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MACROPOROUS SCAFFOLDS FOR BONE ENGINEERING

Studies on Cell Culture and Ectopic Bone Formation

by

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ABSTRACT

Ville Meretoja. Macroporous scaffolds for bone engineering. Studies on cell culture and ectopic bone formation. Department of Prosthetic Dentistry and Biomaterials Science, Institute of Dentistry, University of Turku. *Annales Universitatis Turkuensis*, Turku, Finland, 2008.

Bone engineering is a rapidly developing area of reconstructive medicine where bone inducing factors and/or cells are combined with a scaffold material to regenerate the structure and function of the original tissue. The aim of this study was to compare the suitability of different macroporous scaffold types for bone engineering applications. The two scaffold categories studied were a) the mechanically strong and stable titanium fiber meshes and b) the elastic and biodegradable porous polymers. Furthermore, bioactive modifications were applied to these basic scaffold types, and their effect on the osteogenic responses was evaluated in cell culture and ectopic bone formation studies.

The osteogenic phenotype of cultured cell-scaffold constructs was heightened with a sol-gel derived titania coating, but not with a mixed titania-silica coating. The latter coating also resulted in delayed ectopic bone formation in bone marrow stromal cell seeded scaffolds. However, the better bone contact in early implantation times and more even bone tissue distribution at later times indicated enhanced osteoconductivity of both the coated scaffold types. Overall, the most promising bone engineering results were obtained with titania coated fiber meshes.

Elastic and biodegradable poly(ε-caprolactone/D,L-lactide) based scaffolds were also developed in this study. The degradation rates of the scaffolds *in vitro* were governed by the hydrophilicity of the polymer matrix, and the porous architecture was controlled by the amount and type of porogen used. A continuous phase macroporosity was obtained using a novel $CaCl_2 \cdot 6H_2O$ porogen. Dynamic culture conditions increased cell invasion, but decreased cell numbers and osteogenicity, within the scaffolds. Osteogenic differentiation in static cultures and ectopic bone formation in cell seeded scaffolds were enhanced in composites, with 30 wt-% of bioactive glass filler.

Keywords: Bone engineering, Cell culture, Bone marrow stromal cells, Ectopic bone formation, Scaffold

TIIVISTELMÄ

Ville Meretoja. Makrohuokoisia tukirakenteita luun kudosteknologiaan. Soluviljely- ja ektooppisen luun muodostuksen tutkimuksia. Hammasprotetiikan ja Biomateriaalitieteen oppiaine, Hammaslääketieteen laitos, Turun yliopisto. *Annales Universitatis Turkuensis*, Turku, 2008.

Luun kudosteknologia on korjaavan lääketieteen nopeasti kehittyvä osa-alue, missä yhdistetään luuta indusoivia tekijöitä/soluja huokoisiin tukirakenteisiin tavoitteena alkuperäisen kudoksen rakenteen ja toiminnan palauttaminen. Tämän tutkimustyön tarkoituksena oli verrata erilaisten makrohuokoisten tukirakenteiden soveltuvuutta luun kudosteknologiaan. Tukirakenteita oli kahta päätyyppiä: a) mekaanisesti luja ja elimistössä säilyvä titaanikuituverkko ja b) joustava ja elimistössä hajoava huokoinen polymeeri. Lisäksi tukirakenteita muokattiin bioaktiivisiksi, ja eri materiaaliversioiden luuperäistä vastetta tutkittiin soluviljelyssä sekä ektooppisen luun muodostuksen kokeissa.

Viljeltyjen luuytimen peruskudoksen solujen luuperäinen ilmiasu voimistui titaanioksidipinnoitetuissa, mutta ei titaanioksidi-piioksidi pinnoitetuissa, titaaniverkoissa. Jälkimmäinen pinnoitetyyppi johti myös viivästyneeseen ektooppiseen luun muodostukseen vastaavilla soluilla ladatuissa tukirakenteissa. Parantunut luukudoskontakti ihonalaisen implantoinnin alkuvaiheessa ja syntyneen luukudoksen tasaisempi jakautuminen huokoisissa tukirakenteissa kuitenkin osoittivat molempien pinnoitteiden hyödyllisyyttä. Kaikenkaikkiaan lupaavimmat tulokset saavutettiin titaanioksidipinnoitetuilla kuituverkoilla.

Tässä työssä myös kehitettiin joustavia ja biohajoavia poly(ε-kaprolaktoni/D,L-laktidi)-peräisiä tukirakenteita. Makrohuokoisten rakenteiden *in vitro* –hajoamisnopeus riippui polymeerin hydrofiilisyydestä, kun taas huokosrakennetta kontrolloitiin huokoistavan aineen määrän ja tyypin avulla. Jatkuvan faasin makrohuokoisuus saavutettiin käyttämällä $CaCl_2 \cdot 6H_2O$ uutena huokoistavana aineena. Kokeissa tutkittu dynaaminen kasvatusmenetelmä lisäsi solujen tunkeutumista tukimateriaalien sisään, mutta vähensi solujen lukumäärää ja luuperäistä vastetta. Bioaktiivisen lasin lisääminen polymeeriin lisäsi solujen erilaistumista staattisissa viljelyissä ja paransi ektooppista luun muodostusta soluilla ladatuissa tukirakenteissa.

Avainsanat: Ektooppinen luun muodostus, Luun kudosteknologia, Luuytimen strooman solut, Soluviljely, Tukirakenne

TABLE OF CONTENTS

ABBREVIATIONS AND DEFINITIONS

ALP	alkaline phosphatase
BAG	bioactive glass
BMSC	bone marrow stromal cell
BSP	bone sialoprotein
CaP	calcium phosphate; (any) ceramic with calcium and phosphate as major components
CFU-F	colony forming unit - fibroblast
cpTi	commercially pure titanium
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
MEPE	matrix extracellular phosphoglycoprotein
OC	osteocalcin
P(CL/DLLA) X/Y	poly(epsilon-caprolactone/D,L-lactide) copolymer with X/Y monomer ratio
PBS	phosphate buffered saline
SBF	simulated body fluid
TiO_2	titania (sol-gel coating)
TiSi	titania-silica (sol-gel coating)

ARF cycle	Bone remodeling cycle starting from osteoclast activation and bone resorption followed by reversal phase and new bone apposition (see Chapter 2.1.2).
Basic (or Bone) multicellular unit	A temporary anatomical structure corresponding to the site of an ARF cycle (see Chapter 2.1.2).
Bioactive material	Biomaterial that is designed to elicit or modulate biological activity (Williams, 1999). In bone engineering this usually refers to direct (biochemical) bone bonding and/or osteoinduction *in vivo*. In addition, the term is often used in reference to bone-like mineral formation in simulated body fluid (Kokubo and Takadama, 2006).
Biocompatibility	The ability of a material to perform with an appropriate host response in a specific application (Williams, 1999). In bone engineering this usually refers to osteoconduction.

Biomaterial	Material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body (Williams, 1999).
Bone engineering scaffold	A growth substrate for osteogenic cells, with three dimensional macroporous architecture and structural integrity enabling manual handling (see Chapter 2.2.1).
Bone marrow stromal cells	Heterogeneous population of adherent, non-haematopoietic, cells of bone marrow (see Chapter 2.3.3).
Chondro-/Osteoblast	(Active) cartilaginous/bone matrix producing cell
Chondro-/Osteoclast	Cartilage/bone resorbing cell
Chondro-/Osteocyte	Terminally differentiated chondro-/osteoblast embedded in cartilaginous/mineralized bone matrix
Ectopic / Heterotopic	Located away from normal position / A type of tissue that is found in an unusual place (Williams, 1999). In bone engineering this usually refers to calcified tissue occurring (within a scaffold) in a soft tissue site.
Osteoconduction	Process of passively allowing bone to grow and remodel over a surface (Williams, 1999).
Osteoinduction	Act or process of stimulating bone formation (Williams, 1999).

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals **I – V**. In addition, some unpublished results are included.

I **Meretoja VV, de Ruijter AE, Peltola TO, Jansen JA and Närhi TO.** Osteoblast differentiation with titania and titania–silica-coated titanium fiber meshes. *Tissue Engineering* 11 (2005) 1489-1497.

II **Meretoja VV, Tirri T, Ääritalo V, Walboomers XF, Jansen JA and Närhi TO.** Titania and titania-silica coatings for titanium: Comparison of ectopic bone formation within cell seeded scaffolds. *Tissue Engineering* 13 (2007) 855-863

III **Meretoja VV, Helminen AO, Korventausta JJ, Haapa-aho V, Seppälä JV and Närhi TO.** Crosslinked poly(ε-caprolactone/D,L-lactide)/bioactive glass composite scaffolds for bone tissue engineering. *Journal of Biomedical Materials Research, Part A* 77A (2006) 261-268.

IV **Meretoja VV, Malin M, Seppälä JV and Närhi TO.** Osteoblast response to continuous phase macroporous scaffolds under static and dynamic culture conditions. *Journal of Biomedical Materials Research, Part A* in press

V **Meretoja VV, Tirri T, Malin M, Seppälä JV and Närhi TO.** Ectopic bone formation in and soft tissue response to biodegradable continuous phase macroporous scaffolds. Submitted manuscript

1 INTRODUCTION

Bone grafts are widely used to reconstruct bone defects, and autogenous grafts are considered as a golden standard in the procedure (Yaszemski *et al.*, 1996; Finkemeier, 2002). However, the use of autogenous bone grafts is restricted by insufficient supplies, donor site morbidity, and the fact that additional surgical procedure under general anesthesia is usually needed for harvesting the graft. This is an important aspect as most of the patients are at an advanced age, and for many elderly the use of autogenous bone grafts is impossible. Furthermore, the use of allografts is restricted by the risk of immune reactions and transmission of disease. The number of the elderly is rapidly increasing, and new strategies to reconstruct bone defects are urgently needed.

Bone engineering is a rapidly developing area of reconstructive medicine where bone inducing factors and/or cells are combined with a scaffold material to regenerate the structure and function of the original tissue (Caplan and Goldberg, 1999; Mendes *et al.*, 2002a; Cancedda *et al.*, 2007). The scaffold acts as a guiding growth substrate for the regenerating tissues, whereas the biological component enhances the healing capacity of the (human) body. This idea of tissue engineering was popularized in a pioneering paper by Langer and Vacanti (Langer and Vacanti, 1993). However, the broader definition by National Science Foundation originates from the first tissue engineering meeting held already in 1988, and it encompasses basic as well as applied research (Nerem, 1991; Viola *et al.*, 2004): "Tissue Engineering is the application of principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue function."

Some basic requirements for bone engineering scaffolds include general biocompatibility and osteoconductivity to allow tissue regeneration on the surface of the implanted material. Furthermore, a macroporous scaffold structure is needed for successful vascularization and bone ingrowth (Burg *et al.*, 2000; Hutmacher, 2000). In this thesis the cell and tissue responses to different scaffold types were evaluated in three dimensional cell cultures and in ectopic bone formation assays.

2 REVIEW OF THE LITERATURE

2.1 Structure and function of bones

Bone tissue, together with cartilage, makes up the skeletal system in vertebrates. It provides support and the sites of muscle attachment for locomotion, and protects bone marrow and vital organs. The bones also have important metabolic function in blood homeostasis (Broadus, 1996), as they form the main reservoir of calcium and phosphate in the body.

2.1.1 Structure of bones

Mature bone tissue consists of mineralized extracellular matrix with embedded cells and blood vessels (Martin and Burr, 1989a; Buckwalter *et al.*, 1995a; Currey, 2002). The composition of dried and defatted bone matrix is ~2/3 of inorganic minerals and ~1/3 of organic material, although the exact proportions vary with the bone type, skeletal location, age, and species (Aerssens *et al.*, 1998; Yeni *et al.*, 1998; Pietrzak and Woodell-May, 2005). The matrix of a living bone tissue also contains 10 to 15 % of water and cells. Some 85 to 90 % of the total bone protein consists of collagen (type I) fibers, which provide flexibility and stiffness to the bone matrix. Collagen fibers also act as a binding site for numerous noncollagenous proteins (Termine and Gehron Robey, 1996; Gorski, 1998), and help to orient the calcium phosphate mineral crystals of bone (Weiner and Traub, 1992). The bone mineral is mostly in the form of carbonated apatite, and it gives strength to the tissue (Wopenka and Pasteris, 2005).

Two types of bone can be defined by their characteristic macroscopic structure. Cortical (or compact) bone comprises about 80 % of the adult bone mass. It forms the outer surface of the bones, and has a dense structure (~10 % porosity) with space only for embedded bone cells (osteocytes) and blood vessels. Trabecular (or cancellous) bone is found in the interior part of the bones, especially in the vertebral bodies, pelvic bones, and at the ends of tubular bones. This type of bone has a sponge-like morphology (50 to 90 % porosity), and the spaces between the trabeculae are filled with bone marrow and blood vessels. Due to the large surface area and high turnover rate trabecular bone has a major metabolic function, as opposed to cortical bone, which is responsible for the mechanical strength of the bones (Jee, 2001).

2.1.2 Normal bone formation

Bones can be formed by two distinct processes called endochondral and intramembraneous ossification (Whitson, 1989; Buckwalter *et al.*, 1995b; Shea and Miller, 2005). The majority of bones (the bones of the limbs, the vertebrae, the ribs) are formed by the endochondral process, which uses cartilaginous tissue as a template for the bones. In the beginning of the process mesenchymal stem cells differentiate into chondroblasts, which proliferate and secrete hyaline cartilage matrix forming the template. The cells inside the matrix further differentiate into non-dividing hypertrophic chondrocytes, produce calcified cartilage, and undergo programmed cell death (apoptosis). Osteoclasts then start to resorb some of the matrix allowing capillaries to penetrate the tissue for the first

time. The invasion of cells to the resorption site ultimately creates a highly vascularized marrow cavity. At this stage a new set of mesenchymal stem cells (from periosteum) begin to differentiate into osteoblasts that proliferate and produce true bone matrix on the cartilage remnants.

Flat bones (parts of the skull, pelvis, scapula, and clavicles) and periosteal bone on the exterior surface of all bones are produced by intramembraneous ossification (Whitson, 1989; Buckwalter *et al.*, 1995b; Shea and Miller, 2005). In this process mesenchymal stem cells aggregate, and start to produce a matrix that also contains blood vessels. The stem cells differentiate into osteoblasts, and the matrix undergoes ossification. No cartilage template is present.

Irrespective of the ossification route the newly formed bone has unorganized structure. Small collagen fibrils in the highly mineralized matrix are randomly oriented. This so called woven bone undergoes controlled remodeling. In the activation-resorption-formation (ARF) cycle mineralized bone is first resorbed by activated osteoclasts, followed by a reversal phase and new bone matrix (osteoid) apposition and subsequent mineralization by osteoblasts. Some of the osteoblasts become osteocytes, some remain on the bone surface as lining cells, and the rest undergo apoptosis. The temporary anatomical structure corresponding to the site of an ARF cycle is called a basic multicellular unit. Remodeling is a continuous process with an approximate annual turnover rate of 3 % and 26 % in adult human cortical and trabecular bone, respectively (Jee, 1988). Remodeled bone has a well organized lamellar structure where thicker collagen fibers (and mineral crystals) have unique preferential orientation in each layer. Individual lamellae 3 to 7 μm thick can be stacked on a flat surface or can form concentric rings around blood vessels in cortical bone, creating a so called Haversian system (or secondary osteon). However, cortical bone of rats (and some other small mammals) is not constantly remodeled, and it is devoid of Haversian systems (Bentolila *et al.*, 1998). The lamellae are organized parallel to physiological loads giving rise to the anisotropic biomechanical behavior of bones (Currey, 1984; Martin and Burr, 1989b; Weiner and Wagner, 1998).

2.1.3 Fracture healing

Bone fracture initiates a three-step process of healing, i.e. inflammation, repair and remodeling (Hulth, 1989; Einhorn, 1998; Thompson *et al.*, 2002). A fracture disrupts local blood vessels resulting in the formation of hematoma. The clotting cascade releases chemoatractants, and the invading inflammatory, endothelial, and mesenchymal cells form granulation tissue. This is the shortest phase of healing. In the repair phase new mineralizing matrix is produced by differentiating mesenchymal cells. Many of these cells come from the periosteum of the fracture site, but also from distant soft tissues via blood stream. The new bone tissue is formed by a combination of endochondral and intramembraneous ossification. Intramembraneous process predominates under low mechanical strain, but cartilaginous matrix is usually produced to stabilize the fracture site. The formed tissue is called a fracture callus, and it is replaced by woven bone in a similar manner to normal endochondral ossification. The final and longest phase of fracture healing is the remodeling of woven bone to restore the lamellar structure of mature bones. The restoration of normal function in uncomplicated long bone fractures

takes ~3 months in humans (Claes *et al.*, 2002; Herrmann *et al.*, 2002) and ~1 month in rats (Einhorn, 1998; Cullinane *et al.*, 2003), but the remodeling process continues much longer (up to several years in humans).

The continuous remodeling and the ability to regenerate the damaged tissue are characteristic properties of bone (together with e.g. epithelium and liver). Most of the other tissues heal by scar formation: the damaged tissue is replaced by a poorly vascularized fibrous connective tissue. Bone regeneration can also fail, resulting in cartilage (pseudoarthrosis) or scar (nonunion) formation if severe inflammation, infection, poor oxygen supply, persistent fracture mobility, or a large defect size hampers the healing process (Jupiter and von Deck, 1998; Munk and Larsen, 2004; Keating *et al.*, 2005). Such bone defects are not spontaneously regenerated even after the primary cause of impaired healing has been eliminated.

2.2 Bone engineering

The goal of bone engineering is to repair bone defects which are difficult or even impossible to treat by conventional methods. This usually involves the use of bone graft substitutes to treat bone losses due to traumatic injury or revision surgery (Yaszemski *et al.*, 1996; Lane *et al.*, 1999). Furthermore, basic research on bone biology and tissue regeneration can also benefit from bone engineering studies. There are several approaches to bone engineering, ranging from inorganic bone fillers (in common clinical use) (Bucholz, 2002) to *in situ* bone induction by growth factors (in limited use) (Lieberman *et al.*, 2002; Westerhuis *et al.*, 2005) to laboratory cultured bone cells and gene therapy (in experimental phase) (Bruder and Fox, 1999; Hutmacher and Garcia, 2005; Kimelman *et al.*, 2007). All these methods, however, have two common requirements: a physical continuity across the damage site has to be provided to guide the bone growth, and scar formation has to be avoided.

2.2.1 Scaffolds

Osteoblasts and their progenitors are adherent cells. This means that they need a solid surface for attachment to be able to survive and function normally (Anselme, 2000). During normal bone formation and repair these cells themselves produce the extracellular matrix to which they adhere, but in bone engineering artificial scaffolds are used to provide the initial supporting surface for the expanding cell population (Salgado *et al.*, 2004).

Dense non-resorbable materials, like conventional PMMA-based bone cements or metallic orthopedic and dental implants, can successfully be used to replace bone, but they do not allow for the regeneration of the original tissue. Thus, the simplest bone engineering “scaffolds” consist of (dense or porous) granular bone graft substitutes, permitting bone growth in inter-particle spaces (Finkemeier, 2002). Furthermore, Guided Tissue Regeneration membranes are often used in oral applications to keep the granules in the defect site, and to inhibit soft tissue ingrowth (Hämmerle and Jung, 2003). For the purpose of the present work a veritable bone engineering scaffold has been defined as a

macroporous three dimensional object with structural integrity enabling manual handling. This definition does not include different hydrogels used to encapsulate osteogenic cells (Alhadlaq and Mao, 2005; Kim *et al.*, 2007a). The scaffolds usually have sponge-like or fibrous, random or oriented, structure depending on the fabrication method (Ma, 2004).

Scaffolds for bone engineering must meet a number of material and fabrication requirements. Often, a resorbable scaffold is desired whereas the material should have adequate strength, and be biocompatible, easy to sterilize, and moldable into specific shapes (Burg *et al.*, 2000; Hutmacher, 2000). The scaffold should also have interconnected macropores from 100 to 500 μm in (average) diameter to allow vascularization and good penetration of tissue (Yang *et al.*, 2001; Karande *et al.*, 2004).

2.2.1.1 Porous architecture

Porous architecture is perhaps the most important property of the bone engineering scaffolds. Bone and vasculature can grow into and through the scaffold only if the pores are interconnected and open to the surface. Early studies by Hulbert and co-workers (discussed in (Hulbert *et al.*, 1972; Whang *et al.*, 1999)) indicated that the minimum scaffold pore size required for bone regeneration (in canine femur) would be ~100 μm, whereas smaller pores favored soft tissue ingrowth. Bone tissue has later been shown to grow through pores as small as 50 μm (Lu *et al.*, 1999; Itälä *et al.*, 2001; Otsuki *et al.*, 2006), but large pores (up to 500 μm) seem to allow for more efficient tissue formation in three dimensions (reviewed in (Karageorgiou and Kaplan, 2005)). In addition to (relatively small) primary porosity, (larger) individual channels/conduits can be manufactured to enhance bone formation within and through the scaffolds (Dutta Roy *et al.*, 2003). It is interesting to note that the Haversian systems of human cortical bone have an approximate cross section of 150 – 250 μm (Jowsey, 1966), while the average trabecular thickness in cancellous bone is 100 – 250 μm (Hildebrand *et al.*, 1999). Furthermore, metabolically active cells do not survive embedded in locations further than ~100 – 200 μm from the nearest nutrient and oxygen supply (Colton, 1995).

Increasing the volumetric porosity of a scaffold has shown to increase the bone formation in some *in vivo* experiments (Lewandrowski *et al.*, 2000; Kruyt *et al.*, 2003). However, as the higher porosity often leads to the increased interconnectivity of the pores, and can change the pore size distribution and the available surface area of the scaffold, the effect of the porosity itself remains unclear. Indeed, other studies have indicated similar bone responses to scaffold porosities differing by as much as 20 vol-% (Fisher *et al.*, 2002; Kujala *et al.*, 2003). Furthermore, mechanical requirements and the production process used to prepare the scaffolds can also set limits to the maximum feasible porosity (Le Huec *et al.*, 1995; Hutmacher, 2000).

2.2.1.2 Materials

An implanted bone regenerative material should form a direct contact with surrounding tissues, without an intervening fibrous capsule (Albrektsson and Johansson, 2001). It has been suggested that this requirement can be met through the use of bioactive materials, on the surfaces of which deposition of bone-like mineral takes place, and specific

proteins are incorporated *in vivo* (Hench, 1998; Ducheyne and Qiu, 1999; Hastings, 2002). Recently, the importance of solution mediated effects on the regeneration process has also been recognized (Hench *et al.*, 2004; Jell and Stevens, 2006).

Materials applied in bone engineering scaffolds can be classified as metals, ceramics and glasses, and polymers. Each of the material groups has some characteristic advantages and disadvantages. Metals are used in load bearing applications due to their superior strength and durability, but they will stay permanently at the implantation site. Certain bioactive ceramics and glasses have shown excellent osteoconductivity and even osteoinductivity, but inferior handling properties and brittleness of these materials are the most important reasons for their limited use as porous scaffolds. More ductile and easy-to-shape, but usually less osteoconductive, scaffolds can be prepared from biocompatible polymers. Many kinds of composite scaffolds have been prepared to combine the strengths, and to alleviate the weaknesses, of the constituent materials.

Metals

Porous metals have high fracture and fatigue resistance (in comparison to e.g. porous ceramics), and this makes them interesting candidates as load bearing bone engineering scaffolds. Such scaffolds have been prepared using several different techniques, like sintering of metal powders and fibers, replication/removal of a space holder (polymer) material, chemical vapor deposition, and rapid prototyping (Ryan *et al.*, 2006). Metallic scaffolds have been applied in two different ways. In the first approach, fully metallic scaffolds are used as such to reconstruct bone (Bobyn *et al.*, 1999; Kujala *et al.*, 2003; Sikavitsas *et al.*, 2003; Otsuki *et al.*, 2006; Deglurkar *et al.*, 2007). In the second approach, metallic mesh or cage works as an outer (possibly load bearing) support for the inner non-metallic scaffold material (Papavero *et al.*, 2002; Warnke *et al.*, 2004; Nienhuijs *et al.*, 2006). In addition, traditional surgical meshes and plates can be used in combination with (autologous) bone grafting (Kuttenberger and Hardt, 2001).

The most commonly used metals in medical implants include commercially pure titanium (cpTi) and titanium alloys, cobalt-chromium alloys, and stainless steel. Of these, titanium (alloys) have the highest corrosion resistance and mechanical properties closest to bone (Van Noort, 1987; Becker and Bolton, 1997), and are therefore the most promising materials to be used as permanent bone engineering scaffolds. Porous tantalum implants have also shown great promise in bone related applications (Levine *et al.*, 2006). In contrast to this, certain magnesium alloys have been proposed as fully degradable metallic scaffold materials (Witte *et al.*, 2007), but too fast corrosion of these alloys has usually resulted in compromised *in vivo* results (Staiger *et al.*, 2006).

Titanium (alloys) and tantalum are considered to be biocompatible materials for bone contact applications, and this has been largely attributed to the corrosion resistant oxide layer forming on the implant surfaces. On the other hand, such passive oxide layer does not allow for direct (biochemical) bone bonding, but only mechanical interlocking is achieved (Albrektsson and Wennerberg, 2004). Proposed methods to increase the bioactivity of titanium metal include alkali-heat treatment (Kokubo *et al.*, 2004; Takemoto *et al.*, 2006), anodic (Liang *et al.*, 2003; Son *et al.*, 2003; Sul *et al.*,

2005) or micro-arc (Sul, 2003; Li *et al.*, 2004; Sul *et al.*, 2006) oxidation, and different sol-gel-derived titania coatings (Li and de Groot, 1993; Ramires *et al.*, 2003; Areva *et al.*, 2004). Surface-reactive ceramic coatings, such as various calcium phosphates (CaP) and bioactive glasses (BAG), have also been used to improve bone contact of titanium implants (Kitsugi *et al.*, 1996; Moritz *et al.*, 2004). Similarly, the alkali-heat treatment (Kato *et al.*, 2000) and CaP coatings (Barrère *et al.*, 2003) have also been used to improve the bone bonding ability of tantalum implants.

Ceramics and glasses

Porous ceramics and glasses are brittle materials, and usually not suitable for load bearing applications. However, such scaffolds can be highly osteoconductive and even osteoinductive (Ripamonti, 1996; Gosain *et al.*, 2002), and are therefore widely used in bone engineering. In load bearing applications, like long bone reconstructions (Petite *et al.*, 2000; Marcacci *et al.*, 2007), additional fixation is always used to support the defect site (this is also the case with polymer and composite scaffolds). At least two steps are required to manufacture ceramic and glass scaffolds. Different approaches, like rapid prototyping methods, compaction molding, and template replication can be used to prepare the so called green body, which is always subsequently sintered to obtain the final porous structure (LeGeros *et al.*, 2003; Chen *et al.*, 2006; Jones *et al.*, 2006; Vitale-Brovarone *et al.*, 2007). In all cases, sintering parameters greatly influence the final mineral phases present, mechanical and resorption properties, and corresponding biocompatibility of the scaffolds (Habibovic *et al.*, 2006).

The majority of bone matrix is composed of inorganic CaP mineral (carbonated apatite) (Wopenka and Pasteris, 2005). Inspired by nature, multiple commercial tricalcium phosphate, hydroxylapatite and biphasic CaP ceramics have been developed to be used as synthetic bone graft substitutes/porous scaffolds (Bucholz, 2002; Ylinen, 2006). Furthermore, recent animal studies have shown superior bone ingrowth and tissue attachment with silicon substituted hydroxylapatite granules in comparison with phase pure ceramic (Patel *et al.*, 2002; Porter *et al.*, 2004). It seems that certain degree of CaP dissolution is needed for optimal bone regeneration (Bodde *et al.*, 2007; Pietak *et al.*, 2007). Bioactive glasses, which form a bone-like mineral layer on their surface *in vivo*, are also in clinical use as dense granules and plates (Hench, 2006; Peltola *et al.*, 2006). However, porous BAG scaffolds have been tested only in small animals (Yuan *et al.*, 2001; Itälä *et al.*, 2003; Goodridge *et al.*, 2007).

Polymers and composites

Polymer and composite bone engineering scaffolds are usually prepared from biodegradable materials (Rezwan *et al.*, 2006). Only few examples of non-degradable porous matrices exist in the literature, and they include malleable high-density polyethylene implants (Frodel and Lee, 1998) and *in situ* porosity forming polymethylmethacrylate bone cements (Bruens *et al.*, 2003; Puska *et al.*, 2006). Furthermore, prefabricated fiber reinforced implants with porous surface have recently been studied in craniofacial applications (Tuusa *et al.*, 2007).

Biodegradable polymer scaffolds have been most often prepared from linear thermoplastic polymers, especially from saturated aliphatic polyesters like poly(glycolic acid), poly(lactic acid), polycaprolactone, and their copolymers (Hutmacher, 2000; Agrawal and Ray, 2001; Laurencin *et al.*, 2006). The mechanical properties, degradation rate, and biological response to such polymer scaffolds vary depending on the molecular weight (distribution), crystallinity, and hydrophobicity of the polymer matrix. These polymers are, in general, well tolerated implant materials which degrade by bulk hydrolysis of the ester bonds, although some enzyme activity can also be involved (Pitt *et al.*, 1981; Athanasiou *et al.*, 1996; Lim *et al.*, 2005). However, the accumulation of slowly resorbing crystalline degradation products has in some cases resulted in adverse host response (Bergsma *et al.*, 1995a; Bergsma *et al.*, 1995b). In addition to saturated polyesters, unsaturated (cross-linkable) polyesters like poly(propylene fumarate) have also been used in bone engineering scaffolds (Vehof *et al.*, 2002; Hedberg *et al.*, 2005a). Other, less often used polymers include tyrosine derived polycarbonates (Simon *et al.*, 2003) and silk fibroin (Meinel *et al.*, 2005; Meinel *et al.*, 2006).

Porous polymer scaffolds have been produced using several different techniques including gas foaming, textile technologies, rapid prototyping, and others (Mikos and Temenoff, 2000; Liu and Ma, 2004). However, solvent casting and particulate leaching is the most common method used, due to its simplicity and cost efficiency. The use of thermoplastic polymer matrices usually necessitates the application of organic solvents in the fabrication process, and generates the risk of toxic residues in the end product. Recently, low molecular weight cross-linkable fumarate (Fisher *et al.*, 2001; Jabbari *et al.*, 2005) and acrylate (Burdick *et al.*, 2001; Vogt *et al.*, 2005) functional oligomers have been used with particulate leaching technology to prepare biodegradable tissue engineering scaffolds. This allows melt processing in low temperatures, and enhances the mechanical properties of such elastic (low-crystallinity) polymers (Helminen *et al.*, 2002; Declercq *et al.*, 2006).

Porous composites of several biodegradable polymers and bioceramic fillers have shown to be bioactive in terms of their apatite forming ability in aqueous solutions (Wang *et al.*, 2001; Maquet *et al.*, 2003; Korventausta *et al.*, 2004; Li and Chang, 2004). Furthermore, enhanced cell seeding efficiencies have been reported with composite scaffolds, and the effect was recently assigned to increased protein adsorption (Woo *et al.*, 2007). CaP (Ma *et al.*, 2001; Montjovent *et al.*, 2005; Causa *et al.*, 2006; Koh *et al.*, 2006) and BAG (Blaker *et al.*, 2003; Lu *et al.*, 2005) fillers or coatings have therefore been used to improve the osteogenic cell response to polymer scaffolds *in vitro*. Such composite scaffolds have just entered the small animal *in vivo* experiments. No long term (>6 months) results are usually available, but short term results have indicated uncomplicated bone regeneration within composite scaffolds (Dean *et al.*, 2003; Hasegawa *et al.*, 2005; Chu *et al.*, 2007; Wang *et al.*, 2007). However, the true advantages of composite scaffolds remain controversial. Two recent studies have demonstrated enhanced bone regeneration using scaffolds with CaP coating or exposed filler particles (Kim *et al.*, 2007b; Lickorish *et al.*, 2007), whereas some other studies have indicated similar or even decreased bone formation within composite scaffolds in comparison to neat polymers (Ekholm *et al.*, 2005; Holmbom *et al.*, 2005; Chim *et al.*, 2006; Yang *et al.*, 2006).

2.2.2 Cells

In cell-based bone engineering strategies, osteogenic cells are incorporated into the implanted scaffolds to supply adequate cells and to produce growth factors needed for tissue regeneration. The most common source of such cells is bone marrow (Krebsbach *et al.*, 1999; Derubeis and Cancedda, 2004). Other potential but less frequently used cell sources include adult stem cells from adipose tissue (Cowan *et al.*, 2004; Conejero *et al.*, 2006; Hattori *et al.*, 2006) and embryonic stem cells (Bielby *et al.*, 2004a; Chaudhry *et al.*, 2004). The adherent stem/progenitor cells (from bone marrow or other sources) can be expanded and induced to osteogenic lineage *in vitro* to obtain suitable cell population for further *in vivo* applications (Pountos and Giannoudis, 2005; Niemeyer *et al.*, 2007).

2.2.2.1 Bone marrow stromal cells

Single-cell preparations of bone marrow form fibroblastic colonies when cultured *in vitro* in the presence of serum. The non-adherent hematopoietic cells are removed during medium exchange, and the remaining cells represent the stromal component of marrow (Westen and Bainton, 1979; Bianco *et al.*, 1993). However, the precise *in vivo* counterpart of the culture expanded cells remains to be elucidated. The term fibroblastic colony-forming unit (CFU-F) is used to indicate the original cell *in vivo* giving rise to an individual clonal colony *in vitro*. The frequency of CFU-F in bone marrow is ~1-5 per 10^5 mononuclear cells (Bruder *et al.*, 1997; Aubin, 1999; Muschler *et al.*, 2001). A series of (animal) studies by Alexander Friedenstein and colleagues in the 1970s, extended by Maureen Owen and colleagues in the 1980s, revealed that CFU-Fs contain at least two types of osteogenic cells (reviewed in (Owen, 1985; Owen and Friedenstein, 1988)). Determined osteogenic precursor cells produced only bone tissue after ectopic *in vivo* transplantation, whereas more primitive inducible osteoprogenitors gave rise to bone, cartilage and adipocytes, and were able to support host-derived hematopoietic cells. However, majority of the transplants produced fibrous tissue or no tissue at all. Determined osteoprogenitors were found only in bone marrow, whereas inducible osteoprogenitors were present also in thymus and spleen. On the other hand, cultured human CFU-Fs (from marrow) have repeatedly failed to form ectopic bone without inductive factors (Haynesworth *et al.*, 1992).

The first demonstration of bone-like tissue formation *in vitro* using CFU-F derived cells was given by Maniatopoulos et al (Maniatopoulos *et al.*, 1988). They added ascorbic acid, Na-β-glycerophosphate and dexamethasone to the serum supplemented culture medium to induce the osteogenic differentiation. This protocol has since become the golden standard for osteogenic cell cultures. Ascorbic acid (vitamin C) is a cofactor of several hydroxylases, and is essential for hydroxylation of proline (and lysine) residues and subsequent assembly of triple-helical collagen structure (Anttinen *et al.*, 1981; Peterkofsky, 1991). Normal collagen synthesis in turn induces differentiation of pre-osteoblasts (Takamizawa *et al.*, 2004; Carinci *et al.*, 2005). Glycerophosphate acts as an organic phosphate source in extracellular matrix mineralization (Tenenbaum and Heersche, 1982; Bellows *et al.*, 1992; Chung *et al.*, 1992), and dexamethasone is a synthetic glucocorticoid inducing osteogenic differentiation of stromal cells (Ohgushi *et al.*, 1996; Aubin, 1999; ter Brugge and Jansen, 2002). Recent proposals to increase the

yield of osteogenic cells from cultured CFU-Fs include the replating of non-adherent cell fractions (Wan *et al.*, 2006), careful selection of the serum lot used, and addition of specific growth factors, namely FGF-2, to the culture medium (Martin *et al.*, 1997; Kotev-Emeth *et al.*, 2002; Sotiropoulou *et al.*, 2006).

It has been demonstrated that cultured CFU-Fs from adult human marrow (Pittenger *et al.*, 1999) or adipose tissue (Zuk *et al.*, 2001) can be induced to multiple mesenchymal lineages, like bone, cartilage, fat, and muscle by simply altering the composition of the culture medium. Such findings have given rise to ambiguous nomenclature, like multipotent adult progenitor cells, mesenchymal stem cells, skeletal stem cells and connective tissue progenitors. These terms are used to describe various more or less purified populations of undifferentiated CFU-Fs (Adassi and Verfaillie, 2004; Bianco and Gehron Robey, 2004; Caplan, 2004; Muschler *et al.*, 2004). In contrast, the term bone marrow stromal cell (BMSC) better describes cultured heterogenous adherent populations whose multipotentiality and self-renewal capacity have not been explicitly demonstrated (Krebsbach *et al.*, 1999).

2.2.2.2 Cell culture models

Osteoblasts and their progenitors are adherent cells, and do not grow in suspension. The most commonly used culture substrate material for the expansion of such cells is surface modified polystyrene. Common polystyrene is non-cytotoxic polymer, but its hydrophobicity can limit cell adherence (van Kooten *et al.*, 2004). Standard polystyrene cell culture vessels have therefore been surface modified to make them more hydrophilic. The exact modification method, and the resulting surface structure and chemistry, may vary by brand. When the expanded cells are seeded into biomaterial scaffolds, non-adherent culture vessels can be used to minimize cell growth outside the scaffolds.

Bone engineering studies have traditionally been performed in static conditions, i.e. the scaffolds have been placed in individual culture wells. In such conditions the nutrient and oxygen supply, as well as metabolic waste product removal, are limited by diffusional constraints, and cell growth is therefore limited to the exterior of the scaffolds (Botchwey *et al.*, 2003; Bilodeau and Mantovani, 2006). As osteogenic cells start to produce mineralizing extracellular matrix the open porosity of the scaffold decreases, and diffusion is further limited. In long term static cultures osteoblasts can penetrate only few hundred microns into the scaffolds (Ishaug *et al.*, 1997; Ishaug-Riley *et al.*, 1998), and the same applies also in spinner flasks (Shea *et al.*, 2000). On the other hand, convection produces shear stress on the cultured cells, which can increase their osteogenicity (Hillsley and Frangos, 1994). Spinner flask cultures can therefore stimulate osteoblast growth and differentiation (in the periphery of the scaffolds) (Sikavitsas *et al.*, 2002; Mygind *et al.*, 2007). Osteoblast cultures in rotating wall bioreactors have been demonstrated to improve cell penetration into the scaffolds, but the resulting osteogenic response has been controversial. Both increased (Qiu *et al.*, 1999; Marolt *et al.*, 2006) and decreased (Sikavitsas *et al.*, 2002) differentiation in comparison to static cultures have been reported. The most promising bone engineering results have been obtained using perfusion bioreactors where mechanical stresses can be strictly controlled (Goldstein *et*

al., 2001; Bancroft *et al.*, 2002; Cartmell *et al.*, 2003). Such cultures can simultaneously enhance both the scaffold colonization and osteogenic differentiation.

2.2.2.3 Ectopic bone formation

Ectopic implantation into small animals is a widely used method for the assessment of biological response to different bone engineering scaffolds and cell-scaffold constructs. Typical targets of observation include the inflammatory and foreign-body reactions to, tissue ingrowth and vascularization of, as well as bone formation (osteoinductivity) within the scaffolds (Hartman *et al.*, 2004; Kruyt *et al.*, 2007). The most common implantation sites include dorsal subcutaneous space and different intramuscular locations (Goshima *et al.*, 1991; Ripamonti, 1996; van Gaalen *et al.*, 2004; Yuan *et al.*, 2006).

Ectopic implantations are commonly performed to screen the potential bone engineering methods and scaffold candidates for further (more laborious and time-consuming) bone regeneration studies (Lin *et al.*, 2007). Such experiments have been used to analyze the importance of e.g. porous design (Kuboki *et al.*, 1998; Nishikawa *et al.*, 2004), and bulk (Ohgushi *et al.*, 1990; Arinzeh *et al.*, 2005) or surface (Takaoka *et al.*, 1996; Vehof *et al.*, 2003) chemistry of the scaffolds on the subsequent bone formation. Effects of the cell seeding method (Wilson *et al.*, 2002; Wang *et al.*, 2006), *in vitro* culture time (van den Dolder *et al.*, 2002; Kruyt *et al.*, 2004a; Zhou *et al.*, 2007), and initial degree of cell differentiation (Yoshikawa *et al.*, 1998; Mendes *et al.*, 2002b; Holtorf *et al.*, 2005) have also been evaluated. However, there are only few studies which have directly compared the results of ectopic and orthotopic implantation in a single experimental set-up (Kruyt *et al.*, 2004b; Kruyt *et al.*, 2007). Those studies have shown that implanted cells can have a greater role in ectopic bone formation than in successful regeneration of large (critical sized) bone defects.

Ectopic bone formation in neat or growth factor loaded scaffolds mostly follows the endochondral route (Kuboki *et al.*, 2001), whereas intramembraneous bone formation predominates in scaffolds seeded with osteogenic cells (Yoshikawa *et al.*, 1992). However, cartilage formation can be favored by hypoxic conditions inside thick cell-scaffold constructs (Claase *et al.*, 2007; Zhou *et al.*, 2007). In slowly resorbing osteoconductive scaffolds ectopic bone formation can persist for months (Yoshikawa *et al.*, 2000; Dong *et al.*, 2002; Hasegawa *et al.*, 2007), whereas very fast resorption of a scaffold can be accompanied by loss of previously formed ectopic bone (Kim *et al.*, 2005a).

3 AIMS OF THE STUDY

The aim of the present study was to compare the suitability of different macroporous scaffold types for bone engineering applications. The two scaffold categories studied were a) the mechanically strong and stable titanium meshes (**I**, **II**) and b) the elastic and biodegradable porous polymers (**III** – **V**). It was hypothesized that bioactive modifications (sol-gel coating and BAG filler) applied to these basic scaffold types would enhance their osteogenic cell and tissue responses.

The specific aims were:

1. To compare the osteogenic activities of cultured BMSCs in titania or titania-silica coated and uncoated titanium meshes. (**I**)

2. To compare ectopic bone formation in titania or titania-silica coated and uncoated implants loaded with BMSCs. (**II**)

3. To develop resorbable elastic scaffolds with controlled porosities suitable for bone tissue engineering applications. (**III**, **IV**)

4. To compare BMSC response to polymer scaffolds and composites with BAG under static and dynamic culture conditions. (**III**, **IV**)

5. To compare the *in vivo* inflammatory tissue response and ectopic bone formation in neat and BMSC seeded, respectively, polymer and composite scaffolds with continuous phase macroporosity. (**V**)

4 MATERIALS AND METHODS

A summary of materials and methods employed in the study is presented below. More detailed information can be found in **I-V**.

4.1 Scaffold preparation

4.1.1 Titanium meshes (I, II)

A sintered titanium fiber mesh (Bekaert N.V., Zwevegem, Belgium) was used with a volumetric porosity of 86 %, a density of 600 g/m^2, a fiber diameter of 40 µm, and an average distance between fibers of 250 µm. Of this mesh, disc-shaped specimens 6 mm in diameter and 0.8 mm thick were cut, and used as such (cpTi), or coated with titania (TiO_2), or a 30:70 mol-% mixture of titania and silica (TiSi), using the sol–gel technique essentially as described by Jokinen et al. (Jokinen *et al.*, 1998).

Briefly, the titanium fiber mesh substrates were cleaned ultrasonically in acetone and ethanol (5 minutes + 5 minutes), and dried in air before each coating cycle. The two different coatings were achieved by dipping the substrates into respective sols, and then withdrawing them at a speed of 0.3 mm/second. The substrates were then heated at 500°C for 10 minutes. The dipping and heating cycle was repeated five times. Uncoated and coated scaffolds were finally cleaned ultrasonically, and sterilized using gamma radiation (25 kGy minimum).

4.1.2 Polymer and composite scaffolds (III – V)

Branched poly(ε-caprolactone/D,L-lactide) macromers [P(CL/DLLA)] with controlled monomer ratios and four methacrylate end groups were synthesized as described by Helminen et al. (Helminen *et al.*, 2002). The macromers were blended with 2 wt-% of cross-linking initiator. For composites, 20-50 wt-% of bioactive glass S53P4 (Vivoxid, Finland) were added to the mixture. The composition of BAG was 53 wt-% SiO_2, 23 wt-% Na_2O, 20 wt-% CaO and 4 wt-% P_2O_5, and the granule size was either <45 µm or 90-315 µm. The constituents were mixed in a Brabender W50EH batch mixer (Brabender, Germany) using 50 rpm at 50-90°C for five minutes. In order to obtain the desired scaffold porosities, 60-90 wt-% of the porogen agent was either added to the batch mixer (NaCl) or manually mixed at room temperature ($CaCl_2 \bullet 6H_2O$). The final mixture was then applied to a mold (6 mm in diameter and 12 mm in height) or cast to a plate (2 mm thick). Either thermal or light curing was used to cross-link the copolymer matrices. The molded specimens in **III** were used for material characterizations. They were made porous by leaching the salt particles with distilled water for one week followed by ethanol rinsing and vacuum drying. For all the other studies, scaffolds were punched out from the cured plate, and soaked multiple times in either water or ethanol. The leaching was done within 2.5 hours in water or within 24 hours in ethanol to avoid the dissolution of the bioactive filler. These scaffolds were dried and sterilized with gamma radiation (25 kGy minimum) before use. A summary of different scaffold types used in **III-V** is given in **Table 1**.

Table 1. Different scaffold types used in **III-V.**

	CL/DLLA ratio	Curing	Bioactive filler	Porogen agent	Scaffold size	Porosity
III	90/10, 30/70	Thermal	20-50 wt-%, 90-315 µm	NaCl, 420–590 µm	Ø 6 mm x 12 mm, Ø 10 mm x 2 mm	43-83 V-%
IV	70/30	Light	30 wt-%, <45 µm	$CaCl_2 \cdot 6H_2O$, 400–500 µm	Ø 6 mm x 2 mm	70 V-%
V	90/10, 70/30	Light	30 wt-%, <45 µm, 90-315 µm	$CaCl_2 \cdot 6H_2O$, 400–500 µm	Ø 6 mm x 2 mm	70-80 V-%

Table 2. Summary of different cell culture modes in **I-V.**

	Rat strain	Primary culture	Seeding method	Seeding density	Secondary culture
I, III	Sprague-Dawley	Osteogenic	Suspension	1.0 x 10^6 cell/ml	Osteogenic
II	Fisher 344	Osteogenic	Suspension	1.5 x 10^6 cell/ml	Implantation
IV	Sprague-Dawley	Non-differentiating	Droplet	3.0 x 10^6 cell/ml	Osteogenic
V	Fisher 344	Osteogenic	Droplet	3.8 x 10^6 cell/ml	Implantation

4.2 Micro-computed tomography (IV)

Porous architecture of the composite scaffolds in **IV** was characterized using micro-computed tomography (μCT 40, Scanco Medical AG, Switzerland) with a resolution of 12 μm (isotropic). System settings were: energy 70 kV, intensity 114 μA, integration time 300 ms, image matrix 1024 x 1024. Quantitative morphometry was performed to assess the pore wall thicknesses and pore sizes as well as the volumetric porosity of the scaffolds. The imaging service was kindly provided by Scanco Medical.

4.3 Scaffold hydrolysis (III)

Porous specimens (five replicates) for hydrolysis study were immersed in 10 mL of phosphate buffered saline (PBS) pH 7.0 in test tubes at 37°C. The buffer solution was changed once a month. The specimens were retrieved after four, eight and twelve weeks of immersion and weighed, and their compressive properties in wet state were tested. Compressive modulus and stress-strain behaviour of polymer matrices were measured using an Instron 4204 universal testing machine (Instron, UK) applying the standard ISO 604-1973(E). The crosshead speed was 3 mm/min, and the elasticity of specimens was evaluated with consecutive runs to 25 % strain. After testing, the specimens were vacuum dried and their mass loss and water absorption were determined.

4.4 Immersion in simulated body fluid (III)

Simulated body fluid (SBF) was prepared by dissolving reagent grade NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, and Na_2SO_4 into deionised water. The solution was buffered to physiological pH 7.40 at 37°C with tris(hydroxymethyl)aminom ethane and hydrochloric acid. The ion composition of the SBF corresponds to inorganic portion of human blood plasma (Kokubo *et al.*, 1990).

Three replicate discoid scaffolds were soaked in SBF to analyze glass dissolution and *in vitro* CaP formation on their surfaces. Each scaffold with 50 mL SBF was closed in test tube and incubated in a shaking water bath at 37°C. The follow-up time was 14 days.

4.5 Cell cultures (I – V)

An outbred Sprague-Dawley rat strain (Hsd:SD, University of Turku, Finland) was used in cell culture studies, whereas implantation studies were performed with inbred Fisher 344 rats (F344/NHsd, Harlan, the Netherlands) to avoid possible rejection of the transplanted cells. Different culture schemes in **I-V** are outlined in **Table 2**.

Rat BMSCs were harvested and cultured as described by Maniatopoulos et al.(Maniatopoulos *et al.*, 1988). Briefly, femora were isolated from five to seven week old male rats. The bones were wiped with 70 % ethanol and immersed twice in serum-free cell culture medium. The condyles were cut off, and bone marrow was flushed out using complete, non-differentiating or osteogenic, culture medium. The resulting suspension

was passed through a 20 G needle. Adherent cell population was then cultured in a humidified 5 % CO_2 atmosphere at 37°C. After seven days of primary culture, the cells were trypsinized and a cell stock was prepared in osteogenic medium. The medium compositions were as follows. Non-differentiating: DMEM culture medium, containing 10 % fetal bovine serum and 100 units/mL of penicillin/streptomycin (Gibco BRL, Life Technologies BV, The Netherlands). Osteogenic: α-MEM culture medium (Sigma Chemical Co., USA) with 15 % fetal bovine serum, and supplemented with 7.0-8.5 mM Na-β-glycerophosphate (Merck, Germany), 50 μg/mL ascorbic acid and 10 nM dexamethasone (Sigma).

The cell culture scaffolds in **II** and **III**, but not in the other studies, were incubated in the presence of serum proteins before cell seeding. The scaffolds (pooled in polypropylene tubes) were immersed in 0.1-0.5 ml of cell suspension, or an individual 50 μl droplet of cell stock was added on top of each scaffold (in culture plates). Cells were allowed to adhere at 37°C for one to three hours. The cultures were subsequently continued in 24-well plates for up to 21 days, with medium replacement (one to two milliliters) every two to three days. The scaffolds for implantation in **II** and **V** were placed into serum-free medium on the second day of culture, and translocated to the animal facility. The scaffolds were kept at 37°C during surgery, and washed with PBS before implantation. The dynamic cultures in **IV** were also initiated on the second day by transferring half of the scaffolds to the rotating wall bioreactors (RCCS-4D rotator base with disposable 50 ml high aspect ratio vessels; Synthecon, USA). The bioreactors were rotated 10 rpm, and they contained four milliliters of medium per scaffold. Half of this medium was replaced every two to three days.

4.6 Implantation procedure (II, V)

For the implantations of cultured cell-scaffold constructs, 18 three-week-old male (25 to 40 g) and 18 nine-week-old female (135 to 155 g) syngeneic Fisher 344 rats (F344/NHsd, Harlan, the Netherlands) were used in **II** and **V**, respectively. Furthermore, three twelve-week-old male (350 to 370 g) outbred Sprague-Dawley rats (Hsd:SD, University of Turku, Finland) were used for the cell free implantations in **V**. The experiments were accepted by the Lab-Animal Care & Use Committee at the University of Turku, Finland (licenses #953/99, #1420/04 and #1624/06). The experiments were conducted in GLP compliant laboratory animal facilities, and national and European regulations for animal experimentation were followed. Operations were performed under general anesthesia induced by subcutaneous injection of fentanyl citrate/fluanisone (Hypnorm®, Janssen Pharmaceutica, Belgium) and midatzolam (Dormicum®, Roche, Switzerland). The operation area was shaved and disinfected with chlorhexidine gluconate solution (Klorhexol®, Leiras Ltd, Finland).

Subcutaneous implantations were performed through longitudinal incisions in the midline of dorsal skin. Individual implant beds were prepared by blunt dissection. Half of the animals in **V** received only one type of cultured implants, whereas all other animals received one implant of each type. The placement of implants (3 – 6 per animal) was randomized using Latin Square scheme. Skin wounds were closed with individual

resorbable sutures (Ethicon® Vicryl 3-0 or Monocryl 5-0, Johnson & Johnson Intl, Belgium). The animals were killed after one, four and twelve weeks respectively, using CO_2 suffocation. Implants were retrieved with 2 to 4 mm soft tissue margin, and fixed in 4 % buffered formalin at 8°C for two weeks.

4.7 Ion concentration analysis (I, III, IV)

In **I**, precipitated calcium within the cultured scaffolds was dissolved in acetic acid. In **III** and **IV**, silica and calcium concentrations in used cell culture medium were analyzed before each medium change. From SBF incubations (**III**) also phosphate concentrations were measured, and the solution was not changed between assayed time points. Three parallel measurements were carried out from each liquid sample.

Colorimetric measurements of silica and orthophosphate concentrations were based on molybdenum blue method. Silicomolybdate complex was reduced with a mixture of 1-amino-2-naphthol-4-sulphonic acid and sulphite, and tartaric acid was used to eliminate interference from phosphate (Fanning and Pilson, 1973). The antimonyphosphomolybdate complex was reduced with ascorbic acid (Murphy and Riley, 1962). Calcium concentrations were determined using ortho-cresolphtalein complexone (OCPC) method (Lorentz, 1982). The assay reagent consisted of OCPC with 8-hydroxyquinol in an ethanolamine/boric acid buffer. Absorbances (820 nm for silica, 700 nm for phosphate and 560 nm for calcium) were measured using either UV-1601 spectrophotometer (Shimadzu, Australia) or Multiskan® MS ELISA plate reader (Labsystems, Finland).

4.8 Cell activity (III, IV)

The amounts of cultured cells were determined using alamarBlue™ assay (BioSource International, USA) in colorimetric format. At predetermined times, four or eight replicate scaffolds were washed with culture medium and transferred to either clean 24-wells or microcentrifuge tubes. Fresh assay solution (DMEM culture medium with 10 % serum and including 10 % alamarBlue™ reagent) was added to the reaction vessels. After three hours of incubation in static (**III**) or dynamic (**IV**) conditions, absorbance readings of the solution were taken at 560 nm and 595 nm using the ELISA plate reader. Measured absorbances were used to determine the reduction of alamarBlue™ reagent in accordance with the manufacturer's instructions. Reductive cell activity of cultured osteoblasts has been shown to correlate with their numbers (Jonsson *et al.*, 1997).

4.9 Amounts of DNA, protein and ALP (I, IV)

At predetermined times, four replicate scaffolds were washed in PBS and placed to microcentrifuge tubes containing 1 mL of either sterile water (**I**) or lysis buffer (**IV**) (25 mM HEPES, 0.1 % Triton X-100, 0.9 % NaCl, pH 7.6). The cells were lysed with freezing-thawing method, followed by ultrasonication. The released amount of either DNA (**I**) or protein **(IV**) and alkaline phosphatase (ALP) activity were measured from supernatant diluted as needed.

Amounts of DNA were measured in 100 µL of supernatant transferred to microtiter plates. Equal amounts of PicoGreen® dsDNA quantitation reagent (Molecular Probes Europe BV, The Netherlands) were added to each well, followed by ten to fifteen minutes of incubation at room temperature, with protection from light. Fluorescence from three replicate wells was measured using a Victor™ 1420 multilabel counter (Wallac, PerkinElmer Life Sciences, Finland), at excitation and emission wavelengths of 490 nm and 535 nm, respectively. Amounts of DNA were read from a lambda phage dsDNA standard curve.

Amounts of protein were measured in 150 µL of supernatant transferred to microtiter plates. Equal amounts of Micro BCA™ working reagent (Pierce, USA) were added to each well, followed by three hours of incubation at 37°C. Mean readings of absorbance from three replicate wells were recorded at 560 nm using the ELISA plate reader. Amounts of protein were read from a bovine serum albumin standard curve.

To measure ALP activity, 50 µL of supernatant were transferred to a microtiter plate, and 200 µL of para-nitrophenylphosphate substrate solution (P7998; Sigma) were added. The plate was incubated at 37°C for one hour, and 50 µL of a 3 M NaOH solution were added to each well to stop the enzymatic reaction. Mean readings of absorbance from three replicate wells were recorded at 405 nm using the ELISA plate reader. Amounts of converted substrate were read from a para-nitrophenol standard curve. ALP activities measured were normalized in relation to amounts of DNA or protein determined.

4.10 Osteocalcin production (I)

Accumulation of osteocalcin (OC) within the cultured scaffolds was determined using rat osteocalcin EIA kit (Biomedical Technologies Inc., USA). At predetermined times, four replicate scaffolds were washed in PBS, and transferred to microcentrifuge tubes containing 1 mL of assay buffer. The cells were lysed with freezing-thawing method, followed by ultrasonication. The amount of OC released into the supernatant was measured in accordance with the manufacturer's instructions.

4.11 RT-PCR (III, IV)

At predetermined times, total cellular RNA was extracted from the culture scaffolds using Trizol® reagent (Gibco). Two to four replicate RNA pools for each scaffold type were formed, and reverse transcribed with either $d(T)_{16}$ or random hexamer primers using GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems, USA). The resultant first-strand cDNA was divided into individual PCR reactions to amplify bone sialoprotein (BSP), osteocalcin (OC) matrix extracellular phosphoglycoprotein (MEPE) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a house keeping gene) transcripts.

Two different methods were used to quantitate the gene expression levels. In **III**, 35 cycles of PCR with self-designed primers were carried out using a GeneAmp® PCR system 9700 (Applied Biosystems). The amplification products were electrophoresed

in agarose gel, and visualized by ethidium bromide staining. Expression levels of BSP, OC and GAPDH were obtained by fluorometric scanning (FluorImager 595, Molecular Dynamics, Germany) and image analysis (ImageMaster 1D Elite v4.0, Amersham Pharmacia Biotech) of the gel. The final results were normalized to GAPDH expression in each RNA pool.

In **IV**, a more accurate real-time PCR method was used. The first-strand cDNA was analyzed in duplicate PCR reactions using iQ™ Supermix kit (Bio-Rad Laboratories, USA) and FAM-labeled TaqMan® Gene Expression Assays (Applied Biosystems). Amplifications were carried out using an iCycler iQ real-time PCR detection system with software version 3.1 (Bio-Rad Laboratories). The threshold cycles (C_T) were automatically calculated using "the maximum curvature approach", and gene expression levels of BSP, OC and MEPE were normalized to GAPDH expression in each RNA pool ($\Delta C_T = C_{T,\ target} - C_{T,\ GAPDH}$). A difference of one unit in ΔCT values corresponds to a two fold difference in gene expression level.

4.12 Scanning electron microscopy (I, III, IV)

Specimen surfaces and cut cross-sections of cultured and non-cultured scaffolds were examined using JSM-5500 or JSM-6335F scanning electron microscope (JEOL Ltd., Japan). Porosity of the scaffolds, cell growth, extra cellular matrix formation, and CaP precipitation were evaluated. Cultured scaffolds were washed with PBS, and fixed with 2 % glutardialdehyde in 100 mM cacodylic acid buffer pH 7.4. Fixed specimens were rinsed in buffer, and dehydrated in increasing alcohol series at ambient temperature. Non-cultured scaffolds were rinsed in water and dried in an oven. The specimens were coated with a thin layer of gold or carbon before examination. The presence of calcium and phosphorus within mineral deposits was verified using EDS analysis (PGT Prism 2000-Si(Li) EDS detector; Princeton Gamma-Tech, USA).

4.13 Histology and histomorphometry (II, IV, V)

Fixed specimens were dehydrated in an increasing ethanol series. In **II**, specimens were embedded in methylmethacrylate for thermal curing, and three 10 μm thick sections were prepared per implant, using a modified sawing microtome technique (van der Lubbe *et al.*, 1988). Sections were double stained with methylene blue and basic fuchsine. In **IV** and **V**, specimens were embedded in a light curing resin (Technovit 7200 VLC, Kulzer, Germany), and two or three 10-20 μm thick sections were prepared per specimen via cut-and-grind method. Sections were double stained with either hematoxylin and eosin or methylene blue and basic fuchsine. Furthermore, paraffin sections were prepared for the cell free implantations in **V**, and stained with either hematoxylin and eosin for routine histology or immunostained for ED1 (macrophages).

In cell cultures (**IV)**, histology was used to evaluate the porous architecture and osteoblast colonization of the scaffolds. Histological sections from implantation studies (**II** and **V**) were qualitatively evaluated for fibrous tissue capsule formation, inflammatory and

foreign body reaction, tissue ingrowth and vascularization, and the amount and quality of ectopic bone formation. Quantitative computer-based histomorphometrical analysis (Leica QWin Standard v3.0 software) was performed in **II** to determine cross-sectional area of the scaffold material and the newly formed bone in the implants. The distribution of bone within scaffolds was analyzed by dividing the implant area into the outermost 200 µm boundary layer and into the interior part. Average of the measurements from three sections per implant was used for the statistical analysis.

4.14 Statistics (I - V)

Statistical analyses were performed using the SPSS software package (SPSS Inc., USA). Data in **I – IV** were analyzed with one-way ANOVA followed by Tukey's post-hoc test. One-sample T-Test was additionally used in **IV** to analyze possible deviations from the initial state (n = 1). In **V**, histological results (bone vs. no bone) were analyzed with two-tailed Fisher´s exact test. Differences were considered significant at 95 % confidence level.

5 RESULTS

5.1 Primary cell cultures (unpublished)

Rat BMSCs for different cell culture studies were harvested from femora of approximately six week old male rats, and adherent cells were typically cultured in osteogenic medium for seven days. A total of 18 such primary cultures, originating from 50 rats, were undertaken during this study. An average yield in those cultures was $2.8 \pm 1.1 \times 10^6$ cells per animal (mean ± standard deviation). Four non-differentiating cultures were also performed, with similar yield (11 rats, $2.1 \pm 0.9 \times 10^6$ cells per animal).

Cells in osteogenic cultures became cuboidal in appearance, and started to form nodule like structures. In some instances, especially in case of prolonged culture time, these nodules started to mineralize, and the cells became hard to detach by trypsinization. Cells in non-differentiating medium had more spindle shaped form, and did not form clear nodules. In both cases the cultured cell populations remained responsive to further osteogenic stimuli, at least in the first passage (**Figure 1**), but osteogenic preculture resulted in more mature osteogenic phenotype.

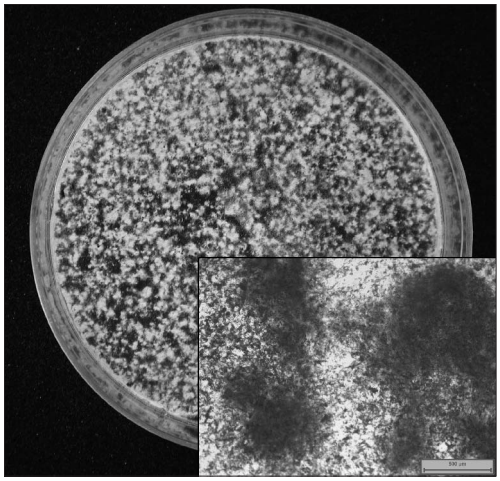

Figure 1. Mineralized nodules on common cell culture plastic (osteogenic culture, passage 1, day 18). The diameter of the culture vessel is 55 mm; the scale bar in the higher magnification image is 500 μm.

5.2 Titanium fiber meshes (I, II)

5.2.1 Preparation of sol-gel coated titanium scaffolds (I, II)

As received titanium mesh had a reported volumetric porosity of 86 % and an average distance between fibers of 250 μm. However, the mesh was compressed in the periphery of the scaffolds due to the cutting process. The smallest pores in coated implants were further occluded by sol-gel aggregates. The amount of aggregates increased in each dipping and heating cycle, and histomorphometry indicated ~10 % reduction in scaffold porosity (from ~65 % to ~55 %) using five consecutive coating cycles. Both coating types showed highly cracked surface morphology in scanning electron microscopy (**Figure 2**). The sol-gel coatings were essentially stable, and showed no evident signs of degradation

in either *in vitro* or *in vivo* conditions. However, TiSi scaffolds exhibited small initial decrease in pH and <10 µg/ml release of soluble silica into culture medium.

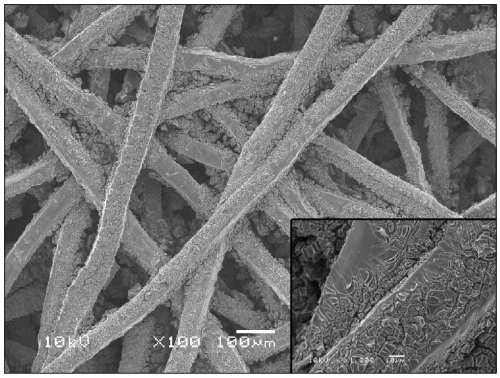

Figure 2. Titanium mesh after five TiO_2 coating cycles. Some of the porosity has been occluded by sol-gel aggregates (larger image with 100 µm scale bar), and the coating is highly cracked (smaller image with 10 µm scale bar).

5.2.2 Osteogenic differentiation cascade (I)

Expansion of seeded cells and specific ALP activities (normalized to amounts of DNA) within different titanium scaffolds were followed for one week. Maximal DNA release and ALP activity with TiO_2 scaffolds were observed after seven days of culture, whereas proliferation and ALP induction with cpTi and TiSi scaffolds had already ceased after three days. The maximal levels of DNA release and ALP activity, however, were similar with all scaffold types. At later times, TiO_2 scaffolds showed increased mineralization and OC secretion in comparison to neat titanium scaffolds, whereas these markers were decreased with TiSi scaffolds. The effect of different sol-gel coatings on the osteogenic differentiation cascade is summarized in **Table 3**.

Table 3. Effect of sol-gel coating on the osteogenic differentiation cascade.

	Cellular DNA				
	Initial	**Peak**	**ALP activity**	**OC**	**Mineralization**
TiO_2	–	Delay	Delay	+	+
TiSi	–	±	±	–	–

+, ± and – denote increase, similarity and decrease, respectively, in comparison to uncoated titanium scaffolds.

5.2.3 General host response (II)

After one week of implantation a few mononuclear leucocytes were observed in and around the scaffolds. All scaffolds were surrounded by loosely aligned connective tissue that formed a 4-10 cell layers thick capsule. Furthermore, the scaffolds were already filled with well vascularized loose fibrous connective tissue. After four weeks, fibrous tissue in and around the scaffolds was more organized and contained more collagen fibers when compared to one week implants. The number of mononuclear inflammatory cells was comparable to that observed before. The appearance of the fibrous capsule and the number of inflammatory and multinuclear cells did not change from four to twelve weeks.

5.2.4 Ectopic bone formation (II)

After one week multiple patches of unorganized mineralizing tissue with scarce amounts of woven bone were seen in all implants. Most of the mineralization was found on the periphery of the scaffolds. The amount of bone like tissue clearly increased from one to four weeks with all scaffold types, and the majority of bone was located in the scaffold interior. Large areas of mineralized matrix with embedded osteocytes were observed (**Figure 3**). The bone apposition occurred in direct contact with sol-gel coated meshes while a thin layer of unmineralized fibrous tissue was often observed surrounding the cpTi mesh fibers. After twelve weeks fibrous tissue inside the scaffolds was mostly replaced by adipose tissue that typically formed discrete areas within mineralized tissue resembling bone marrow. The structure of bone was further matured containing larger areas of remodeled lamellar bone. With all scaffold types direct bone–titanium contact was observed when titanium mesh fibers were embedded within bone. More even distribution of bone like tissue was observed within sol-gel coated scaffolds compared to the uncoated scaffolds, and three (out of five) TiO_2 and all TiSi scaffolds showed some bone formation also on their outer surface. The amount of bone within the whole implant area, as well as within the open porosity (**Table 4**), did not show substantial differences between scaffold types at any time. However, TiSi scaffolds were the only ones showing further increase in bone area by twelve weeks.

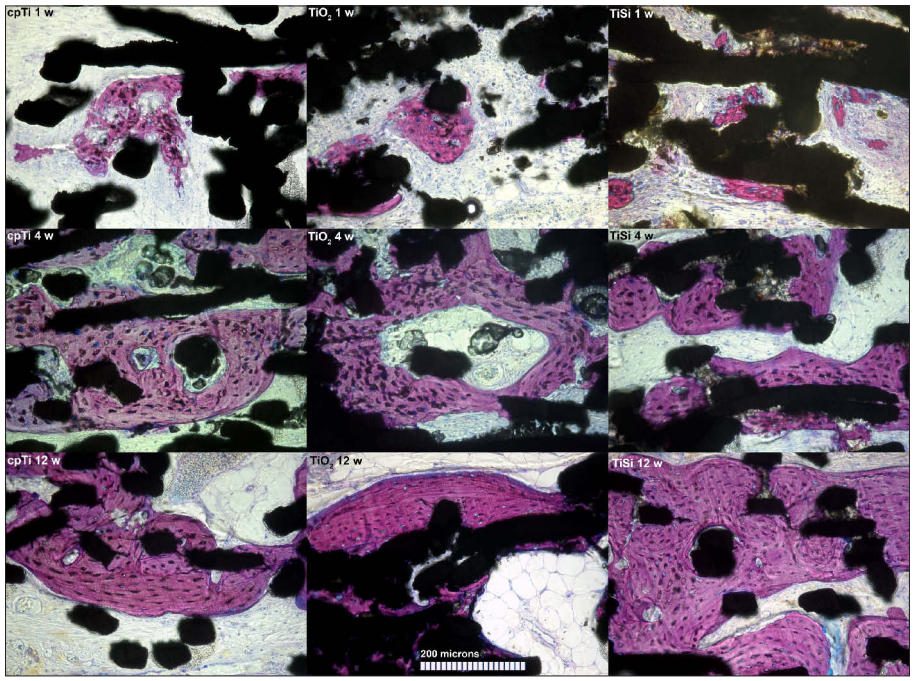

Figure 3. Typical patterns of bone formation within subcutaneously implanted cpTi (left column), TiO_2 (center column) and TiSi (right column) scaffolds. After one week multiple patches of unorganized mineralizing tissue with scarce amounts of woven bone were seen in all implants. The amount of bone-like tissue clearly increased from one to four weeks. After 12 weeks the structure of bone, with bone-marrow-like tissue, was further matured and mesh fibers were embedded in lamellar bone. Scale bar 200 µm.

Table 4. Cross-sectional area of bone within open porosity during 12 weeks of implantation

	cpTi	TiO_2	TiSi	p<0.05
1 week	5.0 ± 0.4%[a]	3.0 ± 2.6%[a]	1.6 ± 1.2%[b]	NS
4 weeks	23.9 ± 5.8%	23.0 ± 5.5%	16.4 ± 3.6%	NS
12 weeks	25.9 ± 1.6%	27.3 ± 7.0%	34.1 ± 5.3%	NS
p<0.01	1 wk < 4 wk	1 wk < 4 wk	1 wk < 4 wk < 12 wk	

n=5 if not otherwise stated; [a]n=3, [b]n=4
NS = Not significant

5.3 Polymer and composite scaffolds (III – V)

5.3.1 Porous structure of the scaffolds (III-V)

The polymer and composite scaffolds were made porous using particle leaching method. Scaffold porosity was controlled by the added amount of porogen agent. In case of NaCl porogen (**III**), scaffolds with 60 vol-% (calculated) porosity were used for cell cultures, and the bisectional dimension of the cubical pores coincided with the size range (420 – 590 μm) of sieved salt crystals used in their preparation. However, the pore interconnections were much smaller (<50 μm). Sieved (400 – 500 μm) calcium chloride hexahydrate crystals (**IV**, **V**), in turn, formed a continuous porogen phase during light curing, resulting in a mean pore size of 220 ± 80 μm in composites with 65 vol-% (measured) porosity. The different scaffold structures are compared in **Figure 4**.

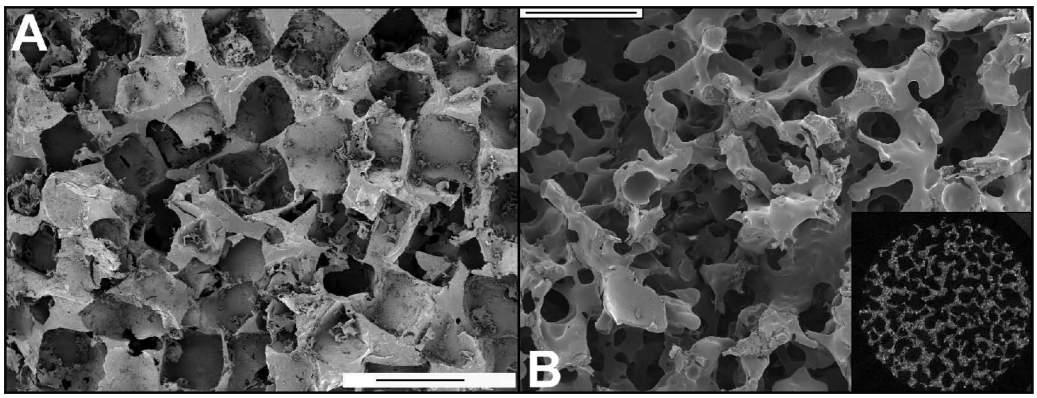

Figure 4. Porous structure of the polymer and composite scaffolds with (A) NaCl and (B) $CaCl_2 \bullet 6H_2O$ porogen. Scale bar in SEM micrographs 500 μm; scaffold diameter in the μCT image 6 mm.

5.3.2 *In vitro* reactions of the scaffold materials (III, IV)

In vitro degradation of the neat copolymer scaffolds was studied in **III**. After leaching, the compressive modulus ranged from 190 ± 10 kPa to 900 ± 90 kPa, indicating that the scaffolds were soft in comparison to high-molecular weight polycaprolactone control (20 MPa). The P(CL/DLLA) 30/70 specimens were quite susceptible to hydrolysis. Such scaffolds mainly lost their mechanical properties in four weeks, and simultaneously showed minor (~5 wt-%) mass loss (**Table 5)**. In addition, the scaffolds absorbed up to 290 wt-% of water, and swelled during leaching and hydrolysis processes. Mechanical

properties of also the P(CL/DLLA) 90/10 specimens started to decrease during hydrolysis, but significant mass losses were not observed. The scaffolds absorbed no more than 110 wt-% of water in twelve weeks, and retained their mechanical integrity.

Table 5. Effect of hydrolysis (PBS, pH 7.0, 37°C) on the cross-linked polymer matrices with different porosities. Measured values represent average ± standard deviation (n = 5).

Matrix	NaCl wt-%	Time weeks[a]	Compressive modulus, kPa	Mass loss wt-%	Water absorption, wt-%
P(CL/DLLA 90/10)	60	0	900 ± 90	-	-
		4	620 ± 80	15 ± 4	60 ± 12
		8	490 ± 50	20 ± 2	58 ± 5
		12	360 ± 70	15 ± 6	66 ± 14
	70	0	390 ± 50	-	-
		4	430 ± 40	2 ± 3	32 ± 6
		8	270 ± 80	0 ± 1	78 ± 11
		12	220 ± 40	2 ± 0	85 ± 6
	80	0	190 ± 10	-	-
		4	190 ± 20	0 ± 1	41 ± 19
		8	180 ± 20	-2 ± 0	103 ± 7
		12	130 ± 20	1 ± 0	108 ± 8
P(CL/DLLA 30/70)	60	0	330 ± 50	-	-
		4	20 ± 10	9 ± 2	170 ± 30
	70	0	310 ± 40	-	-
		4	13 ± 4	3 ± 1	200 ± 20
	80	0	270 ± 40	-	-
		4	190 ± 20	3 ± 2	290 ± 20
		8	-	8 ± 1	540 ± 60

[a] Week 0 represents the properties of porous samples after 1 week of salt leaching.

The reactivity of P(CL/DLLA) 90/10 – BAG composites in SBF was further studied. Water based salt leaching process in **III** changed the ion dissolution profile of the BAG filler, decreasing the initial calcium and phosphate release from the scaffolds. However, it was shown that BAG rendered the composite scaffolds bioactive in terms of their CaP formation ability (**Figure 5**). Biomimetic CaP layer started to form on the pore walls during the first 24 hours of incubation in SBF. In contrast, none of the scaffold materials were able to form such a biomimetic layer in complete culture medium, containing 15 % of serum. Composite scaffolds in **III** and **IV** exhibited a constant 0.1-0.2 unit increase in culture pH. Furthermore, high (~120 μg/ml) initial amounts of calcium and silica were observed. The reactivity of BAG filler diminished with time, and the amounts of dissolution products decreased in each medium exchange. The intrinsic reactivity of the composite scaffolds was further masked by expanding cell populations that covered the scaffolds, and started to produce mineralizing ECM.

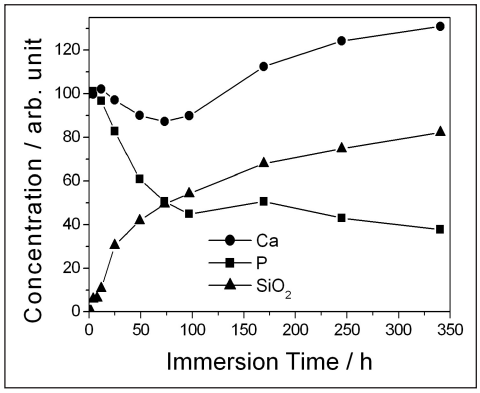

Figure 5. Evolution of Ca, P, and silica concentrations in SBF for P(CL/DLLA 90/10)-BAG composite scaffolds. Composite samples were measured with P(CL/DLLA 90/10) as a background. Coefficient of variations are less than 5% (n = 3). (Reproduced from study **III**)

5.3.3 Osteogenic differentiation cascade (III, IV)

Composite scaffolds in **III** showed increased cell adhesion in comparison to neat polymers. Stronger proliferation was observed with neat polymers, however, and the cell activities were similar in both scaffold types after one week of culture. In contrast, equal cell adhesion was observed in **IV**, whereas composite scaffolds sustained higher cell activities after one week of static culture. Cell activities in late cultures were not increasing anymore, and no difference between scaffold types was observed. Furthermore, cell activities in dynamic cultures remained at the initial level still after three weeks.

After osteogenic preculture in **III**, cells already expressed clear osteogenic phenotype, and no further change in gene expression was observed during the first week of secondary culture. After two weeks, BSP expression had turned to decline, whereas OC expression was increasing with both scaffold types. The cell stock used in **IV** indicated only weak signs of osteogenicity after non-differentiating preculture. Strong induction of osteoblast markers was subsequently observed after one week of osteogenic secondary culture, and the markers were further induced after three weeks. After one week, composites showed higher ALP activity and BSP expression level, but OC expression was lower than with neat polymers. No other differences were seen between scaffold types. All markers were higher in static than in dynamic cultures after three weeks.

Composite scaffolds in static cultures showed increased mineralization compared to neat polymers, irrespective of the preculture method. However, only minimal calcium precipitation occurred under dynamic culture conditions, even with the composites. The effect of BAG filler on the osteogenic differentiation cascade in static cultures is summarized in **Table 6**. In dynamic cultures, all the studied parameters were similar with both scaffold types.

Table 6. Effect of BAG filler on the osteogenic differentiation cascade in static cultures.

	Cell activity		Gene expression, 7d				
	Initial	7d	BSP	OC	MEPE	ALP, 7d	Mineralization
III	+	±	±	±	ND	ND	+
IV	±	+	+	–	±	+	+

+, ± and – denote increase, similarity and decrease, respectively, in comparison to neat polymer scaffolds.
ND = not determined

5.3.4 Scaffold colonization *in vitro* (III, IV)

Osteoblasts in static cultures grew on the surface of the scaffolds. The cells were able to penetrate only to the outermost pore layer, irrespective of the porogen agent used. At late cultures a thick collagen rich matrix covered the scaffolds entirely, masking much of the surface porosity. Dynamic culture conditions, in turn, allowed cells to colonize the scaffold interior, whereas the scaffold surface was devoid of cells (**Figure 6**). Furthermore, cells grew as a monolayer still after three weeks. No differences in scaffold colonization were observed between polymer and composite scaffolds in either culture mode.

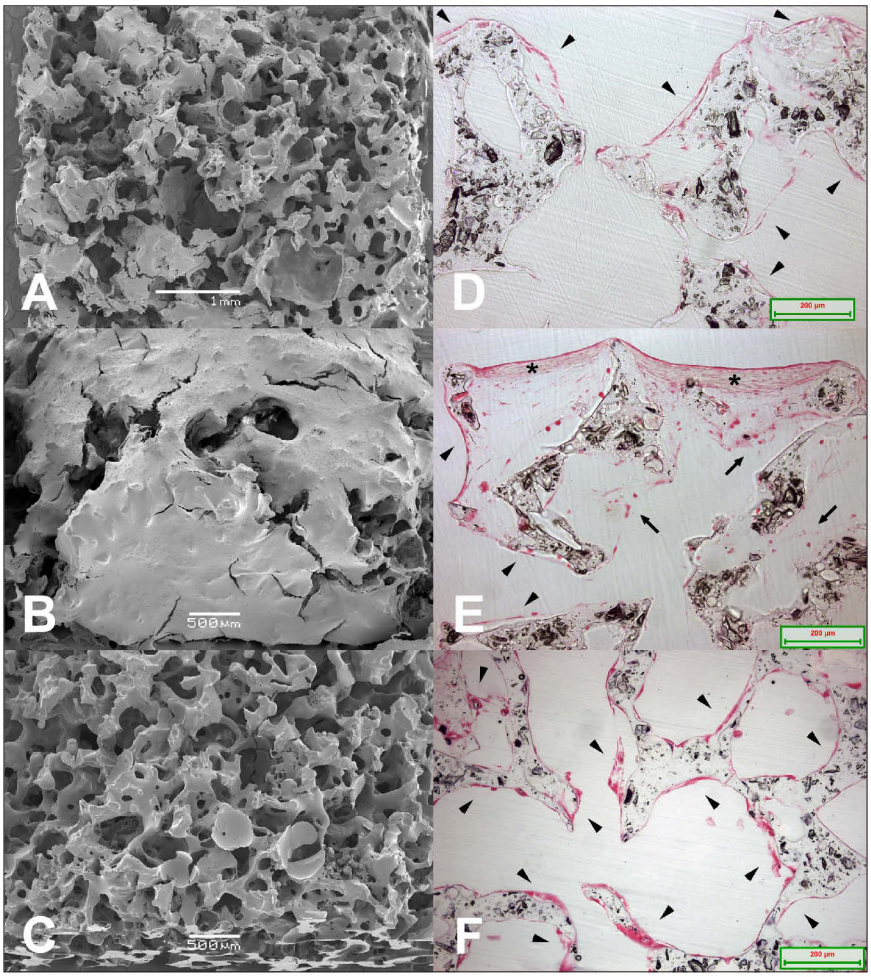

Figure 6. Scaffold colonization in composites with $CaCl_2 \bullet 6H_2O$ porogen. SEM images from the surface of the scaffolds after 7d (A; bar = 1 mm) and 21d (B; bar = 500 µm) of static culture, and after 21d (C; bar = 500 µm) of dynamic culture. Corresponding HE-stained histological sections (bar = 200 µm) from the scaffold periphery after 7d (D) and 21d (E) of static culture, and from the scaffold interior after 21d (F) of dynamic culture. Markings in D-F: arrowheads = flattened osteoblasts on the pore surfaces; arrows = matrix embedded rounded (possibly apoptotic) cells within the scaffold porosity; asterisk = thick mineralized extracellular matrix on the surface of the scaffolds. (Reproduced from study **IV**)

5.3.5 Cell free implantations (V)

Similar tissue reactions were observed for all cell free scaffold types after four weeks of implantation, irrespective of the polymer matrix or BAG filler. All scaffolds were well integrated into host tissues, and were filled with well organized fibrous connective tissue characterized by good vascularization (**Figure 7**). Scattered ED1 positive multinucleated giant cells were observed on the polymer surface throughout the scaffold in each histological section. Furthermore, occasional accumulations of inflammatory cells and areas of immature fibrous connective tissue were observed in all scaffold types. No signs of mineralizing tissues were ever seen.

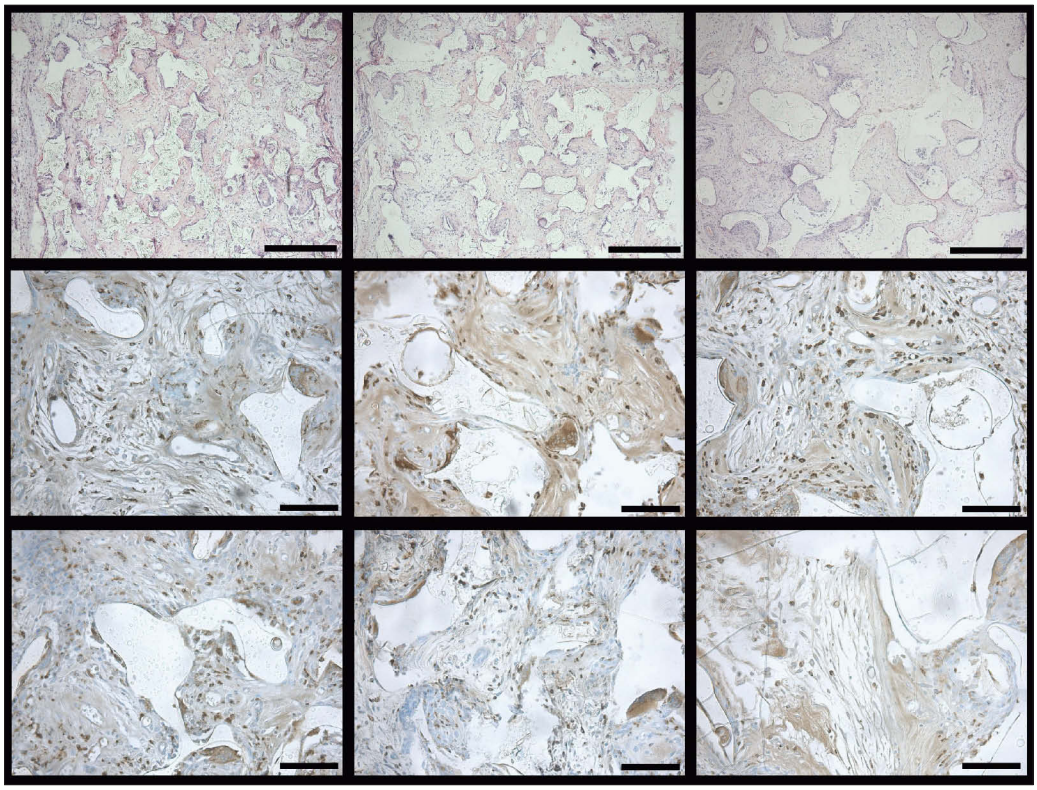

Figure 7. Soft tissue reaction to subcutaneously implanted scaffolds: composite with 90-315 µm (left column) and with <45 µm (middle column) BAG, and polymer (right column). Differences in scaffold structure can be seen in low magnification images (top row, 70/30 matrix, HE stain, bar = 500 µm). Well vascularized fibrous connective tissue had fully invaded the scaffolds by four weeks. Similar tissue reaction was observed with all scaffold types, irrespective of the polymer matrix or BAG filler (middle and bottom row: 70/30 and 90/10 matrix, respectively; ED1 immunostaining, bar = 100 µm). Scaffold matrix does not stain, and appears white/grayish; * = multinuclear giant cells, + = large capillaries. (Reproduced from study **V**)

5.3.6 Implantation of cell-scaffold constructs (V)

After one week of implantation loose immature fibroconnective tissue with capillaries filled the periphery of the cell-seeded scaffolds. The innermost 30 % of the scaffold cross-section was characterized by the high amounts of empty pores with clusters of

erythrocytes. After four weeks, tissue ingrowth was observed throughout the scaffolds which were mostly filled by well organized fibrous connective tissue with good vascularization. Several loci of immature mineralizing tissue were seen. Furthermore, small amounts of mature bone tissue was also observed in two polymer scaffolds and four composites ($p = 0.61$) (**Table 7**). After twelve weeks of implantation mature bone was observed in only one polymer scaffold but in seven composites ($p = 0.01$). Occasionally bone had remodeled into lamellar structures following the contour of scaffold pores. However, the amount of bone had not significantly increased from that of four weeks, covering the maximum of 7 % of open porosity (**Figure 8**). Bone tissue was found both in the periphery and in the middle of the scaffolds.

Table 7. Occurence of ectopic bone formation in BMSC loaded scaffolds.

	4 weeks		12 weeks	
	Bone	**No Bone**	**Bone**	**No Bone**
Polymer	2	6	1	7
Composite	4	4	7	1
	$p = 0.61$		$p = 0.01$	

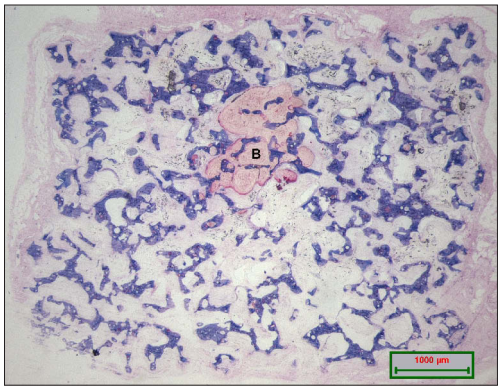

Figure 8. Ectopic bone formation in subcutaneously implanted cell-scaffold constructs. Composite scaffold at 12 weeks. Scaffold matrix stains blue. Methylene blue - basic fuchsine stain, scale bar = 1000 µm, B = mature bone tissue.

6 DISCUSSION

The general aim of the present study was to compare the different macroporous scaffold types in BMSC cultures and subcutaneous implantation studies. Physical and structural properties of the scaffolds were also characterized. The two scaffold categories studied were a) the mechanically strong and stable titanium meshes (**I**, **II**) and b) the elastic and biodegradable porous polymers (**III** – **V**). Furthermore, bioactive modifications were applied to these basic scaffold types, and their effect on the cell and tissue responses was evaluated. Certain methodological issues possibly affecting the obtained results manifested themselves in the course of this work, and are also discussed below.

6.1 Primary cultures (I-V, unpublished)

Fresh laboratory expanded rat BMSC were used as a cell source in this study, and scaffolds were always seeded with passage 1 cells. The cell yield and their response to dexamethasone were well in accordance with the literature (Rickard *et al.*, 1994). However, non-selected serum lots were used, adding one possible source of variation to the cell behavior (Caplan, 2004). The primary cultures were conducted with osteogenic supplements, except in **IV**, to increase the osteogenic potential of the expanded cell populations (ter Brugge and Jansen, 2002). Correspondingly, mature mineralizing phenotype was observed after one week of secondary culture with all scaffold types in **I** and **III**. Non-differentiated primary cells were used in **IV** to get a more pronounced response to different scaffold types and culture conditions, and indeed mineralization occurred only with composite scaffolds in static culture conditions.

The initial phenotype of seeded cells is also known to affect the amount of ectopic bone formation (Yoshikawa *et al.*, 1998; Holtorf *et al.*, 2005). This can possibly explain the much higher ectopic bone formation with uncoated titanium scaffolds in **II** (15 % to 18 %) in comparison to previous research using similar experimental protocol (less than 10 %) (Hartman *et al.*, 2004; Hartman *et al.*, 2005). No definitive answer can be given, however, and it emphasizes the difficulties in comparing results from different studies, and the importance of using relevant control samples in all experiments.

6.2 Titanium fiber meshes (I, II)

It has been previously shown that cell-loaded titanium fiber scaffolds have bone-forming potential in ectopic (van den Dolder *et al.*, 2002) and orthotopic rat models (Sikavitsas *et al.*, 2003; van den Dolder *et al.*, 2003a). Titanium meshes that were not loaded with cells had only minor osteoconductive properties in those studies. *In vivo* data suggest that sol-gel TiO_2 coating can increase both bone and soft-tissue attachment to titanium implants (Li and de Groot, 1993; Areva *et al.*, 2004). Under two-dimensional culture conditions, cellular response was further enhanced by incorporation of a reactive silica phase in the coating (Areva *et al.*, 2007). It was therefore hypothesized that non-resorbable bioactive coatings based on titania, with or without a silica phase, might enhance the performance of titanium fiber scaffolds in bone engineering applications.

6.2.1 Preparation of sol-gel coated titanium scaffolds

In previous studies thin sol-gel derived TiO_2 and TiSi coatings on planar titanium surfaces have been prepared using a dipping method (Jokinen *et al.*, 1998; Ääritalo *et al.*, 2007). The method, however, is not optimal for porous substrates because the high viscosity of the coating sols may result in uneven coating thickness on scaffold surfaces. This was also noticed in the present work, as a high amount of aggregated coating material was found inside the TiO_2 and TiSi scaffolds. The phenomenon was most pronounced at the edges of the scaffolds, where the porosity was already decreased due to the cutting process. In fact, it would have been beneficial to apply the coatings on neat fiber mesh instead of individual scaffold specimens. Although the aggregates did not seem to result in particle-induced inflammation, they did alter the pore size distribution and the porous structure of the scaffolds. This reduces the permeability of the scaffold, and occludes some of the smallest pores. More controlled coatings are thus preferred. Spin coating could possibly be used to remove the excess sol by centrifugal force before heat treatment (Gomez-Vega *et al.*, 2001; Kim *et al.*, 2005b). Furthermore, coatings prepared using single dipping in low-viscosity sol are currently under investigation (unpublished).

6.2.2 In vitro performance of titanium scaffolds

Uncoated and TiSi coated scaffolds showed clear cell proliferation for only three days of culture, whereas proliferation with TiO_2 scaffolds continued for a week. However, maximal DNA release was no greater than with the other scaffold types. An overall short proliferation phase corroborates the literature, and can be explained on the basis of the high concentration of cells (1×10^6 cells / ml; ~1×10^5 cells / scaffold) used to seed the scaffolds (Holy *et al.*, 2000; Wilson *et al.*, 2002; van den Dolder *et al.*, 2003b). The prolonged proliferation with TiO_2 substrates was reflected in slower ALP induction. Similar results have been obtained when cell differentiation has been delayed due to an absence of osteogenic supplements during osteoblast pre-culture (Ishaug *et al.*, 1997; Ishaug-Riley *et al.*, 1998). In those experiments proliferation of cells inside porous scaffolds continued for several weeks, as long as ALP activity was increasing. All of the findings are in accordance with the notion that there is a reciprocal relationship between proliferation and differentiation of osteogenic cells derived from bone marrow and calvaria (Stein *et al.*, 1990; Malaval *et al.*, 1994). In comparison with uncoated scaffolds, the OC production and extracellular matrix mineralization were increased with TiO_2 scaffolds, but decreased with TiSi scaffolds. The results with TiO_2 scaffolds are consistent with literature, indicating enhanced osteoblast differentiation but not proliferation on bioactive titania surfaces (Nishio *et al.*, 2000; Kim *et al.*, 2005b). There is only one previous osteoblast culture study with titania-silica coatings (Areva *et al.*, 2007). Similar to the current study, it indicated slightly enhanced ALP induction in comparison with pure titania coating, whereas no difference in mineralization was observed in qualitative terms.

6.2.3 In vivo performance of titanium scaffolds

There were no substantial differences in the amount of bone within the implants, but the kinetics of ectopic bone formation differed between scaffold types. Practically every specimen showed ingrowth of well vascularized connective tissue through the scaffold

already at one week. This is very important for the survival of seeded cells and for the subsequent bone formation (Kruyt *et al.*, 2003; Pelissier *et al.*, 2003). Mineralization began from the periphery of scaffolds where the amount of seeded cells was likely to be the highest, and diffusion limitations the lowest. Later bone formation was most evident in the middle of the scaffolds. The maximal amount of bone with cpTi and TiO_2 scaffolds was reached already after four weeks of implantation, whereas bone formation with TiSi scaffolds continued throughout the experiment. Light microscopy indicated that bone formation within cpTi scaffolds usually started away from the titanium surface, and individual fibers were mostly surrounded by a thin layer of fibrous unmineralized tissue still at four weeks. In contrast, bone formation occurred directly on the TiO_2 and TiSi coated titanium. Such behavior resembles ectopic bone formation within porous CaP ceramics, and indicates good attachment of seeded cells to the underlying substrate (Goshima *et al.*, 1991; Yoshikawa *et al.*, 1992). As bone formation proceeded, and mesh fibers were embedded in bone matrix, all materials showed good bone contact. Coated scaffolds indicated enhanced osteoconductivity, having more dispersed bone formation than the uncoated ones. Bone formation on the outer surface of only the sol-gel coated scaffolds further advocates their increased osteoconductivity. Again, this finding resembles ectopic bone formation within ceramic scaffolds (Dong *et al.*, 2002; van Gaalen *et al.*, 2004).

6.2.4 Potential applications and future research

Fully or only surface porous load bearing metallic implants are currently used in several biomedical applications as e.g. spinal fixation devices and orthopedic and cranio-facial implants (Ryan *et al.*, 2006). The current study showed that TiO_2 coated bone engineering scaffolds enhanced osteogenic differentiation *in vitro*, and also indicated increased osteoconductivity in comparison to uncoated titanium scaffolds *in vivo*. In contrast, TiSi coated scaffolds resulted in decreased BMSC differentiation, and resulted in delayed although well dispersed ectopic bone formation. These results indicate that sol-gel derived TiO_2, but not TiSi, coatings could possibly be used to enhance *in vivo* bone response to titanium implants. However, there are multiple competing techniques to achieve this aim (Son *et al.*, 2003; Albrektsson and Wennerberg, 2004; Kokubo *et al.*, 2004; Sul *et al.*, 2005), and it is unclear whether TiO_2 coatings can have major commercial potential in bone related clinical applications. On the other hand surface modified titanium is only rarely reported to enhance soft tissue bonding (Areva *et al.*, 2004; Wu *et al.*, 2006). TiO_2 coatings could therefore have better competitive edge in applications requiring good contact with soft tissues, e.g. percutaneous or -mucosal devices (van der Pouw *et al.*, 1999; Rompen *et al.*, 2006), and the studies with TiO_2 coatings in our research group is currently focusing in this direction. An additional benefit of (modified) TiO_2 coatings is that they can be applied on a large variety of metallic, ceramic and polymeric substrates, further expanding the possible application areas.

6.3 Polymer and composite scaffolds (III – V)

Biodegradable bone engineering scaffolds have been traditionally prepared from linear thermoplastic polymers. This usually necessitates the use of organic solvents in the fabrication process, and generates the risk of toxic residues in the end product. Recently

low molecular weight cross-linkable fumarate (Fisher *et al.*, 2002; Grijpma *et al.*, 2005; Jabbari *et al.*, 2005) and acrylate (Burdick *et al.*, 2003; Hedberg *et al.*, 2005b; Vogt *et al.*, 2005; Declercq *et al.*, 2006) functional oligomers have been used to prepare biodegradable tissue engineering scaffolds, enabling melt processing in low temperatures and even *in situ* curable porous systems (Trantolo *et al.*, 2003). Furthermore, cross-linking can enhance the form stability and mechanical strength of the corresponding linear polymers (Helminen *et al.*, 2002). Bioactive fillers, in turn, have been used to improve the osteogenic response to porous polymer scaffolds *in vitro* (Ma *et al.*, 2001; Lu *et al.*, 2003; Montjovent *et al.*, 2005). Based on these premises different types of biodegradable scaffolds with elastic properties were prepared and analyzed for bone engineering.

6.3.1 Scaffold preparation

The volumetric porosity of the scaffolds was determined by the amount of porogen used. Increasing the porosity decreased the pore wall thickness, and eventually compromised the crosslinking efficiency and mechanical integrity of the scaffolds (**III**). Literature indicates that the optimal proportion of BAG in composite osteoblast culture substrates would be around 30 wt-% (Lu *et al.*, 2005; Yao *et al.*, 2005a). However, addition of bioactive filler further decreases the relative amount of polymer in porous scaffolds. Consequently, it was not practical to produce polymer and composite scaffolds with porosities greater than ~85 % and ~75 %, respectively.

Scaffold colonization is governed more by pore size distribution than volumetric porosity. Recent reviews have emphasized the importance of interconnected macroporosity, approximately 100 – 500 μm in (average) diameter, to allow vascularization and good penetration of tissue into bone engineering scaffolds *in vivo* (Karande *et al.*, 2004; Karageorgiou and Kaplan, 2005). It was possible to produce large cubical pores using conventional NaCl porogen (**III**), but the size of interconnections was at the lower limit for successful bone ingrowth (Lu *et al.*, 1999; Itälä *et al.*, 2001; Otsuki *et al.*, 2006). Thus, NaCl was replaced with a novel $CaCl_2 \bullet 6H_2O$ porogen agent (**IV, V**). This salt melts at 30°C forming a continuous porogen phase during light curing of the scaffolds, and the resulting pore architecture was much closer to the aim. In addition, this porogen can be leached with either water or ethanol, allowing strict control over the dissolution behavior of the bioactive filler.

Crosslinked P(CL/DLLA) copolymer matrices with three different molar macromer ratios were studied. The lactide rich 30/70 matrix had inferior mechanical properties in hydrolysis conditions, whereas caprolactone rich 90/10 matrix was deemed to be excessively hydrophobic and slow to degrade (**III**). In later studies (**IV**, **V**), 70/30 matrix was chosen as a more hydrophilic but still mechanically sound alternative (Helminen *et al.*, 2002). Moreover, the macromer size was decreased to enhance the crosslinking density in highly porous scaffolds.

Porous specimens for biological evaluation, but not those for material characterization, were sterilized by gamma radiation. Irradiation is an efficient and widely used method to sterilize biomedical products (Kowalski and Morrissey, 1996). It is especially useful way to sterilize materials, like many polymers, which do not tolerate high temperatures

and humidity. However, high energy radiation can cut polymer chains and create free radicals, causing structural changes at the molecular level (in dose dependent manner) (Haugen *et al.*, 2007). This, in turn, may alter the mechanical and degradation properties of the scaffolds (Plikk *et al.*, 2006).

6.3.2 In vitro performance of polymer and composite scaffolds

6.3.2.1 Early response in static cultures

Porous composites of several biodegradable polymers and bioceramic fillers have shown to be bioactive in terms of their apatite forming ability in aqueous solutions (Wang *et al.*, 2001; Maquet *et al.*, 2003; Korventausta *et al.*, 2004). For the current cell cultures composite scaffolds with 30 wt-% BAG in P(CL/DLLA) copolymer matrix were prepared, and their bioactivity in SBF was confirmed in **III** and (Malin *et al.*, 2008). On the other hand no calcium precipitation was observed in complete cell culture medium, where serum proteins inhibit CaP formation (Radin *et al.*, 1997; Areva *et al.*, 2002; Combes and Rey, 2002). The scaffolds with NaCl porogen were leached with water and soaked before cell seeding, allowing BAG filler to react during the processes. Although the time scale did not allow for biomimetic CaP formation (Jokinen *et al.*, 2001), it is quite likely that some silica and calcium released from BAG had reabsorbed on the composites, resulting in modification of scaffold surface properties. In contrast, $CaCl_2 \bullet 6H_2O$ porogen was leached with ethanol, and BAG filler remained essentially inert at this stage. Furthermore, small filler particles were fully embedded in the polymer matrix whereas large granules were more exposed. Thus, the initial material surfaces were similar with both scaffold types and resulted in equal cell adhesion in **IV**, whereas enhanced cell adhesion with composites was observed in **III**. In both instances BAG remained reactive upon immersion into culture medium releasing high amounts of silica and calcium, whereas only a small increase in culture pH was observed.

Increased silica (Xynos *et al.*, 2000; Bielby *et al.*, 2004b; Valerio *et al.*, 2004) and calcium (Sugimoto *et al.*, 1994; Maeno *et al.*, 2005) concentrations have been reported to induce osteoblast proliferation. Accordingly, higher cell activity was observed with composites than with neat polymers after seven days of static culture in **IV**. Early osteoblast markers, ALP activity and BSP expression level, were also more induced indicating faster osteogenic differentiation with composites. Both findings are in concert with recent literature reporting positive osteoblast response to the BAG filler in the scaffolds (Blaker *et al.*, 2003; Yao *et al.*, 2005b). In contrast, the culture conditions in **III** allowed only minimal cell expansion after seeding, and therefore neat polymer scaffolds (with lower amount of adhered cells) showed higher proliferation than composites. Furthermore, the initial cell stock was already highly differentiated, and the semi-quantitative analyses method did not reveal any significant changes in gene expression during the first week of culture. The beneficial effect of BAG filler was, however, demonstrated by earlier onset of mineralization.

6.3.2.2 Late response in static cultures

No further increase in cell activity was ever observed after the first week of culture. Similar results have been reported previously (Wilson *et al.*, 2002; Xie *et al.*, 2006),

showing that there is an upper limit to the amount of cells capable to expand and to survive on a given scaffold type and culture conditions. The highly differentiated cells in **III** expressed mature osteogenic phenotype after two weeks of culture (Malaval *et al.*, 1994). BSP expression had already turned to decline with polymer matrices, and possibly with composites, whereas both scaffold types showed increased OC expression and strong mineralization. Normal osteoblast response was seen also in **IV**. Proliferation had ceased, ALP activity was increased, and late markers of osteogenic gene expression, OC and MEPE, were strongly induced after three weeks of culture. The lack of mineralization in the neat polymer scaffolds was most likely due to the omission of dexamethasone from the primary cultures. This is known to decrease the osteogenic potential of cultured BMSCs, even if dexamethasone is supplemented in the subsequent cultures (ter Brugge and Jansen, 2002). On the other hand the mineralizing phenotype was rescued in the composites, a further indication of the osteogenic effects of the BAG filler.

6.3.2.3 Dynamic cultures

The size of pore interconnections was improved using the novel porogen agent in **IV**. This did not, however, enhance the scaffold colonization under static culture conditions. Still only few cells had penetrated into the interior of the scaffolds, even if the interconnected porosity remained unoccluded. This was a clear indication of diffusional constraints inside millimeter scale porous scaffolds (Botchwey *et al.*, 2003). Forced fluid flow through the scaffolds was supposed to both enhance nutrient and waste transport, and provide mechanical stimuli for the osteoblasts (Sikavitsas *et al.*, 2005; Müller *et al.*, 2006). However, scaffolds in dynamic cultures did not tumble about freely, as intended (Qiu *et al.*, 1999; Yu *et al.*, 2004), but tended to aggregate under the handling ports of the bioreactors. This exposed the cells to high shear stresses caused by constant collisions and convection. The cells on the scaffold surfaces were consequently washed away, and less cells were present than in static cultures. The dynamic cultures resulted in compromised osteogenicity, and no mineralization occurred with either polymer or composite scaffolds. It is unclear whether this was due to the harsh culture conditions within the bioreactors or an indirect consequence of decreased cell numbers. Anyhow, similar findings have also been reported by others using the same type of bioreactor (Sikavitsas *et al.*, 2002). In the current study the cells were able to colonize the interior of 2 mm thick scaffolds only in dynamic cultures, evidencing that culture conditions are as important as scaffold porosity for the successful *in vitro* bone engineering. Perfusion bioreactors allow strict control over the shear stresses, and could therefore offer optimal culture conditions for osteoblasts (Goldstein *et al.*, 2001; Bancroft *et al.*, 2002; Cartmell *et al.*, 2003). Recently, such cultures have also been used to coculture osteogenic and hematopoietic cells, and to improve ectopic bone formation within bone engineered constructs (Braccini *et al.*, 2005).

6.3.3 In vivo performance of polymer and composite scaffolds

In the first part of this experiment, six different macroporous polymer-BAG combinations were screened for their inflammatory response and tissue ingrowth in a rat subcutaneous implantation model. The copolymer matrices differed by their caprolactone/lactide monomer ratios, the 70/30 matrix being more hydrophilic and more susceptible to

hydrolytic degradation than the 90/10 matrix (Helminen *et al.*, 2002). Both scaffold types were prepared as such, and as composites with 30 wt-% of either <45 µm or 90 – 315 µm BAG granules. All scaffold types gave similar tissue response at four weeks. Scattered foreign body giant cells observed probably indicate sites of active polymer matrix resorption (van Tienen *et al.*, 2002; Xia *et al.*, 2004). In contrast, no signs of gross inflammation were ever observed. Further evaluation over time would be needed to make detailed conclusions about the scaffold degradation. The current findings were in good agreement with the literature reporting good biocompatibility of caprolactone and lactide based porous scaffolds (Taira *et al.*, 2003; Rücker *et al.*, 2006). The scaffolds were fully invaded by well vascularized soft connective tissue, a clear indication of their biocompatibility and good porous architecture. All the tested scaffold types showed an appropriate host response in subcutaneous tissues, and were therefore considered as potential candidates for bone engineering.

The more hydrophilic polymer matrix and smaller BAG filler size were chosen for the further ectopic bone formation assay. The scaffolds were loaded with differentiating BMSCs and implanted subcutaneously in syngeneic rats. Excluding bone formation the host response was considered similar to that with cell-free scaffolds. After twelve weeks most of the composites, but only one polymer scaffold, contained mature bone. Thus, in this animal model with relatively large implants osteopromotive signal was better retained in composites with BAG than in neat polymer scaffolds. This finding was consistent with the reports about increased *in vitro* osteogenicity of composite scaffolds (Lu *et al.*, 2005; Yao *et al.*, 2005a). However, such positive *in vitro* results have not always been transferable into the *in vivo* situation (Holmbom *et al.*, 2005; Yang *et al.*, 2006). The interior of the millimeter scale scaffolds were devoid of tissue growth and vascularization after one week of implantation, which has likely decreased the amount of living BMSCs in the scaffolds (Colton, 1995; Karande *et al.*, 2004). This may be one reason for the less-than-expected amount of bone formation in this experiment (Wilson *et al.*, 2002; Kruyt *et al.*, 2003). On the other hand, the random distribution of bone within the implants indicates good scaffold structure, supporting angiogenesis and osteoconductivity. In conclusion, plain polymer and composite scaffolds supported the ingrowth of well vascularized fibroconnective tissue. Furthermore, cell seeded composites with BAG filler showed enhanced ectopic bone formation in comparison to neat polymer scaffolds.

6.3.4 Potential applications and future research

There are several ceramic bone substitute materials / porous scaffolds currently on the market, but inferior handling properties, variable resorption rates, and lack of bioactivity have limited their wider clinical use (Bucholz, 2002; Ylinen, 2006). Better control over degradation times and mechanical properties can be achieved with polymers, but they tend to have lower bone bonding ability (Mano *et al.*, 2004). There is therefore a trend in bone engineering scaffolds towards moldable, biodegradable and highly osteoconductive polymer-ceramic composites (Rezwan *et al.*, 2006), and the scaffolds of the current study are one example of this research line. Though bioactive filler particles and the concomitant release of Ca, P and Si ions can enhance bone formation (Kim *et*

al., 2007b; Lickorish *et al.*, 2007) and vascularization (Day *et al.*, 2005; Andrade *et al.*, 2006; Leach *et al.*, 2006), such composite scaffolds are not osteoinductive. Presence of osteoprogenitor cells was mandatory for ectopic bone formation (**V**). The studied materials are ultimately intended as temporary osteoconductive scaffolds for bone reconstructions, with or without cells. Accordingly, the bone regeneration ability of the neat scaffolds will be tested in rabbits in the near future. However, as with the titanium scaffolds there are multiple competing materials/technologies available, and therefore the scaffolds will need further development.

The scaffold matrix in **IV** and **V** was hardened through crosslinking of end-functionalized copolymers by photopolymerization in room temperature. The same principle might be used also in stereolithography to produce even more controlled pore architectures in large scale (Lee *et al.*, 2007). On the other hand, particle leaching is a promising method for *in situ* porosity formation, although the porogen agent needs to adsorb high amounts of water relatively fast after implantation. Water containing and easily decomposing porogens, like $CaCl_2 \bullet 6H_2O$ used in this study, might solve this problem. Their performance in injectable bone graft materials is not known, but it is likely that they are functional also in contact with the interstitial fluids and blood *in situ*. Besides salt hydrates, some fast degrading polymers like polyesteranhydrides have good potential to provide interconnective porous structures in the *in situ* hardening materials (Korhonen *et al.*, 2006; Rich *et al.*, 2007). An additional benefit of low temperature processing is the possibility to add biomolecular signals such as proteins and drugs into the scaffold structure (Lieberman *et al.*, 2002; Malafaya *et al.*, 2002). All of these aspects have been included in our research plan for the next three years.

7 CONCLUSIONS

The dip coating process used in **I** and **II** was not optimal for porous substrates because the high viscosity of the sols resulted in the uneven coating thickness on fiber surfaces and high amounts of aggregated coating material within the scaffold porosity. However, the coated scaffolds were suitable for tissue engineering studies, and no adverse effects related to coating materials were observed. More controlled coatings are currently developed by our collaborators.

The current study confirmed the previous findings that cpTi fiber mesh supports the attachment, growth and differentiation of rat BMSCs *in vitro*, as well as BMSC driven ectopic bone formation *in vivo*. More importantly, the osteogenic phenotype of cultured cell-scaffold constructs was heightened with a sol-gel derived TiO_2 coating, but not with a TiSi coating. TiSi coating also resulted in delayed ectopic bone formation, and was therefore deemed inferior in comparison to TiO_2 coating. The amount of ectopic bone formation with both the BMSC seeded TiO_2 and TiSi coated scaffolds was comparable to that with uncoated titanium scaffolds. Furthermore, the better bone contact in early implantation times and more even bone tissue distribution at later times indicated enhanced osteoconductivity of the coated scaffolds. Overall, the most promising bone engineering results were obtained with TiO_2 coated fiber meshes.

Elastic and biodegradable P(CL/DLLA) based scaffolds suitable for bone tissue engineering were also developed in this study. The degradation rates of the scaffolds *in vitro* were governed by the hydrophilicity of the polymer matrix, and the porous architecture was controlled by the amount and type of porogen used. Traditional NaCl porogen resulted in only minimally interconnected porosity, whereas a continuous phase macroporosity was obtained using a novel $CaCl_2 \bullet 6H_2O$ porogen.

Rat BMSC differentiation within the biodegradable scaffolds *in vitro* was enhanced in composites, with 30 wt-% BAG filler. A dynamic culture model was needed to support cell invasion into the macroporous scaffolds, but harsh mechanical stresses in the rotating wall bioreactors resulted in the decreased cell numbers and inhibition of the differentiation process irrespective of the scaffold type. All the tested polymer and composite scaffolds showed an appropriate host response in rat subcutaneous tissues, with a moderate foreign body reaction and no signs of gross inflammation. The millimeter scale scaffolds supported ingrowth of well vascularized tissues. Furthermore, ectopic bone formation in BMSC seeded composites was enhanced in comparison to neat polymer scaffolds.

The applied primary rat BMSC culture and subcutaneous implantation protocols were useful models to evaluate biological response to different tissue engineering scaffolds in a comparative way. The beneficial effect of bioactive scaffolds was clearly shown. However, further implantation studies in orthotopic site will be needed to confirm the suitability of these scaffolds for real bone regeneration applications.

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Ville Meretoja

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