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REVIEW ARTICLE

Materials in particulate form for tissue engineering. 2. Applications in bone

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Abstract

Materials in particulate form have been the subjects of intensive research in view of their use as drug delivery systems. While within this application there are still issues to be addressed, these systems are now being regarded as having a great potential for tissue engineering applications. Bone repair is a very demanding task, due to the specific characteristics of skeletal tissues, and the design of scaffolds for bone tissue engineering presents several difficulties. Materials in particulate form are now seen as a means of achieving higher control over parameters such as porosity, pore size, surface area and the mechanical properties of the scaffold. These materials also have the potential to incorporate biologically active molecules for release and to serve as carriers for cells. It is believed that the combination of these features would create a more efficient approach towards regeneration. This review focuses on the application of materials in particulate form for bone tissue engineering. A brief overview of bone biology and the healing process is also provided in order to place the application in its broader context. An original compilation of molecules with a documented role in bone tissue biology is listed, as they have the potential to be used in bone tissue engineering strategies. To sum up this review, examples of works addressing the above aspects are presented.

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1. Materials in particulate form and bone tissue engineering (TE)

Regarding materials for use in bone TE, several approaches have been shown to be effective in stimulating bone regeneration, and ceramics especially excel in this regard(Degroot, 1993; Hench, 1998; Ducheyne and Qiu, 1999). Notwithstanding the stimulatory effect of bioactive ceramics on bone tissue formation, there is a continuous need to explore avenues in which materials, cells and biologically active molecules are combined. This is critical, since cells and growth factors are the two key elements when discussing bone biology/healing, their interaction being fundamental for an effective regeneration process. Although continuous progress is being made in understanding osseous healing process, these new insights have not readily found their way into effective TE approaches. The combination of materials, cells and growth factors seems to be the recipe for a truly effective bone TE strategy. Therefore, the present review focuses on the role that particle-based systems can play in bone TE, emphasizing the combination of materials with cells and their role as carriers for biologically active molecules.

2. Requirements for an effective bone TE strategy

The skeletal system has been described as a dynamic, mineralized, vascular tree that serves as a metabolic

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reservoir of calcium as well as a structural scaffold for neurovascular distribution and muscular function(Roberts and Hartsfield, 2004). Important properties that are part of the skeletal system (Canalis, 1983; Hauschka, 1990; Tenembaum, 1990; Yaszemski *et al.*, 1996; Roberts and Hartsfield, 2004) are:

- It is the reservoir of calcium in the body, containing 99% of the body's calcium.
- Its homeostasis is regulated to a large degree by systemic influences expressed through the endocrine system, but also controlled at the local level.
- Its structural function derives from its nature as mineralized tissue.
- It is an anisotropic material (the mechanical properties vary according to the direction).
- Its physiological efficiency is evidenced by maximal strength with minimal mass.
- It has a relative high turnover (remodelling) rate in young individuals.

The ultimate goal of bone TE is to recapitulate the structure and function of the native tissue it is designed to replace (Schneider *et al.*, 2003). Therefore, the following principles apply to scaffolds for bone tissue engineering:

1. Bone TE scaffolds require not only a material with adequate composition, but also mechanical stability, precise shapes and tailored pore distribution (Gross and Rodriguez-Lorenzo, 2004; Rodríguez-Lorenzo and Ferreira, 2004). Osseous tissue is an exquisitely structured composite material: it is composed of organic and inorganic components and also contains water. The inorganic component is apatitic calcium phosphate, which comprises 60–70% of the bone dry weight. The organic component contains materials such as collagen, extracellular matrix proteins (osteocalcin, osteonectin, bone sialoprotein), tissue-specific cells and water (Jain and Panchagnula, 2000). Having this in mind is crucial for the design and fabrication of an adequate scaffold. The adult skeleton consists of cortical (or compact) and trabecular (or cancellous, spongy) bone, which are present in various ratios and geometries to form the individual bones of the body (Buckwalter *et al.*, 1996; Mundy, 2000; Davies, 2003). Both cortical and trabecular bone tissue types are essential for the ability of skeleton to provide structural support that can simultaneously withstanding torsion and bending. A minimum pore size is required for tissue growth, interconnectivity for access to nutrients and transport of waste products, pore shape and roughness for better cell spreading and pore throat size for passage of tissue throughout the scaffold (Ranucci and Moghe, 1999; Zeltinger *et al.*, 2001; Gross and Rodriguez-Lorenzo, 2004). The lack of adequate porosity can lead to failure, as inner areas of the scaffold will lack adequate nutrient and oxaemic conditions to allow cells to populate those areas (Gross and Rodriguez-Lorenzo, 2004).
2. The material should act as a permissive environment into which bone cells would be enticed to migrate and begin the process of depositing bone matrix in the carrier template (Li and Wozney, 2001). Bone, being a mineralized tissue that is incapable of internal expansion or contraction, can only be remodelled along the surface via anabolic and catabolic modelling (Roberts *et al.*, 2004). Bone is resorbed by osteoclasts and formed by osteoblasts, and the coupling of these two processes underlies bone remodelling. Figure 1 depicts the bone healing process, which the repair using scaffold materials attempts to mimic. Briefly, upon fracture and formation of a blood clot, the fibroblast layer of the periosteum begins a period of active division in order to generate enough cells to close the gap at the surface. In the central zone of the bone, haematopoietic precursors in the bone marrow differentiate into osteoclasts that start the process of resorbing the end bone of the defect, and mesenchymal cells within the bone marrow are stimulated to migrate to the healing site. These cells originate chondrogenic cells that produce an intermediate cartilaginous matrix that mineralizes. This cartilaginous phase is then replaced by new bone synthesized by osteoblasts. This newly formed bone is the so-called 'woven bone', which possesses an unorganized structure and still needs to be remodelled by the normal osteoclast–osteoblast process (Davies, 2003; this scheme does not incorporate the vascularization process). To be successful, a scaffold material must be capable of allowing a similar process to occur. Ideally, the scaffold would degrade at a similar rate to that at which the tissue is healing, and the new tissue would fully replace the space once occupied by the scaffold.
3. A system designed for bone repair would ideally combine osteoconductive and osteoinductive properties, in a way that new bone formation can be enhanced through an adequately shaped three-dimensional (3D) scaffold (osteoconduction) and by a biological stimulus (osteoinduction) (Luginbuehl *et al.*, 2004). Ceramic materials, due to their inorganic nature and ionic composition, are adequate for bone applications. Examples of ceramic materials are calcium phosphates, such as hydroxyapatite, tricalcium phosphate and bioactive glasses, known for their ability to bond to and stimulate bone regeneration (Ripamonti, 1991, 1996; Klein *et al.*, 1994; Ducheyne and Qiu, 1999; Yuan *et al.*, 2001). From these, bioactive glass has been shown to stimulate osteogenesis (Jun Yao, 2005; Radin, 2005) via surface-mediated and solution-mediated mechanisms (Radin *et al.*, 1997). Other materials besides bioactive glasses have been extensively used, such as β-tricalcium phosphate (TCP) (Zerbo *et al.*, 2005) and hydroxyapatite (Paul and Sharma, 1999; Sari *et al.*, 2003), but there are also some reports of the use of composite materials (ceramic–polymer) (Shikinami and Okuno, 1999). Composite ceramic–polymer materials have the advantages of combining bioactivity,

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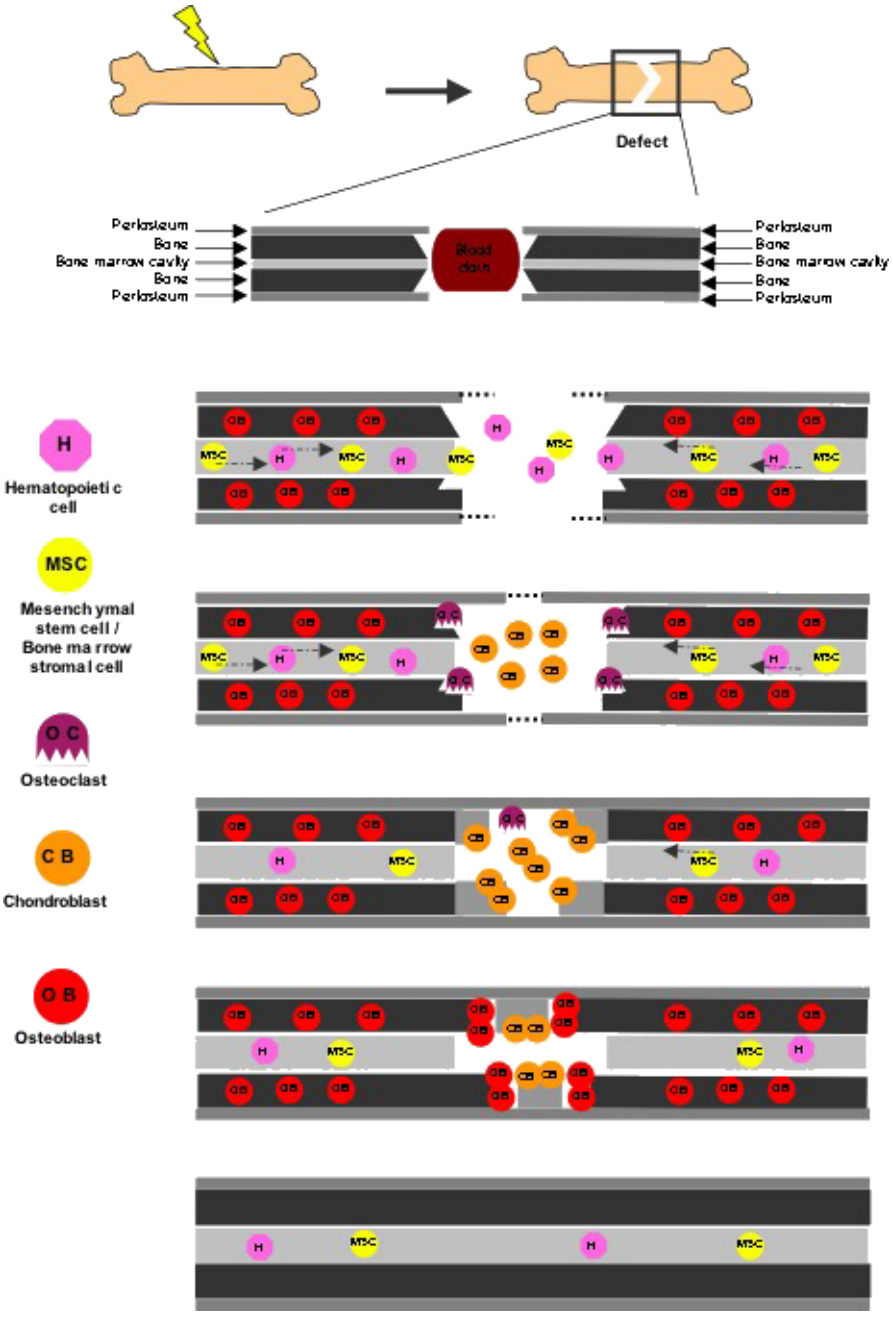

Figure 1. Healing process of bone, depicted in a simplified diagram. After the defect and formation of a blood clot, haematopoietic precursors (H) in the bone marrow differentiate into osteoclasts (OC), which start the process of resorbing the end bone of the defect. Mesenchymal cells (MSCs) within the bone marrow are stimulated to migrate to the healing site. These cells originate chondrogenic cells (CB), which produce an intermediate cartilaginous matrix that progressively mineralizes. This cartilaginous phase is then replaced by new bone synthesized by osteoblasts (OB). Not depicted is the role of vascularization. Based on Simmons and Grynpas (1990) and Rydziel *et al.* (1994)

ability of adequate control of the scaffold degradation rate, and enhancement of the mechanical properties and structural integrity of scaffolds (Day *et al.*, 2004).

4. Some biologically active molecules act locally and therefore must be delivered directly to the site of regeneration via a carrier matrix (Li and Wozney, 2001). The system should be able not only to provide structural support but also to serve as carrier for biologically active agents that can enhance the regenerating potential of the system. These agents can be of different natures, as listed in Table 1. Since the identification of bone morphogenetic proteins (BMPs) by Urist (1965), several other growth factors, as well as hormones and other biologically active agents, have been identified as acting in bone, and have recently been of interest for bone tissue engineering strategies.

Two groups of molecules (growth factors and steroids) with well-documented effects over bone, and considered relevant to the field of bone tissue engineering, are described below.

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2.1. Growth factors

Among all available growth factors, PDGF, IGF, VEGF, TGFβ and BMPs appear to have the closest association with bone regeneration. PDGF plays an important role in inducing the proliferation of undifferentiated cells in mesenchymal tissues. It can enhance bone regeneration in conjunction with other growth factors, viz. IGF, TGFβ or BMP, but is unlikely to provide entirely osteogenic properties itself (Schliephake, 2002). IGFs have an important role in general growth and maintenance of the body skeleton, and appear to integrate and extend the effects of both BMPs and TGFβs (McCarthy *et al*., 2000). Equally important is VEGF, which couples ossification

Table 1. Some molecules and trace elements with a brief description of their role/effect on bone, compiled in the scope of this review

Molecule	Role/effect on bone tissue	Reference
Bone morphogenetic proteins (BMPs): BMP-2, BMP-4, BMP-3, BMP-5, BMP-6, BMP-7(OP-1)	Expressed in bone generation, regeneration, modelling and remodelling. Stimulate differentiation of osteoblasts and inhibit differentiation of muscle cells. Induce endochondral bone formation in ectopic sites	(Urist, 1965, 1997; Urist *et al*., 1979; Cheifetz *et al*., 1996; Yeh *et al*., 1997; Wada *et al*., 1998; Wozney and Rosen, 1998; Chen *et al*., 2001; Reddi, 2001)
Epidermal growth factor (EGF)	Stimulates chondrocyte proliferation while decreasing the ability of cells to synthesize matrix components	(Caplan and Boyan, 1994)
Basic fibroblast growth factor (bFGF)	Mitogenic effects on cells from the mesenchymal lineage. Promotes proliferation and inhibits differentiation. Involved in fracture repair	(Pitaru *et al*., 1993; Caplan and Boyan, 1994; Lockin *et al*., 1999; Mundy, 2000)
Insulin-like growth factor (IGF)	Enhances osteoblast activity and chemotaxis, type I collagen production, decreases collagen degradation, stimulates growth in various cell types and blocks apoptosis. Induces bone formation. Enhances VEGF expression in osteoblasts	(Goad *et al*., 1996; Mundy, 2000; Meinel *et al*., 2001)
Platelet-derived growth factor (PDGF)	Potent mitogen and chemotactic factor for cells of mesenchymal origin. Anabolic action on bone formation *in vivo*	(Kim and Valentini, 1997; Hsieh and Graves, 1998; Park *et al*., 2000)
Transforming growth factor-β (TGFβ)	Mitogenic and chemotactic effects; increase in collagen and extracellular matrix synthesis. New bone formation. Involved in fracture repair. May promote osteoclast apoptosis. Overexpression leads to osteoclast-mediated resorption. Potent inhibitor of terminal differentiation of epiphyseal plate chondrocytes	(Marcelli *et al*., 1990; Centrella *et al*., 1994; Erlebacher and Derynck, 1996; Hugues *et al*., 1996; Kim and Valentini, 1997; Ripamonti *et al*., 1997; Duneas *et al*., 1998; Lockin *et al*., 1999; McCarthy *et al*., 2000; Mundy, 2000; Schmidmaier *et al*., 2003; Kahai *et al*., 2004; Li *et al*., 2005)
Hepatocyte growth factor (HGF)	Contributes to fracture repair by upregulating the expression of BMP receptors	(Imai *et al*., 2005)
Vascular endothelial growth factor (VEGF)	Induces vascularization	(Mohle *et al*., 1996; Vu and Werb, 1998; Asahara *et al*., 1999; Gerber *et al*., 1999)
Calcitonin	Secreted by the thyroid gland. Controls the levels of calcium and phosphorous in the blood. When administered, inhibits bone resorption by decreasing the number of osteoclasts and their resorptive activities. Effectively inhibits the manifestations of metabolic bone disorders, such as Paget's disease and osteoporosis by frequent and relatively high dosage	(Overgaard and Christiansen, 1991; Lee and Sinko, 2000; Patton, 2000; Inzerillo *et al*., 2002)
Melatonin	Increased proliferation of osteoblastic cells and increased procollagen type I c-peptide production. Augmented gene expression of sialoprotein and other bone marker proteins, e.g. alkaline phosphatase and osteocalcin in bone cells. Modifies bone remodelling after ovariectomy in close relation with estradiol	(Roth *et al*., 1999; Ladizesky *et al*., 2001)
Parathyroid hormone (PTH)	In low dose causes increase in bone density and cancellous/trabecular bone volume without impairing normal bone architecture and has a direct effect on recruitment/proliferation of osteoblasts	(Stewart, 1996; Morley *et al*., 1997; Watson *et al*., 1998; Mohan *et al*., 2000; Patton, 2000; Rattanakul *et al*., 2003; Schneider *et al*., 2003)
Thyroxin	Thyroid hormone which stimulates osteoclastic bone resorption	(Buckwalter *et al*., 1996)
Cortisol	Influences PTH-responsiveness of bone. Inhibitor of the stimulatory effect of IGF-I	(Ng and Heersche, 1978; Tam *et al*., 1979; Chyun *et al*., 1984)
Interleukin-6 (IL-6)	Stimulates the differentiation of osteoclasts from haematopoietic precursors	(Ishimi *et al*., 1990; Migliaccio *et al*., 1991)

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Table 1. (*Continued*)

Molecule	Role/effect on bone tissue	Reference
Interleukin-1 (IL-1)	Stimulates the effect of IL-6. Most potent inducer of bone resorption	(Gowen *et al*., 1985a, 1985b; Hoffmann *et al*., 1987; Hauschka, 1990)
Tumour necrosis factor (TNF)	Stimulates the effect of IL-6. Stimulates bone resorption and suppresses its formation	(Bertolini *et al*., 1986; Bockman *et al*., 1987; Canalis, 1987; Stashenko *et al*., 1987)
Prostaglandin E2 (pE2)	Potentates the effect of IGF-I. Concentration-dependent actions (regulation of the expression of other molecules). Increases expression of BMP-7 (OP-1)	(Chyun and Raisz, 1982, 1984; Dewhirst *et al*., 1987; Paralkar *et al*., 2002)
Interferon-β (IFN-β)	Suppresses osteoclastogenesis and bone resorption	(Nakamura *et al*., 2005)
Interferon-γ (IFN-γ)	Suppresses bone resorption induced by IL-1	(Nakamura *et al*., 2005)
Bi-phosphonates Etidronate Clodronate Pamidronate Alendronate Ibandronate Risedronate Zolendronate Tiludronate YH 529 Icadronate Olpadronate Neridronate EB-1053 TRK-300	Considered stable analogues of pyrophosphate, a physiological regulator of calcification and bone resorption. Decrease bone resorption/increase bone mass Decreases the level of tumour necrosis factor alpha (TNFα) in the bone marrow of rats with adjuvant arthritis	(Ezra and Golomb, 2000; Patton, 2000; Roschger *et al*., 2001) (Iwase *et al*., 2002)
Ipriflavone (Isoflavone)	Synthetic flavonoid derivative that improves osteoblast cell activity inhibiting bone resorption	(Brandi, 1993; Civitelli, 1997; Perugini *et al*., 2003)
Anthraquinones	Anti-inflammatory and anti-osteoclastic activity	(Savarino *et al*., 2005)
Vitamin D and analogueues	Regulates osteoblast differentiation by either activating or repressing transcription of numerous bone phenotypic genes. Increases TGFβ levels	(Brandi, 1993; Drissi *et al*., 2002)
TAK-778 [(2*R*,4*S*)-(−)-*N*-(4-diethoxyphosphorylmethyl-phenyl)-1,2,4,5-tetrahydro-4-methyl-7,8-methylenedioxy-5-oxo-3-benzothiepin-2-carboxamide]	TAK-778, a benzothiepin derivative, increased cellular alkaline phosphatase activity, an index of bone formation, in a culture of rat bone marrow stromal cells, and enhanced the action of BMP in mouse osteoblastic cell line MC3T3-E1	(Hoshino *et al*., 2000)
TP508 (thrombin peptide)	Activates angiogenesis-related genes during femoral fracture healing. Regulates BMP-2 and -7 expression by human osteoblasts. Enhances bone formation	(Bi *et al*., 2001; Wang *et al*., 2001, 2002; Li *et al*., 2003; Sheller *et al*., 2004)
Indomethacin	Found to inhibit osteoclasts and to decrease the resorptive area	(Adachi *et al*., 1991)
Corticosteroids (glucocorticoids)	Excess generally associated with net bone loss, due to decrease in bone formation and increase in bone resorption	(Heersche and Aubin, 1990)
Statins	Generally used for inhibiting HMG Co-A reductase (rate-limiting step in cholesterol synthesis). Enhance transcription of BMP-2 in bone cells	(Mundy, 2000)
Oestrogen/testosterone	Deficiency results in high turnover of bone remodelling in which the accelerated bone resorption and formation simultaneously occur, but with resorption exceeding formation. Protective effect on bone tissue mass	(Caplan and Boyan, 1994; Kaye *et al*., 1997; Ladizesky *et al*., 2001; Sikavitsas *et al*., 2001)
Trace elements		
Fluoride	Anabolic effects on bone, but has a narrow toxic-therapeutic window	(Simmons and Grynpas, 1990; Brandi, 1993; Mundy, 2000)
Strontium	Potential increase in bone mass	
Aluminium	Causes mineralization deficit by inhibiting hydroxyapatite crystal formation. Interferes locally with osteoblast maturation	
Boron Tin	Deficiency causes osteopenia. Intervene in magnesium metabolism. Interact with calcium and other ions	
Zinc	Significant for coupling–uncoupling of the remodelling process	

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and angiogenesis during bone formation (Gerber *et al*., 1999; Street *et al*., 2002). BMPs are thought to have their major effects on early precursor bone cell replication and osteoblast commitment. In contrast, TGFβs are thought to be the most potent inducers of committed bone cell replication and osteoblast matrix production (McCarthy *et al*., 2000).

2.1.1. Bone morphogenetic proteins

Growing interest in the clinical use of BMPs as means of promoting bone formation has led to extensive studies on this group of growth factors. In brief, BMPs are hydrophobic, low molecular weight, dimeric molecules with two polypeptide chains held together by a single disulphide bond (Ozkaynak *et al*., 1990; Wang *et al*., 1990; Reddi, 2001). The name stems from the demonstration of a hydrophobic non-collagenous glycoprotein that induced mesenchymal-type cells to differentiate into a spherical ossicle with a medulla containing haematopoietic bone marrow (Urist *et al*., 1979).

This family of secreted growth factors forms a subgroup of molecules within the transforming growth factor-β (TGFβ) superfamily. The history of BMP evolved from observations of allogenic bone matrix-induced cartilage and bone development in mammalian species. In embryogenesis, BMPs appear to be omnipresent, being observed in nearly all developing visceral and somatic organs (Urist, 1997). At least two distinct pathways mediate BMP signalling: the Smad pathway and the mitogen-activated protein kinase (MAPK) pathway (Yoon and Lyons, 2004).

2.1.2. Platelet-derived growth factor

Effects by platelet-derived growth factors (PDGFs) are generally limited to situations associated with inflammation and repair (McCarthy *et al*., 2000). However, PDGFs have been shown to be involved in the chemotaxis of osteoblast precursors to the site of bone regeneration (Mundy *et al*., 1982; Hsieh and Graves, 1998). *In vitro*, they have been shown to stimulate migration and to increase the proliferation rate of osteoblasts, reducing alkaline phosphatase activity and inhibiting bone matrix formation (Centrella *et al*., 1989, 1991; Hock and Canalis, 1994).

There are three isoforms, characterized by the combination of A- and B-chains, featuring two homodimeric (PDGF-AA and PDGF-BB) and one heterodimeric isoform (PDGF-AB) (Hock and Canalis, 1994; Rydziel *et al*., 1994). PDGF-BB and PDGF-AB are systemically circulating isoforms contained in α-granules of platelets, whence they are released after adhesion of platelets to injured sites of vessel walls, whereas PDGF-AA is secreted by unstimulated cells of the osteoblastic lineage (Canalis *et al*., 1992; Rydziel *et al*., 1994).

The biochemical effects of the different isoforms appear to be graded according to their binding characteristics to the surface receptors. In osteoblast-enriched environments, receptors that favour binding of PDGF-BB chains preferably mediate these effects (Centrella *et al*., 1991). PDGF may thereby contribute to recruitment of bone cells during remodelling and repair, as it is deposited in bone matrix, from where it is released during matrix degradation (Fuiji *et al*., 1999).

The effectiveness of PDGFs on osteoblasts is rapidly modulated by inflammatory cytokines, causing changes in specific PDGF receptors (McCarthy *et al*., 2000). The activated receptors lead to activation of the MAPK cascade, resulting in the transcription of important genes related to bone formation (Schlessinger, 1993).

2.2. Corticosteroids

Corticosteroids are a class of steroid hormones that are produced in the adrenal cortex. They are involved in a wide range of physiological systems, such as stress response, immune response and regulation of inflammation, carbohydrate metabolism, protein catabolism, blood electrolyte levels, and behaviour. This class of molecules is often used as part of the treatment for a number of different diseases, such as severe allergies or skin problems, asthma or arthritis. Within corticosteroids there are mineralocorticoids and glucocorticoids, and a brief description of the latter follows.

2.2.1. Glucocorticoids

Glucocorticoids such as cortisol control carbohydrate, fat and protein metabolism and are anti-inflammatory by preventing phospholipid release, decreasing eosinophil action and a number of other mechanisms.

Physiological amounts of glucocorticoid tend to have permissive effects on osteoblasts (Caplan and Boyan, 1994). However, either when endogenously in excess or when administered exogenously, glucocorticoids lead to a dramatic decrease in bone mineral density. Whereas chronic glucocorticoid exposure suppresses bone formation and disrupts resorption and the bone remodelling cycle, major detrimental effects on the skeleton occur from a decrease in osteoblast replication, bone matrix protein synthesis, marked decrease in osteoblast gene transcription and skeletal tissue loss (McCarthy *et al*., 2000; Kumar, 2001). Pharmacological doses of the glucocorticoids cortisol and dexamethasone directly lower basal IGF-I expression (McCarthy *et al*., 1990), and *in vitro* studies have revealed that high excess glucocorticoid suppresses the expression of IGF-I and the type TGFβ receptor (TGFβRI) by osteoblasts, consistent with decreases in specific aspects of osteoblast function (McCarthy *et al*., 2000).

Dexamethasone is a synthetic member of the glucocorticoid class of hormones. It acts as an anti-inflammatory and immunosuppressant, with potency about 40 times that of hydrocortisone (Barnes and Adcock, 1993; Almawi *et al*., 1998; Saklatvala, 2002). *In vitro*, dexamethasone

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has been employed as a differentiation agent for bone marrow cells to progress into the osteoblastic lineage (Maniatopoulos *et al*., 1988). Within this last role, strategies employing the incorporation of dexamethasone in polymeric materials to be used as carriers for the differentiation of cells into the osteoblastic lineage have been described in the literature (Silva *et al*., 2005), which confers on dexamethasone a highlighted role in bone TE approaches.

3. Materials in particulate form: towards bone TE

In recent years there has been interest on the fabrication of 3D systems using a microsphere-based approach for a TE scaffold possessing a porous interconnected structure (Devin *et al*., 1996; Botchwey *et al*., 2001), with the incorporation of ceramics to control the mechanical properties of the sintered scaffold (Borden *et al*., 2002a, 2002b). This is an extremely interesting strategy, as it provides a potential to overcome normally encountered problems associated with porosity of the scaffold. Additionally, with particle-based systems shaped as scaffolds, the surface area for more chemical and biological reactions to take place is greatly increased (Mushipe *et al*., 2002).

The formation of 3D scaffolds from materials in particulate form creates the potential for these systems to be used either in an acellular strategy (implanting of the scaffold and colonization of it by surrounding cells) or combining it with cells *in vitro*, creating a hybrid cell–material construct. Simultaneously, these scaffolds can also be used as delivery systems, having a multifunctional purpose – support and release of bioactive agents – enhancing the regenerative potential of the system.

3.1. Microparticle-based systems in 3D scaffolds

Materials in particulate form in bone applications have as first examples the filling applications of ceramic particulate materials. Schepers *et al*. (1991, 1993) and Schepers and Ducheyne (1997) described the ability of bioactive glass particulates within a narrow size range to act as fillers for bone lesions. When implanted in the jaws of beagle dogs, the particulates were capable of acting as nucleation sites for further bone repair, eliciting bone tissue formation throughout 5 mm defects in the beagle mandible as soon as 1 month after implantation (Schepers *et al*., 1991, 1993; Schepers and Ducheyne, 1997).

However, as cells in the body grow in three dimensions, anchored onto a network of extracellular matrix, a scaffold is needed to recreate the 3D environment (Yu *et al*., 2004). Classical examples of materials shaped for bone tissue engineering involve 3D porous structures obtained by conventional processing methods that, in an conductive approach, are implanted at an injury site and allow progenitor cells from the surrounding tissue to populate the wound site (Nof and Shea, 2002).

Given that porosity, pore size and interconnectivity are very important parameters for the success of a bone TE system, the strategy based on m-sized particles for fabrication of 3D scaffolds seems to be promising, as a means of achieving more control over the above parameters. So far, the following strategies have been studied to fabricate scaffolds from materials in particulate form:

- *Combining particulate materials with gels/glues*. In bone reconstruction, the combination of particulate ceramics and fibrin glue may result in the synergy of their properties, as the physical properties of the composite can be enhanced. The initial stability of the ceramic–fibrin glue composite may be achieved through its adaptation and adhesion to the walls of the bone defect. The biological properties might also be enhanced due to fibrin, which acts positively on angiogenesis, cell attachment and proliferation (Le Nihouannen *et al*., 2006). The problem associated with this type of approach is the lack of porosity. Although cell adhesion would be greatly enhanced by fibrin glue, the penetration of cells into the interior of the scaffold is limited by this lack of porosity.
- *Dispersing microparticles within ceramic phases for posterior creation of porosity*. Other strategies have focused on dispersing microparticles within ceramic phases, where the rationale for this is that the microspheres will initially stabilize the graft but can then degrade to leave behind macropores on the calcium phosphate cement (CPC) for colonization by osteoblasts. The CPC matrix could then be resorbed and replaced with new bone (Simon *et al*., 2002). This relies on the degradation of the microparticles, which depends greatly on the material from which the microparticles are produced, as well as the implant site. It creates difficulties for osteblast colonization, particularly to the inner areas of the scaffold, as the particles might not degrade as fast as necessary to avoid the failure of the implant. An interesting way of overcoming these problems might be the incorporation, within the matrix of microparticles, of enzymes that can degrade them and thus speed the process of pore formation, as described by other researchers (Martins *et al*., 2004a, 2004b).
- *Incorporating polymer microspheres with polymeric scaffolds*. This approach permits the incorporation of growth factor-containing polymeric microspheres during polymer scaffold fabrication (Meese *et al*., 2002). The basic principle of this approach is to transiently protect the microspheres with a water-soluble coating that resists the organic solvents used during scaffold fabrication. The incorporation of microspheres in scaffolds not only allows the protection of the growth factor during fabrication of the scaffold, but also allows the scaffold to provide both structural support and controlled release properties.

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- *Sintering microspheres together*. The previous approaches have paved the way for the use of microparticles as scaffolds. Microparticles can be used to form 3D scaffolds by utilizing the heating energy of a laser beam to sinter polymer microparticles, allowing the fabrication of 3D scaffolds with a controlled architecture and a fully interconnected network (Botchwey *et al*., 2001; Ciardelli *et al*., 2004; Yao *et al*., 2005). By modifying processing parameters, such as sphere diameter and heating time, it is possible to tune the properties of the scaffold. It was found that increased microsphere diameter resulted in decreased modulus, as well as a positive correlation between sphere diameter and pore diameter (Borden *et al*., 2003). Heating time modifications showed that compressive modulus was dependent on the period of heating, with longer heating times resulting in higher moduli, while the heating time did not affect the pore structure (Borden *et al*., 2003). These scaffolds can be further tested, not only in static but also in dynamic conditions, such as those found in bioreactors.

3.2. Microparticle-based systems in hybrid cell–material constructs

Materials in particulate form have been used for combination with cells in two main approaches: the encapsulation of cells for site-specific delivery, or the combination of scaffolds and cells in hybrid constructs in *in vitro* approaches.

Examples of the former include the encapsulation of specific quantities of cells together with bioactive glass into alginate beads (Keshaw *et al*., 2005). Alginate beads have been extensively used for the encapsulation of several cell types (Shoichet *et al*., 1996; Chandy *et al*., 1999; Papas *et al*., 1999; Lu *et al*., 2000; Read *et al*., 2001; Orive *et al*., 2003; Zimmermann *et al*., 2005). The study in question (Keshaw *et al*., 2005) showed that the encapsulated cells remained viable and secreted significantly more VEGF compared with beads containing no glass particles. This demonstrates that cells can be encapsulated for delivery and with the appropriate stimuli (here conferred by bioactive glass) can serve at the same time as the delivery vehicles for growth factors. With further optimization, this technique offers a novel delivery device for stimulating therapeutic angiogenesis, the lack of which in bone TE has been regarded a contributory factor for implant failure (Keshaw *et al*., 2005).

Temporary encapsulation of cells in microparticles may protect the cells from short-term environmental effects, such as those associated with the delivery to the regeneration site. To overcome certain problems encountered in cell therapy, particularly cell survival and lack of cell differentiation and integration in the host tissue, Tatard *et al*. (2005) developed pharmacologically active microcarriers (PAM). These biodegradable particles, made with poly(D,L-lactic-coglycolic acid) (PLGA) and coated with adhesion molecules, may serve as a support for cell culture and may be used as cell carriers, presenting a controlled delivery of active protein (Tatard *et al*., 2005). They can thus support the survival and differentiation of the transported cells as well as their microenvironment (Tatard *et al*., 2005).

However, for bone applications, approaches that use the materials in particulate form, not only to deliver and temporarily protect the cells, seem to be more adequate, as they can also provide structural support while necessary. Ceramic materials, such as hydroxyapatite particles (both dense and microporous), have been evaluated both *in vitro* and *in vivo* as carriers in an injectable tissue-engineered bone filler (Fischer *et al*., 2003). After seeding and culturing goat mesenchymal progenitor cells on the different types of particles, several layers of cells and ECM held the particles together in a 3D arrangement. The subcutaneous implantation of the constructs (with individual particle size of 212–300 m) in nude mice revealed abundant bone formation by 4 weeks (Fischer *et al*., 2003).

An important issue in bone TE concerns the possibility of limited tissue ingrowth in TE constructs because of insufficient nutrient transport (Yu *et al*., 2004). To overcome such limitations, Ducheyne and co-workers (Qiu *et al*., 1998, 1999, 2000, 2001) envisioned a strategy using the HARV bioreactor and microcarriers to engineer constructs that could be used for bone TE purposes. In a first approach, the authors used bioactive glass, Cytodex-3 beads and rat stromal cells for assessing the feasibility of culture using a HARV bioreactor (Qiu *et al*., 1998). It was observed that 3D multicellular aggregates consisting of multiple cell-covered Cytodex-3 microcarriers bridged together, as well as mineralization taking place, and the expressions of alkaline phosphatase activity, collagen type I, and osteopontin were shown (Qiu *et al*., 1998). The authors further developed bioactive ceramic hollow microspheres with an apparent density in the range 0.81.0 g/cm^3 as microcarriers for 3D bone tissue formation in rotating-wall vessels (RWV). Cell culture studies using rat bone marrow stromal cells and osteosarcoma cells showed that the cells attached to and formed 3D aggregates with the hollow microspheres in a RWV. Extracellular matrix was observed in the aggregates (Qiu *et al*., 1999). Similarly, polymer–glass–ceramic composite microspheres, composed of modified bioactive glass (MBG) powders in a polylactic acid (PLA) matrix, were shown to possess adequate properties for bone TE purposes (Qiu *et al*., 2000). Yu *et al*. (2004) have used a similar approach, but mixing lighter-than-water (density <1 g/ml) and heavier-than-water (density >1 g/ml) microspheres of 85 : 15 poly(lactide-*co*-glycolide) and constructing the scaffold prior to cell seeding by sintering of the microspheres. When rat primary calvarial cells were cultured on the scaffolds in bioreactors for 7 days, the 3D dynamic flow environment affected bone cell distribution and enhanced cell phenotypic expression and mineralized matrix synthesis within the tissue-engineered constructs, compared with static conditions (Yu *et al*., 2004). It has been found that with the stress stimulation inside the

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fluid in the RWV, the active expression of ALP can be increased and the formation of mineralized nodules can be accelerated (Song *et al*., 2004). These studies show that 3D fabrication of engineered bone seems an adequate strategy.

3.3. Microparticle-based systems as scaffolds and carriers for bioactive molecules

By far the major field of application of particle-based systems (in both the micro- and the nano-range) is as drug delivery systems, as described in detail in the first part of this review (Silva *et al*., 2006). Their small size but high surface area renders them attractive for a whole range of applications, including bone TE.

In bone tissue regeneration, the use of conductive scaffolds in combination with the delivery of bioactive factors to direct cellular responses and subsequent tissue formation is a very attractive strategy to enhance regeneration (Nof and Shea, 2002), but parameters such as instability and rapid clearance (short plasma half-life) of these molecules after *in vivo* bolus delivery have led to the need for advanced vehicles for localized release (Baldwin and Saltzman, 1998; Li and Wozney, 2001; Norton *et al*., 2005). The physicochemical properties of many peptides and proteins make their entrapment difficult, because inactivation is possible during their incorporation (Couvreur and Puisieux, 1993). Stability, solubility and sensitivity to light, heat, moisture and pH, intermolecular interactions following co-precipitation or gelling, and adsorption and interaction with excipients are parameters that should be investigated in order to succeed in producing a stable association of peptides with particle-based systems (Couvreur and Puisieux, 1993). While encapsulation of peptides and small molecules into biodegradable microspheres can be achieved using several techniques and with different polymers, the encapsulation of proteins still poses major difficulties with respect to obtaining 'infusion-like' or continuous-release profiles with minimal initial burst and sufficient protein loading within the microspheres (Kissel *et al*., 1996; Morlock *et al*., 1998).

Drug delivery systems for bone applications have been mainly focused on 3D porous scaffolds processed by conventional techniques, which present additional difficulties, due to the possibility of destroying the bioactive agent. Some researchers have focused on the incorporation of microparticles loaded with bioactive agents into 3D scaffolds, in an attempt to protect the bioactive agent and still maintain the 3D structure of the scaffold, as described by Mikos and co-workers, which have added poly(D,L-lactic-*co*-glycolic acid)/poly(ethylene glycol) (PLGA/PEG) microparticles loaded with the osteogenic peptide TP508 to a mixture of poly(propylene fumarate) (PPF), poly(propylene fumarate)–diacrylate (PPF–DA) and sodium chloride (NaCl), for the fabrication of PPF composite scaffolds that could allow for tissue ingrowth as well as for the controlled release of TP508 when implanted in an orthopaedic defect site (Hedberg *et al*., 2002). Other authors have used a 3D chitosan scaffold, which was combined with transforming TGFβ1-loaded chitosan microspheres (Lee *et al*., 2004a).

However, the incorporation of bioactive agents into m-sized systems and using them simultaneously as scaffolds and release systems seems an extremely interesting alternative. Examples include the use of dextran-derived materials, which possess hydrophilic properties and the ability to control drug dissolution and permeability. Dextran–glycidylmethacrylate (Dex–GMA)/poly(ethylene glycol) (PEG) microspheres with entrapped recombinant human bone morphogenetic protein-2 (rhBMP-2) showed full preservation of its biological activity. rhBMP-2 microspheres have good biological effects on cultured periodontal ligament cells, and could achieve a longer action time than concentrations of rhBMP-2 solution. These properties make those microspheres interesting osteoconductive BMP carriers, allowing the amount of implanted factor required for tissue regeneration to be decreased (Chen *et al*., 2005, 2006). Similarly to BMPs, insulin-like growth factor I (IGF-I) exerts an important role during skeletal growth and bone formation. Therefore, its localized delivery appears attractive for the treatment of bone defects. To prolong IGF-I delivery, this molecule was entrapped into biodegradable poly(lactide-*co*-glycolide) microspheres and the system evaluated in two defect models of ovine long bones, a metaphyseal drill hole and a segmental tibia defect. New bone formation was observed within 3 weeks in the drill hole and bridging of the segmental defect within 8 weeks. The authors showed that the IGF-I delivery system down-regulated inflammatory marker gene expression at the site of bone injury, induced new bone formation and reduced bone resorption (Meinel *et al*., 2001).

Other approaches try to combine further properties within a single system, such as the one in which *in situ* hardening composites are formed, based on an alginate hydrogel matrix formulated with β-TCP granules and poly(lactide-*co*-glycolide) microspheres loaded with the osteoinductive growth factor insulin-like growth factor I (IGF-I) (Lee *et al*., 2004b; Luginbuehl *et al*., 2005). This approach combines release properties, structural support and a ceramic material with osteoconductive properties for enhanced bone regeneration. Materials such as collagen–chitosan composite microgranules were fabricated as bone substitutes for the purpose of obtaining high bone-forming efficacy. The microgranules have the flexibility to fill various types of defect sites with closer packing. The interconnected pores formed spaces between the microgranules, which allowed new bone ingrowth and vascularization. In addition, TGFβ1 was incorporated into the microgranules in order to improve bone-healing efficacy. The TGFβ1-loaded microgranules demonstrated a higher bone regenerative capacity in rabbit calvarial defects after 4 weeks than the TGFβ1-unloaded microgranules (Lee *et al*., 2006).

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4. Conclusions

Bone repair has been the subject of intensive research. Approaches in clinical use aim to regain function, using materials that replace the damaged tissue rather than regenerating it. Currently, the approach of research regarding bone TE is to induce regeneration rather than just functional repair. Thus, TE can now be simply defined as the 'science of persuading the body to heal by its intrinsic repair mechanisms' (Agrawal and Ray, 2001).

The complexity of skeletal tissues has been hindering the development of an effective regeneration system. Nevertheless, huge steps are being taken regarding the use of progenitor/stem cells, adequate scaffold materials and growth factors/bioactive agents. The combination in a single system of such properties – structural support, cell support and controlled release – is the way to go, and materials in the particulate form have all the potential needed for achieving such a goal.

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