed radioactivity in BAM sections before and after lyophilization. Results demonstrated that the diffusional loading of the GF solution results in a gradient concentration of GF in BAM. The following dehydration step, in that case a freeze-drying, contributed to improve GF distribution in the scaffold.

In this type of systems, desorption is the main process controlling delivery rate and scaffold composition is of utmost importance. Extensive literature on the use of GF-loaded crosslinked gelatin scaffolds has well illustrated this aspect (Tabata, 2006). GF was incorporated in an acidic gelatin hydrogel, previously crosslinked with glutaraldehyde, based on GF–gelatin intermolecular interactions. GF was released only when the hydrogel was degraded to water-soluble fragments. Various basic GFs (bFGF, TGF-β1, PDGF) were found to interact with acidic gelatin (p*I* = 5) due to electrostatic interactions (Ikada and Tabata, 1998). Release rate of GFs could be manipulated by adjusting gelatin hydrogel biodegradability, i.e. by changing the degree of crosslinking. An extensive review on the results obtained by using this approach in tissue engineering has been recently published (Tabata, 2005, 2006). On the other hand, films prepared by crosslinking of gelatin with dextran dialdehydes (weight ratio 2:1) demonstrated to provide a medium-term sustained delivery of EGF (Draye et al., 1998). Furthermore, also GF physico-chemical properties play a fundamental role in controlling release rate. Park et al. (2000a, 2000b) highlighted the effect of scaffold composition on PDGF-BB release rate from physically crosslinked chondroitin-4-sulfate (CS)/chitosan sponges. GF release was completed in about 4 days whereas it resulted much more modulated from chitosan/CS sponges although the addition of CS increased scaffold porosity. Ionic interactions occurring between PDGF-BB and CS were hypothesized to account for this effect. Kanematsu et al. (2004) demonstrated that different GFs (bFGF, HGF, PDGF-BB, VEGF, IGF-1, HB-EGF) were released from both a BAM and a sponge matrix of porcine type 1 collagen at rates strictly dependent on GF physico-chemical properties. The release of VEGF, HB-EGF and IGF-1 from collagen sponge, in particular, was poorly controlled and practically complete after only 1 day.

Recently, some studies have been carried out also on biodegradable dextran hydrogels and their potential for protein and GF delivery has been discussed in a recent review by Van Tomme and Hennink (2007). Sustained release from EGF- and bFGF-loaded dextran-epichlorohydrin hydrogels (Dogan et al., 2005) as well as from TGF-β1 or BMP-2 impregnated anionic dextran pellets (Maire et al., 2005a, 2005b) was demonstrated.

In analogy to gel-like systems, GF loading by impregnation on polyester preformed scaffolds is in theory feasible. Bryan et al. (2000) reported the production of PLGA foam guides prepared by lyophilization and then loaded with GGF by immersing blocks of foam in aqueous physiological saline GF solutions. The solution was forced into the void volume by first degassing and then repressurizing to 1 atm by admitting air. Analogously, biodegradable scaffolds of PCL or PCL/20% PCL–TCP were recently produced by fused deposition modeling (Rai et al., 2005). Impregnation of BMP-2 in the preformed scaffold was promoted by adding a fibrin Tisseel® sealant solution. Fluorescence and scanning electron microscopy revealed sparse clumps of BMP-2 particles, non-uniformly distributed on the rod surface of PCL–fibrin composites. In contrast, individual BMP-2 particles were evident and uniformly distributed on the rods' surface of the PCL–TCP–fibrin composites. PCL–fibrin composites demonstrated a triphasic release of BMP-2 whereas a biphasic release profile was observed for PCL–TCP–fibrin composites. The addition of TCP caused a delay in BMP-2 release. SDS-PAGE and alkaline phosphatase assay verified the stability and bioactivity of eluted BMP-2 at all time points.

More recently, proteins were loaded in macroporous polymeric scaffolds made of poly(ether-ester) multiblock copolymers by an emulsion-coating method (Sohier et al., 2003). In this process, a water-in-oil emulsion, from an aqueous protein solution and a polymer solution, was forced through a prefabricated scaffold by applying vacuum. After solvent evaporation, a polymer film, containing the protein, was deposited on the porous scaffold surface. The effect of processing parameters on the emulsion coating characteristics, scaffold structure, protein release rate and stability was studied. Protein release rate could be regulated by adjusting the coating emulsion parameters. The method also allowed the creation of a double coating, where each layer contains a specific protein (Sohier et al., 2006).

Impregnation is the commonly employed technique to load GFs inside inorganic scaffolds. Due to the high affinity of the ceramics for proteins, one can load these scaffolds just by absorption of a GF-containing fluid onto the ceramic and release them by desorption. The release pattern of most loaded ceramics seems to consist of an initial *burst* release (elution of unbound protein) followed by a release phase dependent on material/protein interactions and scaffold porosity. In case the material is highly porous, its capillary action allows the solution to be uniformly and quickly distributed in the material by dropping (Ziegler et al., 2002). GF adsorption extent was strongly dependent on the material and the physico-chemical properties of GFs. In the study of Ziegler et al. (2002), the adsorption behaviour of either rxBMP-4, bFGF or VEGF and their release characteristics from biodegradable implants, i.e. porous α-TCP, a neutralized glass-ceramics (GB9N), a composite (PLGA/GB9N) and solvent dehydrated human bone (SDB) as carriers were studied. The results indicated that the synthetic scaffolds had a greater ability (about 60–90%) to bind GFs than the SDB (about 30–45%). The limited loading efficiency of SDB was ascribed to its higher porosity or lower overall surface as compared to the synthetic substitutes. In general, GF release was very poorly modulated as demonstrated also for human growth hormone loaded in a macroporous biphasic calcium phosphate matrix (Guicheux et al., 1998). Degradation of GFs in time was also experience for all GFs tested (Ziegler et al., 2002). Pre-treatment of CPC after setting with rat serum albumin was found to accelerate GF delivery due to shielding GF–cement interactions (Ruhe et al., 2006).

The incorporation of GFs before scaffold preparation is feasible too. When dealing with hydrophilic materials, an important formulation challenge in fabricating hydrogels for protein delivery is the choice of the crosslinking method, which must not involve steps potentially detrimental for GF stability. Ionic crosslinking is one of the most widespread methods in this sense. VEGF and bFGF were incorporated in alginate scaffolds with the aim to encourage the rapid development of a vascular network within 3D tissue engineering matrices (Lee et al., 2003b). A preliminary study of VEGF and bFGF incorporation in alginate was performed on beads prepared by dripping a GF/alginate solution in a calcium chloride solution. Release profile of ^{125}I–GF was characterized by an initial *burst* followed by a slow release phase which was better modulated for VEGF as compared to bFGF. An incomplete release was observed after 12 days (about 60%). Alginate disks crosslinked with $CaSO_4$ and loaded with different amounts of VEGF were tested *in vivo* for ability to promote new blood vessel formation in the subcutaneous tissue of severe combined immunodeficient mice. Results showed a dose–response effect on thickness of a granulation tissue layer formed around the gel and the number of blood vessels, while the density of new blood vessels resulted unaffected. In another study, multi-protein endothelial cell growth supplement containing GFs of varying sizes (10–250 kDa) was released from alginate scaffolds (Tilakaratne et al., 2007). The complete release of the entrapped GFs occurred within the first day. Again,

the implantation of polypropylene hollow fibers containing bioactivated alginate gel gave a two-fold increase in neovascularization at the device–tissue interface with respect to the control for the period between 7 and 17 days post-implantation. Moreover, it is worth to underline that alginate scaffolds are able to offer an on-demand release of entrapped GF following mechanical stimuli which can occur in "mechanically stressed environments" (Lee et al., 2000). To better control release rate of VEGF from gels, nanocoating with polyelectrolyte multilayer films of chitosan and dextran sulfate has been proposed (Matsusaki et al., 2007). Results highlight how non-coated gels release all their VEGF content within 6 h by gel collapse whereas the nano-coated hydrogels release VEGF continuously, even for a month, without any initial *burst* release.

Natural materials crosslinked by enzymes have been considered too. Wong et al. (2003) tested biomatrix prepared from Tisseel®, a Fibrin Sealant, loaded with the angiogenic GFs bFGF, VEGF-165, and VEGF-121. In this type of system, GF interactions with scaffold materials are pronounced and a variety of release behaviour of different GFs can occur. No significant release of bFGF was observed in the time frame of the *in vitro* study (4 days) whereas VEGF-165 was released at a rate lower than VEGF-121. These data were directly related to the binding affinity of GF for fibrin(ogen)/fibronectin. Interestingly, the biomatrix developed was able to reproduce GF binding to ECM and provided the formation of normal neovessels *in vivo* (CAM model) as compared to biomatrix releasing only one GF which gave leaky and immature vessels with an hemorrhagic behaviour. Similar fibrin matrices crosslinked with $CaCl_2$ at different thrombin concentrations ± heparin, offered a very wide variety of bFGF release rates while preserving its bioactivity (Jeon et al., 2005).

By exploiting newer techniques for scaffold production, protein-loaded fibers from interfacial polyelectrolyte complexation were recently developed with the aim to overcome the issue of decreased bioactivity and low encapsulation efficiency for delicate compounds (Liao et al., 2005). Chitosan–alginate fibers were produced by pulling from the interface between two polyelectrolyte solutions at room temperature. Depending on the component properties, the release time of encapsulated components from these fibers can range from hours to weeks. The fibers were able to release PDGF-BB in a steady fashion for over 3 weeks without an initial *burst*. Furthermore, the bioactivity of PDGF-BB was retained over this period. Release kinetics could be controlled by the inclusion of heparin due to its binding activity to GFs. By varying the alginate/heparin ratios in the anionic polyelectrolyte solution, the release of PDGF-BB could be significantly altered. On the same line, Li et al. (2006) demonstrated that it is possible to obtain electrospun silk fibroin fibers bioactivated with BMP-2. Although the extent of BMP-2 stability in the formed fibers was not demonstrated, *in vitro* study on cell cultures showed that bioactivated fibers support higher calcium deposition and enhanced transcript levels of bone-specific markers than in the controls, indicating that nano-fibrous electrospun silk scaffolds were an efficient delivery platform for BMP-2.

Chemical crosslinking is the alternative method to physical crosslinking to prepare scaffolds from water-soluble polymers, taking into account that toxicity due to the presence of residues of crosslinking reagent can occur. Collagen crosslinked with glutaraldehyde in the presence of FGF-2 or TGF-β gave increased angiogenesis and enhanced epithelialization although unloaded collagen scaffolds elicited a higher inflammatory response (Pandit et al., 1998, 1999). A similar approach to deliver VEGF was attempted by Tabata et al. (2000). Analogously, osteoinductive and neuroactive GFs were successfully photoencapsulated in crosslinked PEG hydrogels networks based on PLA–PEG–PLA macromers (Burdick et al., 2002, 2006). GFs were added before crosslinking and released at a rate which could be finely controlled by changing in both the structure (i.e. macromer concentration) and chemistry (i.e. number of degradable units) of the starting macromer. Recent data indicate that UV-crosslinked PVA is effective in entrapping and slowly releasing PDGF-BB (Bourke et al., 2003). In particular, protein permeability of these hydrogels can be tailored by modifying the solid content of the matrix and by incorporating hydrophilic fillers (i.e. non-functionalized PVAs).

Much more difficult is to entrap GFs in a non-gel-like scaffold, generally comprising hydrophobic polymers such as biodegradable polyesters where specific scaffolding methods provide the scaffold with suitable porosity. In most cases, scaffold processing methods work in the presence of an organic/aqueous solvent interface (e.g. emulsion techniques), elevated temperatures (e.g. polymer melt processing) or stirring which are all conditions unfavourable for proteins. A further challenge in producing these scaffolds is the control of morphology, i.e. generation of a pore size distribution that promotes cell infiltration and/or a controlled porosity to tailor polymer degradation and, in parallel, to control the release rate of entrapped proteins.

It has been shown the ability to incorporate and deliver bioactive macromolecules from PLGA scaffolds with controlled microarchitecture prepared by emulsion freeze-drying process. In a study where BSA was used as a model protein (Whang et al., 2000), it was found that protein concentration strongly affected scaffold porosity whereas protein release was incomplete and characterized by a quite high initial *burst*. Nevertheless, BMP-2-loaded scaffolds demonstrated their ability to induce bone formation in a rat ectopic bone induction assay (Whang et al., 1998).

At the beginning of the century, the routine introduction of supercritical fluid technology for producing tissue engineering scaffolds from hydrophobic materials was certainly a relevant advance also to attain GF integration. In a development of the prototype technique to process polyester scaffolds by supercritical fluids (Mooney et al., 1996a), Murphy et al. (2000) fabricated 3D porous scaffolds of PLGA/alginate including VEGF by a gas foaming/particulate leaching process. VEGF was incorporated into PLGA scaffolds with an efficiency of 44 ± 9%. A biphasic release profile without erosion phase was obtained and a GF amount of about 80% of the total scaffold content was released in 10 days. Modulation of release properties was attributed to the alginate component. Bioactivity data indicated that released VEGF was over 70% active for all time intervals of release. This technique was employed in following studies to incorporate two GFs in the scaffold and attain a dual release at different rates (Richardson et al., 2001; Chen et al., 2007b). In a similar attempt to expand methods working in mild conditions, Howdle et al. (2001) developed a $scCO_2$ fluid mixing technology to load scaffolds with solvent-sensitive and thermolabile molecules. Powdered samples of polymer and bioactive guest (for example GFs) were placed in the autoclave and $scCO_2$ added. Neither the guest, nor the polymer, dissolved in $scCO_2$. At temperatures in close proximity of room temperature, the $scCO_2$ swollen polymer leads to homogeneous distribution of the guest particles throughout the polymer matrix. The vessel is then depressurised to produce scaffolds of different porosity. Ribonuclease A, catalase and β-D-galactosidase processed by this technique were found to keep their stability upon the process. Also bioactive factors (BMP-2 or VEGF) incorporated into biodegradable PLLA scaffolds by this technology were found to retain their biological activity (Yang et al., 2004; Kanczler et al., 2007). VEGF release from the scaffold showed a controlled release over a 21-day period. Although no data was provided on GF bioactivity during release, VEGF-loaded PLA scaffolds were found to increase the blood vessel network in the *in vivo* CAM assay (4 days) as compared to non-medicated scaffolds (Kanczler et al., 2007). Techniques using $scCO_2$ were also integrated to classical emulsion techniques to increase scaffold porosity. Hile et al. (2000)

developed a method for the production of microporous PLGA foams containing encapsulated proteins using supercritical carbon dioxide. Foams were generated from an emulsion of an aqueous protein solution (bFGF and BSA used in a sacrificial lamb approach) in a polymer solution (dichloromethane) which was saturated with CO_2 at supercritical conditions and then suddenly supersaturated at ambient conditions causing bubble nucleation and precipitation of the polymer. Despite the use of BSA, a significant loss of FGF activity was experienced probably due to the harsh conditions realized during emulsification step and CO_2 expansion.

To better control release rate properties, natural and synthetic materials have been functionalized with species potentially able to interact with GFs. The repeated binding of GF to these anchoring sites slows down their diffusion, thereby prolonging release rate. The number of binding sites, the affinity of GF for the immobilized recognizing species, and the degradation rate of the hydrogel are key determinants of the amount of immobilized GF, as well as its release rate. As interacting site, heparin has been widely used to this purpose due to the fact that several GFs possess a heparin-binding domain. Affinity-based scaffolds of fibrin modulated the release rate of NGF, BDNF, and NT-3, although these GFs bind heparin with low affinity (Sakiyama-Elbert and Hubbell, 2000a, 2000b; Lee et al., 2003a; Taylor et al., 2004). Heparinazed collagen gels (Wissink et al., 2000; Steffens et al., 2004) as well as heparin-immobilized hydrogels based on thiol-functionalized PEGs (Nie et al., 2007) were synthesized and tested as GF delivery platform. More recently, heparin-immobilized macroporous PLGA scaffolds for the release of bFGF have been developed (Yoon et al., 2006).

At last, the potential of use of pure diffusion-controlled systems based on non-biodegradable polymers such as ethylene-*co*-vinyl acetate copolymer (EVAc) for the controlled release of GFs has been considered although their use in protein release is relatively rare in pharmaceutical field. In applications where the introduction of GFs is only necessary for a short duration to stimulate or augment the healing process, it may be possible to provide a reservoir or coat an existing device for local controlled delivery of GFs. PDGF-BB/EVAc dip-coated stainless-steel wires have been demonstrated to effectively stimulate osteoblast proliferation *in vitro* (Walsh et al., 1995). Dual GF delivery could be achieved from EVAc polymer matrices prepared by extrusion of EVAc/drug mixtures and subsequent coating with EVAc alone (Kim and Valentini, 1997). It was demonstrated that these polymer rods effectively released PDGF-BB, inducing osteoblast proliferation, and TGF-β2, resulting in a marked increase in protein synthesis. Furthermore, activation of non-biodegradable synthetic nerve guidance channels of EVAc for nerve regeneration through rods loaded with neurotrophic factors such as NGF, BDNF, NT-3 and GDNF is also reported in the literature (Bloch et al., 2001; Fine et al., 2002; Barras et al., 2002).

3.2. *Growth factor-loaded delivery systems in tissue engineering*

One of the first investigation on GF delivery from a polymeric DS reports the use of albumin gels containing EGF (Murray et al., 1983). Only in 1997, a carboxymethylcellulose-based topical gel medicated with PDGF-BB (Regranex®) for the treatment of diabetic foot ulcers was approved from Food and Drug Administration as the first GF system for tissue regeneration. GF-bioactivated gels are still applied in wound healing with success to accelerate the repairing process (Alemdaroglu et al., 2006). At the end of 1980s it was finally clear that *in vivo* tissue engineering could benefit of a controlled delivery of GFs and the number and complexity of DS tremendously raised (Table 2). Several DS prepared from EVAc, gelatin and collagen were developed as implants in that period (Buckley et al., 1985). However, the most investigated DS was based on alginate beads ionically crosslinked with ions (Downs et al., 1992) through which several GFs were delivered. An extensive study on the parameters which can be adjusted with the aim of controlling GF delivery rate from alginate beads was conducted on VEGF (Peters et al., 1998). A detailed examination on the effect of different formulation parameters on the release characteristics of VEGF-loaded alginate beads has been reported also in a recent paper (Gu et al., 2004). Beside controlling calcium ions concentration, modulation of VEGF release was obtained by introducing heparin agarose beads in the alginate system (Edelman et al., 1993; Peters et al., 1998; Sellke et al., 1998). Many studies demonstrated that alginate beads delivering bFGF were able to induce therapeutic angiogenesis (Harada et al., 1994; Sellke et al., 1998; Huwer et al., 2001). A cochlear delivery system for the rescue of auditory neurons after ototoxicity-induced deafening through NT-3 releasing alginate beads has been proposed (Noushi et al., 2005). Alginate beads were successively supplemented with smaller alginate microparticles which offer the important advantage of easy injectability in the body (Ko et al., 1995). Recent research on BDNF-releasing alginate microspheres for peripheral nerve regeneration highlights that it is possible to release the protein at a controlled rate for at least eight weeks (Vogelin et al., 2006).

Injectable gelatin microspheres based on ionic interactions acid between gelatine and GFs were developed by the group of Tabata on the basis of previous studies on hydrogel scaffolds (Tabata et al., 1999). Biodegradable microspheres were prepared through glutaraldehyde crosslinking of gelatin followed by bFGF adsorption. Approximately 30% of incorporated bFGF was released from the acidic gelatin microsphere within the initial 3 h, followed by no substantial release. Initial *burst* was ascribed to the free bFGF fraction which did not ionically interact with acidic gelatin. Similarly to crosslinked gelatin discs described in the previous section, gelatin microspheres were effective in a number of tissue engineering strategies being able to induce *in vivo* angiogenic effect through bFGF delivery (Tabata et al., 1999) and histologically cure liver fibrosis in rats through HGF delivery (Oe et al., 2003). In following papers, gelatin microspheres entrapping TGF-β1 (Holland et al., 2003, 2004; Park et al., 2005; Fan et al., 2006), TGF-β1/IGF-1 (Holland et al., 2007), EGF (Ulubayram et al., 2001) and FGF (Vashi et al., 2006; Deng et al., 2007) were integrated in tissue engineering scaffolds of different nature which will be described below. Another type of photocured, styrenated, gelatin-based microspheres (SGMs) with different drug release rates of immobilized angiogenic (bFGF) and adipogenic factors (IGF-1) was developed to accelerate *de novo* adipose tissue engineering (Masuda et al., 2004). Results highlighted that neovascularization and spontaneous accumulation of preadipocytes and subsequent differentiation to mature adipocytes could be obtained by a single injection of SGMs simply regulating GF release rate.

More recently Chen et al. (2005) prepared BMP-2-loaded dextran-based hydrogel microspheres for tissue engineering and guided tissue regeneration by double-phase emulsified condensation polymerization. BMP-2 was entrapped with high encapsulation efficiency and ensured a controlled release for more than 10 days. The same group proposed novel hydrogel microspheres based on both gelatin and dextran acrylate derivatives (Chen et al., 2006) able to entrap positively charged IGF-1 by swelling due to polyionic complexation with negatively charged acidic gelatin. A slow release of IGF-1 could be maintained for more than 28 days, and relevantly constant protein release rate without a significant *burst* was achieved.

In mid-1990s, research in the technological field focused on strategies for protein delivery and body of literature was published on protein encapsulation in biodegradable microspheres. In fact, thermoplastic aliphatic poly(ester)s (PLA and PLGA derivatives) generated tremendous interest due to their excellent biocompat-

Table 2
Delivery systems incorporating growth factors developed for tissue engineering

Delivery system	GF	Application[a]	Reference
Albumin gel	EGF	Wound healing	Murray et al. (1983)
Chitosan gel	EGF	Wound healing	Alemdaroglu et al. (2006)
Carboxymethylcellulose gel (Regrenex®)	PDGF-BB	Wound healing	
Alginate beads	VEGF	Angiogenesis	Downs et al. (1992), Edelman et al. (1993), Peters et al. (1998) and Gu et al. (2004)
	bFGF	Therapeutic angiogenesis	Harada et al. (1994), Sellke et al. (1998) and Huwer et al. (2001)
	NT-3	Rescue of auditory neurons	Noushi et al. (2005)
Alginate microspheres	BDNF	Nerve regeneration	Vogelin et al. (2006)
	ECGF	–	Ko et al. (1995)
	Pegylated EGF	Hepatocyte engrafment	Kim et al. (2002a)
Gelatin microspheres	bFGF	Angiogenesis	Tabata et al. (1999)
	HGF	Liver fibrosis	Oe et al. (2003)
Gelatin microspheres	bFGF, IGF-1,	*De novo* adipose TE	Masuda et al. (2004)
Dextran microspheres	BMP2	Guided tissue regeneration	Chen et al. (2005)
Dextran/gelatine microspheres	IGF-1	Periodontal regeneration	Chen et al. (2006)
PLGA microspheres	CNTF	Neurological diseases	Maysinger et al. (1996)
	EGF	Hepatocyte engrafment	Mooney et al. (1996b) and Kim et al. (2002a)
	VEGF	Neovascularization	King and Patrick (2000), Cleland et al. (2001) and Kim and Burgess (2002b)
	NGF	Neuronal diseases	Cao and Schoichet (1999), Saltzman et al. (1999), Pean et al. (2000), Gouhier et al. (2000) and Lam et al. (2001)
	BMP-2		Isobe et al. (1999), Shrier and DeLuca (2001) and Weber et al. (2002)
	IGF-I	Insulin resistance	Lam et al. (2000)
		Bone healing/bone formation	Meinel et al. (2001, 2003)
	TGF-β1	Bone healing	Peter et al. (2000) and Lu et al. (2000)
	IGF-I and TGF-β1	Bone healing	Elisseeff et al. (2001)
		Bovine chondrocyte delivery	Chun et al. (2004)
	GDNF	Neurodegenerative disorders	Aubert-Pouessel et al. (2004)
PCL/PLGA microspheres	NGF (mouse)	Axonal regeneration	Cao and Schoichet (1999)
PCL microspheres	NGF (mouse)	Axonal regeneration	Cao and Schoichet (1999)
PLGA/PEG microspheres	insulin and IGF-I	Increase of inguinal adipofascial flaps	Yuksel et al. (2000a, 2000c)
	insulin, IGF-I and bFGF	Fat graph survival	Yuksel et al. (2000b)
PLLA/Starch MS	PDGF	Not indicated	Silva et al. (2007)

ibility as well as the possibility to tailor their biodegradability by varying composition (lactide/glycolide ratio), molecular weight and chemical structure (i.e. capped and uncapped end-groups). FDA approval of PLGA use in humans led to the availability of copolymers characterized by a wide range of *in vivo* life-times, ranging from 3 weeks to over 1 year. Drug microencapsulation within PLGA copolymers, in form of micro- and nanoparticles, was regarded as a powerful mean to achieve sustained release for long time-frames and, in the case of labile drugs such as proteins, effectively protect the molecule from *in vivo* degradation occurring at the administration site. Lupron Depot®, a PLGA microsphere-based injectable depot for the sustained release of leuprolide acetate in prostate cancer treatment, was the first protein long-acting formulation to be introduced in the market. After it, several PLGA formulations for the controlled release of protein therapeutics have been introduced in routine clinical practice. Protein encapsulation in PLGA microspheres is a challenging task due to stability issues occurring during microsphere-processing, shelf-life and protein release. Techniques to entrap protein in PLGA microspheres feature partly competing and partly complementary characteristics (Sinha and Trehan, 2003; Tao and Desai, 2003; Varde and Pack, 2004; Tamber et al., 2005; Freitas et al., 2005) and are all joined by the common aim of realizing experimental conditions as mild as possible. *A priori*, all the strategies employed to stabilize proteins in PLGA microspheres (van de Weert et al., 2000, 2005; Benoit et al., 2008) can be well transferred to GFs.

In the first works along this direction, CNTF and EGF were microencapsulated in PLGA microspheres by the multiple emulsion-solvent evaporation technique (Maysinger et al., 1996; Mooney et al., 1996b). These studies provided a basis for the systemic use of PLGA microspheres in tissue engineering applications. Several other examples of successful delivery of GFs through PLGA microspheres are reported in the literature for VEGF (Murphy et al., 2000; King and Patrick, 2000; Cleland et al., 2001; Kim and Burgess, 2002b), bFGF (Perets et al., 2003), NGF (Cao and Schoichet, 1999; Saltzman et al., 1999; Pean et al., 2000; Gouhier et al., 2000; Lam et al., 2001), GDNF (Aubert-Pouessel et al., 2004), BMP-2 (Isobe et al., 1999; Shrier and DeLuca, 2001; Weber et al., 2002), IGF-I (Lam et al., 2000; Meinel et al., 2001, 2003), TGF-β1 (Peter et al., 2000; Lu et al., 2000), IGF-I and TGF-β (Elisseeff et al., 2001; Moioli et al., 2006), insulin and IGF-I (Yuksel et al., 2000a, 2000c), insulin, IGF-I and bFGF (Yuksel et al., 2000b). Some of these particle formulations were integrated in tissue engineering scaffolds and are discussed in the following section.

To overcome shortcomings of high *burst* and GF inactivation during release, microspheres made of PLGA/PEG blends (Yuksel et al., 2000b, 2000c), a PLLA/starch blend (Silva et al., 2007) and polyphosphoesters (Xu et al., 2002) were developed too. On the other hand, the pegylated form of EGF was synthesized and entrapped inside PLGA microspheres (Kim et al., 2002a). Results highlighted that pegylation results in a more physically stable derivative undergoing a lower extent of aggregation during emulsification step although no direct evidence of pegylated EGF biological activity was provided.

A recent study by Chun et al. (2004) also demonstrated that unloaded PLGA microspheres can act themselves as an injectable microcarrier for *in vivo* cell delivery. Surface properties of the microspheres were found to affect strongly cell attachment, growth, and function. Bovine chondrocytes cultured on PLGA microspheres with a positive surface showed the highest functionality as compared to neutral and negatively charged microspheres.

Table 3
Growth factor-loaded delivery systems integrated in tissue engineering scaffolds

Delivery system	Scaffold material	Growth factor	Application	Reference
Cholesterol/methylcellulose/ lactose pellets	Polyvinyl alcohol	EGF	Wound healing	Buckley et al. (1985)
Gelatin microspheres	Gelatin	EGF	Wound healing	Ulubayram et al. (2001)
	OPF	TGF-β1	Cartilage repair	Holland et al. (2003, 2004) and Park et al. (2005)
	OPF	TGF-β1/IGF-1	Cartilage repair	Holland et al. (2007)
	Collagen	bFGF	Adipose tissue formation	Vashi et al. (2006)
	Gelatin–chondroitin–hyaluronate	TGF-β1	Cartilage repair	Fan et al. (2006)
		bFGF	Cartilage repair	Deng et al. (2007)
Chitosan–gelatin microspheres	Chitosan–gelatin	bFGF	Skin repair	Liu et al. (2007)
Chitosan microspheres	Collagen/chitosan/glycosaminoglycan	TGF-β1	Cartilage repair	Lee et al. (2004)
	Chitosan	TGF-β1	Cartilage repair	Kim et al. (2003)
PLGA microspheres	Alginate	bFGF	Neovascularization	Perets et al. (2003)
	Alginate (injectable)	IGF-1	Bone regeneration	Luginbuehl et al. (2005)
	Collagen I	BMP-2[a]	Cell cultivation	Gavenis et al. (2007)
	Collagen tubes	NGF	Nerve regeneration	Rosner et al. (2003)
	Chitosan/chitin tubes	EGF	Nerve regeneration	Goraltchouk et al. (2006)
	Carboxymethylcellulose	BMP-2	Bone regeneration	Woo et al. (2001)
	PEG	IGF-I and TGF-β1[b]	Cartilage formation	Elisseeff et al. (2001)
	PEG	TGF-β1	Cartilage repair	DeFail et al. (2006)
	PLA–PEG–PLA	NT-3/CNTF	Spinal chord injury	Burdick et al. (2006)
	Poly(2-hydroxyethyl methacrylate), poly(1-vinyl-2-pyrrolidinone), PEG	VEGF[c]	Coatings of implantable bio-medical devices	Norton et al. (2005) and Patil et al. (2007)
	PLA	VEGF/PDGF	Vascularization	Richardson et al. (2001)
	PLA	VEGF	Vascularization	Ennett et al. (2006)
	Tubes from microspheres	NGF	Nerve regeneration	Yang et al. (2005)
	Ca–P cements	BMP-2	Bone regeneration	Ruhe et al. (2005)
	Scaffold from microspheres	IGF-I and TGF-β1	Cartilage TE	Jaklenec et al. (2007)
PDLLA microspheres	Scaffold from microspheres	BMP-2[d]	Zonal protein release	Suciati et al. (2006)
PLGA (nanoparticles)	PLLA nano-fibrous scaffolds	PDGF-BB, BMP7	Bone regeneration	Wei et al. (2006, 2007)

[a] Human osteoarthritic chondrocytes were embedded in the scaffold.
[b] Bovine articular chondrocytes were embedded in the scaffold.
[c] VEGF was tested in the presence of dexamethasone in the scaffold.
[d] Trypsin and horseradish peroxidase were also studied as model proteins.

3.3. Scaffolds bioactivated with growth factor-loaded delivery systems

Bioactivation of a tissue engineering scaffold with GF-loaded DS has been especially focused on particulate systems (Table 3). Incorporation of particles in gel-like scaffolds does not present special issues and is accomplished both after and before scaffold preparation whereas their integration in non-gel-like scaffolds is much more difficult to attain and requires special technologies. Nevertheless, techniques to assess protein release directly within the scaffold are strongly suggested since they could provide distinctive information on the evolution in time and space of biological GF cues. Recently, a CLSM-assisted method to follow protein release from microspheres embedded in gel scaffolds has been proposed (Ungaro et al., 2006).

In a very early study, the hypothesis that long-term exposure to GFs could be beneficial to cell growth was tested by incorporation of EGF-loaded cholesterol/methylcellulose/lactose pellets embedded on edge in the center of PVA sponges (Buckley et al., 1985). The scaffold accelerated the process of wound repair, specifically neovascularization, organization by fibroblasts and accumulation of collagen confirming that the effect of GFs can be modulated by controlling their availability to cell surface receptors. A clear evidence that incorporation of a GF delivery system in a gel experienced superior potential as compared to a GF-loaded gel can be found in a study where this comparison is carried out on EGF in wound healing (Ulubayram et al., 2001).

Body of literature has been published on the use of crosslinked gelatin microspheres embedded in different scaffold types. TGF-β1-loaded gelatin microparticles, as developed by Tabata group, have been introduced in OPF hydrogels with the dual functionality of a digestible porogen and a delivery vehicle (Holland et al., 2003, 2004). Besides offering a better control over GF release rate, OPF hydrogels integrated with microspheres were more promptly degraded in the presence of collagenase as compared to OPF hydrogels, offering the novel ability to tailor both TGF-β1 release and material degradation rates. This system was further tested *in vivo* by implantation into osteochondral defects in a rabbit model (Holland et al., 2005) resulting in excellent tissue filling and integration and demonstrating its potential in cartilage repair strategies. Embedding of bovine chondrocytes in the same systems was achieved too (Park et al., 2005). Gelatin microspheres delivering TGF-β1 and bFGF have been also incorporated in fibrillated collagen (Vashi et al., 2006) and gelatin–chondroitin–hyaluronate scaffolds (Fan et al., 2006; Deng et al., 2007). GF-loaded gelatin microspheres were loaded in gelatin–chondroitin–hyaluronan hybrid scaffolds by impregnation of preformed crosslinked/freeze-dried scaffold with a microsphere dispersion in ethanol. It was observed that microspheres (20–160 μm) perfectly integrated within the collagen gel, due to their strong retention in the carrier matrix (Vashi et al., 2006). *De novo* formation of adipose tissue was achieved through controlled release of FGF-2 pointing out a new strategy to overcome the use of Matrigel® in this type of application. An attempt to entrap FGF before glutaraldheyde crosslinking of

gelatin microspheres can be found in a recent paper by Deng et al. (2007).

GF-loaded chitosan microspheres, prepared according to an emulsion/ionic crosslinking method using TPP as crosslinker, were incorporated in chitosan scaffolds (Kim et al., 2003). Proteins (TGF-β1 or BSA as a model protein) were solubilized in an acidic aqueous solution and added to a chitosan solution. Submicron microspheres were loaded in preformed scaffolds by impregnation, as described previously for gelatin microspheres. Both BSA and TGF-β1 were released at a modulated rate, faster for BSA, due to the occurrence of specific interactions between positively charged TGF (p*I* = 8.59) and TPP. Other examples of chitosan microspheres integration in chitosan-containing scaffolds are available in the literature (Kim et al., 2003; Lee et al., 2004).

Integration of PLGA microspheres in tissue engineering scaffolds is a very powerful strategy to control GF levels released. bFGF-loaded PLGA microspheres embedded in alginate scaffolds demonstrated that the exerted control of bFGF delivery effectively enhanced matrix vascularization after *in vivo* implantation (Perets et al., 2003). Microsphere incorporation was accomplished before scaffold-crosslinking and did not alter scaffold porosity or pore size. *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. Analogously, IGF-1-loaded PLGA microspheres were integrated in injectable scaffolds made of alginates physically crosslinked through a calcium carbonate-induced gelation at slightly acidic pH occurring in the presence of TCP granules (Luginbuehl et al., 2005). Reduced swelling and gelation time and an increase in alginate hydrogel stiffness was observed upon TCP addition. Results on osteoblast-like cells highlighted an up to sevenfold increased proliferation rate of cells for microsphere-integrated scaffolds as compared to systems without particles. TGF-β1-loaded PLGA microspheres were also incorporated into biodegradable hydrogels of PEG crosslinked by the non-toxic agent, genipin to obtain cartilage repair (DeFail et al., 2006). Release studies indicated that TGF-β1 was released over 21 days from the delivery system, and the *burst* release was decreased when the microspheres were embedded in the hydrogels. The concentration of TGF-β1 released from the gels could be controlled by both the mass of microspheres embedded in the gel and the concentration of genipin. Additionally, the scaffold permits containment and conformation of the spheres to the defect shape.

Microspheres/cells 3D constructs were also developed. Gavenis et al. (2007) integrated PLGA microspheres loaded with BMP-7 in 3D collagen gel cultures of condrocytes and the construct tested *in vitro*/*in vivo*. Results highlighted that PLGA microspheres are a functional device for the delivery of GFs during the cultivation of articular chondrocytes leading to an increased content of type-II collagen and proteoglycan in the extracellular matrix. In a similar approach, Elisseeff et al. (2001) entrapped IGF-I and TGF-β1-loaded PLGA microspheres together with bovine articular chondrocytes in PEG hydrogels before UV crosslinking. Microsphere concentration in the pregel was found to affect photopolymerization which did not occur with excessive microsphere amounts.

PLGA microspheres loaded with NGF have been recently tested in neural tissue engineering. NGF-releasing microspheres were encorporated in magnetically aligned collagen tubes intended to treat peripheral nerve injuries (Rosner et al., 2003). Sustained release of NGF for 75 days was obtained and a mathematical model to predict NGF distribution in the overall system developed. Recently, microsphere-integrated nerve guidance channels (NGCs) which promote axonal regeneration after transection injury of the peripheral nerve or spinal cord were designed (Goraltchouk et al., 2006). Specifically, PLGA microspheres were physically entrapped in the annulus between two concentric tubes, consisting of a chitosan inner tube and a chitin outer tube. Taking advantage of the extensive shrinking that the outer chitin tube undergoes with drying, >15 mg of microspheres were loaded within the tube walls. Using BSA-encapsulated microspheres as a model drug delivery system, BSA was released from microsphere-loaded tubes for 84 days. EGF, co-encapsulated with BSA, was released for 56 days with a profile similar to that of BSA. Released EGF was found to be bioactive for at least 14 days as assessed by a neurosphere forming bioassay.

In a particular application PLGA microspheres loaded with dexamethasone and VEGF were integrated in anti-fouling hydrogel coatings for the purpose of improving device biocompatibility (Norton et al., 2005; Patil et al., 2007).

A challenging task is to accomplish the integration of PLGA microspheres in biodegradable scaffolds made of lipophilic polymers, due to the fact that DS and scaffold material present similar physico-chemical profiles. Along this direction, the approach of Mooney to obtain a GF bioactivated scaffold of biodegradable polyester (PLA or PLGA) involves processing combinations of polymer particles, free GFs and GF-loaded PLGA microspheres by a gas-foaming/particulate leaching technique (Richardson et al., 2001; Ennett et al., 2006). In a scaffold prepared from free VEGF and PLGA particles, VEGF was positioned predominantly adjacent to scaffold pores and was released rapidly (40–60% in 5 days). Pre-encapsulation in PLGA microspheres led to VEGF being more deeply embedded in the scaffold providing a delayed release. Results also highlighted that GF-loaded microspheres were partly modified during scaffolding, losing their initial architecture and shape. Furthermore, it was found that VEGF release rates from the scaffolds evaluated *in vitro* were very close to those observed *in vivo* (Ennett et al., 2006). Gas-foaming/salt leaching was employed also to form mono o multichannel tubes for nerve regeneration by means of NGF-loaded PLGA microspheres (Yang et al., 2005).

Biodegradable microparticles were also integrated in inorganic scaffolds with different aims. PLGA microspheres were firstly introduced inside CPC resulting in a moldable paste (Simon et al., 2002). This approach was considered in following studies where PLGA microspheres with an average size of 20–40 μm were put inside an injectable CPC so that a macroporous scaffold could be formed after microsphere progressive degradation (Habraken et al., 2006; Ruhe et al., 2006). The introduction of a 20% PLGA microspheres in the scaffold decreased the compression strength, injectability and increased cement setting time, but within manageable ranges. BMP-2 loaded or adsorbed PLGA microspheres were also incorporated in calcium phosphate composites and tested *in vivo* (Ruhe et al., 2005). Release kinetics assessed by scintigraphic imaging highlighted that proper formulation conditions can be useful to accelerate GF release by modulating its interactions with the surrounding cement.

PLGA nanoparticles have been recently tested in tissue engineering applications as reported by Wei et al. (2006, 2007). A technology was developed to immobilize PLGA nanoparticles onto the porous surface of macroporous and nano-fibrous PLLA scaffolds. Single or multiple biological factors were released in a spatially and temporally controlled fashion and release kinetics of each factor could be individually controlled by adjusting formulation conditions. BMP-7-loaded nanospheres incorporated in a nano-fibrous scaffold were successfully used for bone regeneration whereas only fibrous tissue was found in the same scaffold pre-soaked in a BMP-7 solution. A similar approach was employed to incorporate PDGF-BB-loaded nanoparticles.

3.4. Spatiotemporal control of growth factor distribution in tissue engineering scaffolds

From a careful examination of the literature, it is soon realized that a step forward could be moving from static cues of one/two GF(s) toward systems that reproduce more closely the dynamically evolving microenvironment occurring in natural ECM. Tissue morphogenesis is driven by the concomitant action of multiple factors, which work in concert on the same process. Despite the encouraging results obtained in preclinical studies by administering bFGF and VEGF in different fashions, recent large scale clinical trials did not lead to significant therapeutic benefit (Cao and Mooney, 2007). This failure suggested that GF-presentation in the context of cellular microenvironment should be the Achilles' heel in this approach and reinforced the idea that timing of different GF presentation is a key issue to obtain a successful bioinspired tissue regeneration. In neovascularization, for examples, complicate interactions among cytokines, enzymes, GFs and different cells chronologically drive vasculogenesis, angiogenesis and arteriogenesis. It has been shown that VEGF and angiopoietin-2 are able to initiate angiogenesis whereas PDGF and angiopoietin-2 act at later stage by promoting vessel maturation (Jain, 2003; Peirce and Skalak, 2003). Furthermore, also directionality of angiogenesis is highly regulated and strongly depends from VEGF spatial gradients naturally occurring due to continuous diffusion, mobilization and degradation of GFs involved. With this concept in mind, research on DS-integrated tissue engineering approaches is moving in two distinct directions aimed at: (i) designing DS able to provide spatially distinct signal cues inside the scaffold; (ii) creating zones of the scaffold with distinct biological activities by patterning with DS.

Making use of the great versatility of DS, dual GF delivery has been successful in different situations. The first report of a platform capable of delivering multiple angiogenic factors with distinct kinetics is from the group of Mooney (Richardson et al., 2001). Porous PLGA scaffolds for the sequential release of VEGF and PDGF were prepared by mixing free VEGF with empty and PDGF-loaded PLGA particles and subsequently assembling them into a porous scaffold by gas foaming–particulate leaching. Dual release of neuroactive GFs was demonstrated also by entrapping NT-3-loaded PLGA microspheres in PLA–PEG–PLA hydrogels containing free CNTF (Burdick et al., 2006). A fast release of CNTF was realized whereas a sustained release with first-order kinetics was obtained for NT-3. Analogously, a similar approach was carried out in bone tissue engineering where a dual release of TGF-β1 and IGF-1 through gelatin-loaded microspheres incorporated in OPF gels was attempted as a potential strategy for improving cartilage repair (Holland et al., 2007). A surprising conclusion of the study was that dual delivery did not result in widespread improvement to the quality of the neo-surface when compared to untreated defects, indicating that findings of *in vitro* TGF-β1 and IGF-1 synergy do not directly correlate with *in vivo* observations. Sequential release of IGF-I and TGF-β1 from scaffolds made of GF-loaded PLGA microspheres for cartilage tissue engineering applications was recently proved (Jaklenec et al., 2007). Microspheres were assembled in a 3D scaffold by exposing microsphere slabs to dichloromethane vapour. Both GFs were released for up to 70 days in bioactive form at rates controlled by microsphere formulation. Three scaffolds with tailored release kinetics could be fabricated by selecting suitable mixtures of microspheres with different release characteristics.

A possible strategy to control spatial gradients of GFs relies on placing one or more type of DS in predetermined position of the scaffold in order to provide known signal cues. Along this direction, indications that spatial control over biological activity of protein-loaded matrices could be obtained by an appropriately designed controlled-release device were obtained by testing a two-layer polymer matrices that simultaneously released NGF and a neutralizing antibody (anti-NGF) from opposite faces (Fleming and Saltzman, 2001). The concept was proved *in vitro* on cell cultures in collagen gels where it was found that sharp boundary of NGF biological activity was created over a small spatial separation. On the other hand, scaffolds of poly(hydroxyethylmethacrylate) containing gradients of NGF and NT-3 have been created by a gradient maker (Moore et al., 2006). More recently, it has been proposed an anisotropic system based on a porous bi-layered PLGA scaffold able to expose only VEGF in one spatial region and deliver VEGF and PDGF in an adjacent region (Chen et al., 2007b). Controlled GF delivery with this system in an animal model of severe ischemia led to spatial control over vessel density, size, and maturity in the tissue within the scaffolds. Finally, neovascular patterning was guided by the spatially segregated presentation of VEGF and PDGF. Along this direction, scaffolds made of PLA microparticles plasticized with PEG were obtained by sintering protein-free and protein-loaded layers. Distinct release of different bioactive molecules restricted to specific regions within the scaffold were obtained (Suciati et al., 2006), thus opening the way to applications in which gradients of GFs are necessary or where zonal tissue regrowth is the final aim.

Finally, it has been suggested that creating spatial gradients from polymeric scaffolds can help to develop *in vitro* models of angiogenesis to quantify the role of GF concentration/gradient on cells (Chen et al., 2007a). This approach was tested in angiogenesis through the delivery of VEGF from a porous bi-layered PLGA scaffold containing different amounts of VEGF in each layer. Based on a mathematical model, a delivery system was designed to provide the desired profile in ischemic mice hindlimbs. *In vivo* results highlighted that, when VEGF was delivered from the device with uniform VEGF distribution, hindlimbs blood flow was enhanced above control, but did not completely restore perfusion to normal levels. Interestingly, hindlimbs implanted with devices designed to deliver a spatial VEGF gradient based on *in vitro* modeling showed accelerated recovery from hindlimb ischemia.

At last, modern 3D techniques for scaffold production are opening a new window toward the use of DS as an element block in creating complex architectures. For example, inkjet printing was able to create immobilized patterns of unmodified FGF-2 at different surface concentrations (Miller et al., 2006). Cells exposed to defined spatial FGF-2 surface concentrations initially grew randomly across the patterned and non-patterned regions whereas a patterned increase in cell density was observed over time when cell migration took place. In addition, the cells on the FGF-2 patterns survived longer than the cells off patterns. The potential of RP techniques to create patterns of DS is however all to be proved yet and further studies are needed to define the dependence of cellular responses on the spatial organization of GF gradients.

4. Closing remarks

Implementation of tissue engineering-based therapies is underway and results obtained so far suggest that delivery technologies will be more and more a key fundamental. A lot of work was done on tissue engineering scaffolds acting as delivery platform in term of optimization of delivery rate by strategies involving GF interspersion as well as physical or chemical immobilization with the aim to mimic naturally occurring events. Since control of both functional scaffold properties and GF delivery rate is very difficult to attain by these strategies, the integration of technological know-how on protein DS, and especially on particles, in tissue engineering practice can be regarded as an interesting option in tracing the next future directions. If appropriately formulated and integrated in 3D constructs, particles can offer, beside protection and tunable release

rates, multiple levels of scaffold complexity useful to design more and more sophisticated tissue engineering approaches. Finally, the concept that particles can be considered as building blocks in “bottom-up” approaches for scaffold processing also opens new perspectives and challenges. Making use of cutting-edge scaffold preparation technologies, highly regulated bioinspired network of signals could be shortly realized through appropriate particle positioning. In perspective, this approach could also contribute to a deeper knowledge of fundamental biological behaviours such as relationships between cell response to signal cues and tissue morphogenesis.

To fulfil the distinct requirements for discrete tissue engineering applications, protein delivery systems have to provide more and more sophisticated strategies for long-term release under adverse conditions. Through the strategies developed up to now, it is not always possible to achieve this ultimate goal. Nevertheless, if a closer look is given at the opportunity available for protein delivery and those applied to deliver GFs in tissue engineering, it is realized soon that a lot of work could be done. I really hope that this contribution can strongly prompt those who are already involved in this type of research to extend their activities and invite the highest number of researchers already working in protein delivery to contribute to advances in this field. The interdisciplinary attitude of tissue engineering, which already represents the confluence of a complex array of knowledge from quite different domains, will be of help in facilitating and promoting this cultural integration.

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