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Maria Persson

3D WOVEN SCAFFOLDS FOR BONE TISSUE ENGINEERING

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Abstract

Bone tissue engineering has become a rapidly expanding research area because it offers a promising new approach for bone repair and regeneration. Compared to traditional autograft and allograft procedures, bone tissue engineering techniques based on the use of scaffolding materials in combination with autogenous stem cells can eliminate problems of donor site morbidity associated with the harvest of bone tissue, and its short supply. Clearly, the choices of material as well as a scaffold design that enhance bone regeneration are major challenges in the tissue engineering approach. Fibers in the micro-range in combination with textile-based technologies are consider as potential routes for the production of complex scaffolds since they can be used to generate a wide range of morphological structures and geometrically varied structures with high precision. Therefore in this thesis the specific objects were to: (i) develop a biocompatible composite fiber from poly(lactic acid) (PLA) and hydroxyapatite (HA) by melt spinning, (ii) design a 3D textile scaffold utilizing weaving and (iii) evaluate the scaffolds' performance as a bone substitute material *in vitro*.

In the present study PLA/HA composite fibers were successfully produced, and found to possess sufficient mechanical strength even at high loading concentrations (i.e. 20wt %), to be useful in a textile process. In addition, the material was shown to be biocompatible and the presence of HA in the PLA composite significantly enhanced the initial cell attachment. In a 3D woven scaffold, bone marrow derived human mesenchymal stem cells (hMSCs) differentiated into osteoblasts and mineralized bone formation *in vitro* was observed through-the-thickness of the scaffold. Taken together, these results indicate the potential feasibility of PLA/HA composite fiber in a 3D woven scaffold for use as a bone substitute material in tissue engineering applications.

Keywords: bone tissue engineering, hydroxyapatite composite, melt spinning, mesenchymal stem cell, poly lactic acid, textile scaffolds

Persson, Maria, Kudotut luukudosteknologiset tukirakenteet.
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Tiivistelmä

Luupuutosten korvaaminen kudosteknologisesti on kehittynyt nopeasti ja tutkimustulokset tarjoavat lupaavia mahdollisuuksia tuottaa uutta luuta luupuutosalueelle. Perinteisiin potilaan omasta luusta tehtyihin luusiirteisiin ja pankkiluusiirteisiin verrattuna potilaan omat kantasolut voivat vähentää ongelmia, joita ovat siirrematerіaalin rajallinen saatavuus ja vieraan kudoksen aiheuttamat reaktiot. On tärkeä etsiä hyviä materiaaleja, joista voidaan valmistaa sellaisia kolmiulotteisia (3D) rakenteita, joilla tehostetaan luun paranemista ja uuden luun muodostumista. Kutomalla tuotetut tukirakenteet mahdollistavat kantasolusiirteille kolmiulotteisuuden, jota voidaan säädellä monipuolisesti ja tarkasti. Tämän väitöstutkimuksen tarkoituksena oli: (i) kehittää bioyhteensopiva kuitu maitohappopolymeeristä poly lactic acid (PLA) ja hydroksiapatiitista (HA) kuituekstruusiolla, (ii) suunnitella ja kutoa tästä kuidusta 3D tekstiilirakenne, ja (iii) tutkia sen toimivuus ja ominaisuudet luunmuodostusta tukevana materiaalina soluviljelyolosuhteissa.

Tämä tutkimus osoittaa, että PLA kuitua voidaan seostaa hydroksiapatiitilla, ja PLA/HA kuidut ovat mekaanisesti kestäviä sisältäessään jopa 20 painoprosenttia hydroksiapatiittia. Siten kuidut ovat tekstiilin valmistuksessa käyttökelpoisia. Lisäksi materiaali osoittautui bioyhteensopivaksi, ja hydroksiapatiitti paransi solujen tarttumista PLA kuituun viljelyn alkuvaiheessa. Ihmisen luuytimestä peräisin olevat sidekudoksen kantasolut (hMSCs) erilaistuivat soluviljelmässä luuta muodostaviksi soluiksi eli osteoblasteiksi, ja tuottivat mineralisoitunutta luun väliainetta kautta koko kudotun tukirakenteen. Johtopäätöksenä on, että PLA/HA yhdistelmäkuitua voidaan kutoa kolmiulotteiseksi tukirakenteeksi, ja sitä on mahdollista käyttää apuna korvattaessa luupuutoksia kudosteknologian keinoin.

Asiasanat: hydroksiapatiitti komposiitti, kuituekstruusio tekstiili, luun kudosteknologia, mesenkymaaliset kantasolut, poly lactic acid

To my mother Annie

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ACL	Anterior cruciate ligament
AFM	Atomic force microscopy
ALP	Alkaline phosphatase
BG	Bioactive glasses
BMPs	Bone morphogenetic proteins
DP	Degree of polymerization
DSC	Differential scanning calorimetry
ECM	Extra-cellular matrix
ESCs	Embryonic stem cells
FA	Focal adhesion
FDA	US Food and Drug Administration
FT IR	Fourier transform infrared spectroscopy
HA	Hydroxyapatite
hMSCs	human Mesenchymal stem cells
MSCs	Mesenchymal stem cells
Mw	Molecular weight
ICM	Inner cell mass
IgG	γ-globulin
ISCT	International Society for Cellular Therapy
PSCs	Pluripotent stem cells
iPSC	induced pluripotent stem cell
microCT	Micro-computed tomography
MDR	Melt draw ratio
PCL	Poly(caprolactone)
PGA	Poly(glycolic acid)
PDLA	Poly(D-lactic acid)
PDLLA	Poly(D,L-lactic acid)
PINP	Procollagen I N-terminal propeptide
PLA	Poly(lactic acid)
PLLA	Poly(L-lactic acid)
RGD	Arginine-glycine-aspartic acid
SEM	Scanning electron microscopy
SSD	Solid state drawing

SSDR	Solid-state draw ratio
TCP	Tricalcium phosphate
TE	Tissue engineering
Tex	Mass in gram per 1000 m
TGA	Thermal gravimetric analysis
Tc	Cold crystallization temperature
Tg	Glass transition temperature
Tm	Crystal melting temperature
VEGF	Vascular endothelial growth factor
wt%	Weight percent

Original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:

I Persson M, Cho SW & Skrifvars M (2013) The effect of process variables on the properties of melt-spun poly(lactic acid) fibres for potential use as scaffold matrix materials. J Mater Sci 48(8): 3055-3066.

II Persson M, Lorite GS, Cho SW, Tuukkanen J & Skrifvars M (2013) Melt spinning of poly(lactic acid) and hydroxyapatite composite fibers: influence of the filler content on the fiber properties. ACS Appl Mater Interfaces 5: 6864-6872.

III Persson M, Lorite GS, Kokkonen HE, Cho SW, Lehenkari PP, Skrifvars M & Tuukkanen J (2014) Effect of bioactive extruded PLA/HA composite films on focal adhesion formation of preosteoblastic cells. Colloids. Surf., B 121: 409-416

IV Persson M, Lehenkari PP, Berglin L, Finnilä MAJ, Risteli J, Skrifvars M & Tuukkanen J (2014). Osteogenic differentiation of human mesenchymal stem cells in a 3D woven scaffold: a way to grow bone. Manuscript

Contents

1 Introduction

A biomaterial is defined as a "*material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body"* (Williams 1999). The use of biomaterials for various medical applications dates back to ancient civilizations, where, for instance, linen, silk, flax, hair, grass and animal gut were used as suture material (Gajjar & King 2014). With increased understanding of biological and human physiology in the medical field more advanced and complex biomaterials have been developed. According to the type of biomaterial used, it is today possible to choose whether the material is biologically stable once incorporated into a biological environment, or whether it is biodegradable over time (Best *et al*. 2008). In view of the biodegradable materials, a new field of implantology or prosthesis science has evolved to replace defective or dysfunctional tissues and organs in the human body. In this field, known as tissue engineering (TE), cells are usually implanted into some form of a supporting structural device, termed a scaffold, which serves as a template for tissue regeneration (Place *et al*. 2009). The scaffold itself is critical to the success of TE, although a complete understanding of the scaffold-property relationship is yet to be achieved. However, it is generally agreed that an ideal scaffold should be biocompatible, biodegradable, stimulate cellular interactions and tissue formation and have proper mechanical and physical properties (Liu & Ma 2004). Several methods have been applied to fabricate three-dimensional (3D) porous scaffolds for TE with various degree of success, but far too little attention has been paid to fibers in the micro range in combination with textile-based technology in this field. The human body is full of fibers, mainly collagen within the connective tissue, but muscle, tendons and nerves are also of fibrous nature and, therefore, cells are used to handling these structures (Doser & Planck 2011). For this reason, the purpose of this thesis was to develop a bioactive fiber with good biological and mechanical properties, as well as to design a porous 3D textile scaffold utilizing weaving for bone TE applications. This thesis both augments our basic knowledge of scaffold-based TE and contributes to this growing area of research by exploring the potential of textile technology in this field.

2 Review of the literature

2.1 Bone

Bone is a dynamic, highly vascularized tissue that continues to remodel throughout the lifetime of an individual. It provides the attachment sites for the muscles and tendons, which are essential for movement, acts as a protective casing of the vital organs and encloses the blood-forming elements of the bone marrow. In addition to these structural functions, bones serve a pivotal role in the homeostatic regulation through its reservoir for calcium and phosphate ions in the blood. (Barrere *et al*. 2006, Feng 2009, Stevens 2008)

Morphologically, bone can be classified into two forms: cancellous, also called spongy or trabecular bone, and compact or cortical bone. Compact bone appears as a solid mass with little porosity (10%) (Sikavitsas *et al*. 2001) and is found mainly in long bones (femur and tibia), short bones (wrist and ankle) and flat bones (skull vault and irregular bones). In contrast, cancellous bone has a highly porous structure (50-90 %), which consists of a trabecular network, separated by interconnecting space containing bone marrow. Cancellous bone is predominantly found in metaphyses of long bone and in the vertebral body. Due to its porous structure, its modules of elasticity and ultimate compressive strength are as much as 20 times less than that of cortical bone. (Salgado *et al*. 2004, Sikavitsas *et al*. 2001) The outer surface of bone is covered by a dense fibrous layer called the periosteum, a layer of specialized irregular connective tissue with osteogenic potential. The inner surface called the endosteum, lines the marrow cavity and the spaces within spongy bone. (Kierszenbaum 2007)

By weight, bone contains approximately 60% mineral, 10% water, and 30% organic matrix (Feng 2009). The mineral phase or the inorganic matrix consists of calcium phosphate or hydroxyapatite, as well as minor trace elements such as magnesium, and carbonate (Barrere *et al*. 2006). Type I collagen constitutes ~90% of the organic matrix, and serves as a template upon which mineral is deposited (Jang *et al*. 2009). Collagen types III, V and XI are also present in small quantities and the remaining 10% is composed of noncollagenous proteins and small proteoglycans, such as osteopontin, osteocalcin, fibronectin, osteonectin, bone sialoprotein II, decorin and biglycan (Sroga *et al*. 2011). In addition, the bone contains enzymes, such as alkaline phosphatase (ALP) (used as a marker for of osteoblast phenotype) and growth factors, such as bone morphogenetic proteins

(BMPs) (Sikavitsas *et al*. 2001). The mechanical behavior of bones is determined by both the mineral part, which is primarily responsible for the compressive characteristics, and the organic matrix, which defines its tensile strength (Stevens 2008).

Bone comprises several cell types, including osteoblast, osteoclast and bone lineage cells, which are located on the bone surfaces, as well as osteocytes, which are located in the interior of the bone. Osteoblasts, osteocytes and bone lineage cells originate from mesenchymal stem cells (MSCs), whereas osteoclasts originate from hemopoietic stem cells. (Downey & Siegel 2006) Osteoblasts are fully differentiated cells and their function is to synthesize the organic component of bone matrix and to regulate the formation of hydroxyapatite crystals in the newly formed bone. Osteocytes are mature osteoblasts trapped in the lacunae within the bone matrix (Jang *et al*. 2009). These cells are responsible for the matrix maintenance and breakdown through osteocytic osteolysis to release calcium and phosphate ions for the homeostasis (Skerry *et al*. 1989). Bone lining cells cover the bone surfaces that are not undergoing bone formation nor remodeling. Similarly to osteocytes, they have less cytoplasm and fewer organelles than osteoblasts (Downey & Siegel 2006). The role of these cells still remains relatively unclear, although it is accepted that they, together with the osteocytes, sense the mechanical load in the bone, which leads to bone remodeling (Jahani *et al*. 2012). Osteoclasts are large, multinucleated cells, which carry out the resorption of bone by dissolving the minerals and digesting the bone matrix. Bone resorption takes place at the osteoclastic cell membrane called the ruffled border. (Salo *et al*. 1997)

2.1.1 Bone repair and regeneration

Bone has a unique capacity to regenerate and remodel without forming a fibrous scar tissue (Sommerfeldt & Rubin 2001). The purpose of remodeling is to preserve bone strength by repairing microcracking (microscopic damage), and to maintain the calcium and phosphate homeostasis. If the volume of resorbed bone is not equal to the amount of bone that is remodeled, the risk of fractures arises. (Rodan & Martin 2000) The bone repair and regeneration, after a fracture, is a rather complex physiological process, which involves a well-defined orchestrated series of biological events (Marsell & Einhorn 2011).

Bone fracture healing can be divided into direct (primary) and indirect (secondary) cortical fracture healing. Direct fracture healing only occurs when

there is anatomical restoration of the fracture fragments and rigidly stable conditions. The bone cortex heals itself after interruption without the formation of a fracture callus, i.e., little or no periosteal response is noted. This healing is, however, less commonly seen, since most fractures heal through indirect fracture healing. (Dimitriou *et al*. 2005) Indirect fracture healing involves both endochondral and intramembranous bone ossification. In endochondral bone formation, the bone tissue replaces a preexisting hyaline cartilage, which become calcified and eventually replaced by bone. In the intramembranous bone formation the bone tissue is laid down directly in primitive connective tissue, without first forming cartilage (Dimitriou *et al*. 2005, Gerstenfeld *et al*. 2006). More specifically, the indirect healing pathways include hematoma formation, acute inflammatory response followed by the formation of a primary cartilaginous callus, which undergoes revascularization, calcification and finally remodeling.

In the initial phase immediately following the bone trauma, blood vessel rupture results in a hematoma within the fracture site (Claes *et al*. 2012). This contributes to initiation of the acute inflammatory response, which has its peak after 24 h and lasts for 7 days and is necessary for the healing to progress. During this time, inflammatory cells, such as macrophages, monocytes, lymphocytes and polymorphonuclear cells, infiltrate the area and secrete inflammatory cytokines and proinflammatory molecules. This response causes the hematoma to coagulate and to form a primary cartilaginous callus consisting of a higher vascular granular tissue, which later on becomes more fibrous. MSCs start to migrate from the periosteum and marrow in the fracture site, due to the secretion of tumor necrosis factor- α (TNF-α), and differentiate into chondrocytes, which secrete collagen and proteoglycans. (Sikavitsas *et al*. 2001) Important interleukins in the acute phase are IL-1 and IL-6, since they promote the production of primary cartilaginous callus, as well as angiogenesis via secretion of vascular endothelial growth factor (VEGF), and the differentiation of osteoblasts and osteoclasts. (Marsell & Einhorn 2011) For chondrogenesis, the transforming growth factor-β (TGF-β) superfamily members are important. This intramembranous ossification is correlated with a simultaneous activation of osteoprogenitor cells in the periosteum and endosteum, which gradually transforms the cartilaginous callus into a hard callus. At this step of the fracture healing, the chondrocytes proliferate, become hypertrophic and the extra-cellular matrix (ECM) becomes calcified. TNF-α further promotes the osteogenic potential of MSCs, but also initiates chondrocyte apoptosis. This step of the fracture healing resembles endochondral ossification, and these processes lead to the formation of woven bone with a

trabecular structure. In the final stage of bone fracture healing, the woven bone is remodeled to lamellar bone by osteon formation, and the levels of inflammatory cytokines are reduced. Remodeling and the resorption process is initiated as early as within 3-4 weeks in animals and humans, but it can take years before complete healing is achieved, depending on the size and location site of the fracture and fracture fixation used. Bone remodeling is also influenced by the mechanical stress, the bone experiences, and the process may occur faster in younger patients. (Claes *et al*. 2012, Marsell & Einhorn 2011)

2.1.2 Clinical need for bone regeneration

Worldwide, the total number of bone surgeries, that require a bone-graft is more than 2.2 million annually (Naveena *et al*. 2012). This number, however, is expected to increase in the near future due to the ageing population, making the treatment of injured/fractured bone economically one of the most important surgical interventions (Amini *et al*. 2012, Boccaccio *et al*. 2011).

A bone graft is required to treat large bone defects, which arise from bone tumor resections and severe nonunion fractures, which lack the template for an orchestrated regeneration (Stevens 2008). Its main function is to give structural support similar to callus for the fracture to prevent post-operative collapse, as well as to improve and accelerate the healing of fractures and nonunions. A bone graft can be obtained from a genetically nonidentical donor of the same species as the recipient (allograft) or from the patient's own bone (autograft), as well as from different species (xenografts). An autogenous bone graft is the gold standard treatment, which involves the harvest of donor bone usually from the iliac crest or from some other non-load-bearing site in the patient (Amini *et al*. 2012). Autologous bone grafts do not cause an immunogenic reaction upon use and have therefore, the best clinical outcome. (Sen & Miclau 2007) Nevertheless, their use is hampered by the limited quantity that can be obtained, and due to donor site morbidity resulting in heamatoma formation, infection, nerve damage, and chronic pain (Giannoudis *et al*. 2005). Allografts circumvent these drawbacks of autografts, but their rate of use is lower than that of autografts. This is related to concerns associated with possible immunological reactions in their recipient, and the potential for pathogen transmission from donor to host (Lord *et al*. 1988, Mankin *et al*. 2005). Therefore, the demand for new technologies for bone tissue replacement is very high. Recent advances in cell biology and in material science have opened up opportunities for new surgical procedures. Instead of

biologically-derived bone grafts, artificial bone grafts can replace injured or diseased bones. Biomaterials are the focus of the new technology known as TE.

2.2 Bone Tissue engineering

TE has evolved as a multidisciplinary field combining biology, medicine, and material and engineering science, which in the case of bone TE has become a rapidly expanding research area. The goal of TE is to replace, repair or enhance the biological function of damaged, absent or dysfunctional tissues, with the help of a cell supporting structure or scaffold. The purpose of the scaffold is to allow transplanted cells to attach, grow, differentiate and remodel the scaffold into a natural, functional tissue (Dvir *et al*. 2011, Langer & Vacanti 1993), as illustrated in Fig 1.

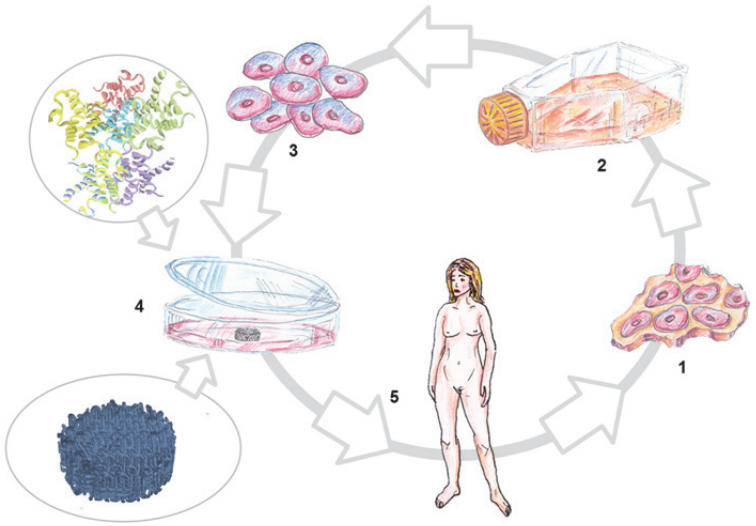

Fig. 1. A bone tissue engineering approach commonly involves cell harvest from the patients' bone marrow (1), which may be cultivated *in vitro* (2) for efficient expansion. The cells are then seeded in a porous scaffold (3), which has the shape of the desired tissue to be regenerated, together with signaling biomolecules, such as growth factors (4). Once the cells have attached and infiltrated the scaffold, the construct is transplanted either in a muscle to confirm bone activity and vascular supply or directly on the defect to induce and direct bone formation (5).

Taking this approach one step further, the ideal scaffold would be one implanted without cells, and which can regenerate the tissue based on the requirement of the native surrounding cells (Place *et al*. 2009). Whichever approach is chosen, the advantage of the TE approach is the reduced number of operations needed, resulting in a shorter recovery time for the patient.

2.2.1 Scaffold design considerations

Many studies have tried to define which properties are required for an optimal synthetic scaffold, in particular in bone tissue regeneration. The requirements are considered complex, and depend mainly on the tissue to be restored, the location and size of the defect to be treated. Nonetheless, a few basic characteristic features of the scaffold have been defined to be essential, which are as follows:

i) Biocompatibility: the scaffold should elicit an adequate response in the host tissue and prevent any adverse response of the surrounding tissue (Morais *et al*. 2010, Williams 2008)

ii) Adequate pore size and interconnected porosity: the scaffold should have open pores with fully-interconnected geometry in a highly porous structure to allow full cell penetration, tissue ingrowth and vascularization. In scaffolds for bone TE the total open porosity should be around 60-70% or more, and a minimum pore size of 100 μm is required (Karageorgiou & Kaplan 2005, Mitra *et al*. 2013, Murphy *et al*. 2010)

iii) Adequate surface characteristics: the scaffold should have suitable surface properties, both chemically and topographically to promote cellular adhesion, spreading and proliferation (Dalby *et al*. 2001). A large surface area is also needed, since most primary cells are anchorage-dependent, i.e., they require attachment to a solid surface for viability and growth (Wei & Ma 2004)

iv) Mechanical performance: the scaffold should have sufficient mechanical strength to withstand the biological forces and maintain cell integrity, which is particularly important for the regeneration of load bearing tissues, such as bone (Moutos *et al*. 2007).

v) Biodegradable/resorbable: the scaffolds should degrade or resorb at a rate that is compatible with the growth rate of the neotissue and be completely degraded upon tissue regeneration (Place *et al*. 2009). If the scaffold resorbs too slowly, new bone formation is hindered and if the scaffold

resorbs too rapidly, fibrous tissue invades the void space and scar tissue formation occurs.

vi) Processability: the scaffold should be designed and manufactured with a technique that promotes high precision and reproducibility (Almeida *et al*. 2013). The scaffold should also be sterilizable for clinical use (Paakinaho *et al*. 2009) and processed economically.

2.3 Biomaterials for bone repair

The selection of the material to produce a scaffold for bone TE applications plays a pivotal role. The materials' properties will to a great extent determine the scaffolds' properties, and hence the functionality of the structure. (Ramier *et al*. 2014) Materials that enhance bone regeneration are preferably bioactive, which refers to the materials' ability to integrate with biological molecules of cells and the surrounding tissue (Kokubo & Takadama 2006). In addition, it is desirable if the material is *osteoinductive*, *osteoconductive* and capable of *osseointegration*. Osteoinductivity refers to the materials' ability to promote differentiation of stem cells or osteoprogenitor cells to an osteoblastic lineage. Osteoconductivity refers to their ability to support the bone growth over the surface of the material and the bone ingrowth into porous implants. Finally, osseointegration is the ability to achieve bone-implant interface without an intervening fibrous layer. (Albrektsson & Johansson 2001, Giannoudis *et al*. 2005)

To date, a variety of materials have been exploited for replacement and repair of damaged and traumatized bone tissue. These materials include metals, ceramics and polymers from both natural and synthetic origins (Liu & Ma 2004). However, the criterion of biodegradable excludes the use of the metals and many ceramics. Although biodegradable metal alloys are a new class of implant materials under investigation, there is concern over their corrosion behavior *in vivo* (Witte *et al*. 2005). Another disadvantage is that the processability is very limited for these materials. Conversely, synthetic polymers are more flexible than metals and ceramics. It is reasonably straightforward to control their mechanical and chemical properties and they are relatively inexpensive to produce. (Naveena *et al*. 2012)

2.3.1 Bioceramics

When biomaterials used for bone repair shifted from being bio-inert to bioactive, bioceramics were first formulated (Best *et al.* 2008). Bioceramics are inorganic materials composed of ionic compounds, and many of them resemble the chemical composition of the mineral phase of bone, e.g., tricalcium phosphate (TCP), $(Ca)_3(PO_4)_2$ and hydroxyapatite (HA), $(Ca)_{10}(PO_4)_6(OH)_2$) (Barrere *et al.* 2006). Owing to their good biocompatibility and osteoconductive properties, they have been widely studied as a bone substitute material. In addition, trace elements in human bone, such as silicon, zinc, strontium or magnesium, are known to induce osteoblast activity by stimulating bone formation (Place *et al*. 2011, Poh *et al*. 2013). Therefore, a range of bioactive glasses (BG) have been developed and widely used in clinical settings to repair bone defects. They have the advantage, in comparison with HA and TCP, that the rate of bone bonding after implantation is rather fast (Oonishi *et al.* 1997). On the other hand, HA has emerged as the most suitable bioceramic for bone replacement therapies because of its excellent ability to adsorb proteins (Kilpadi *et al.* 2001, Ramier *et al.* 2014). Protein adsorption is an important characteristic feature since that mediates the initial cell response to the material (Dos Santos *et al.* 2008, Yanagida *et al.* 2009).

Concerning the degradation of bioceramics, it is either solution or cell-mediated; where the solubility behavior is the dominant factor, which determines the degradation rate. In general, TCP is a readily soluble ceramic, where HA is a very slowly soluble ceramic (Nakamura *et al.* 2013). Depending on the chemical composition of BG, the dissolution rate can be altered. However, in a 14-year follow-up study of BG as a bone graft substitute in benign bone tumors, remnants of glass granulates were still observed (Lindfors *et al.* 2010), which indicates that BG may not be completely eliminated from the body. Cell-mediated resorption can appear through osteoclasts, if the ceramics favor osteoclastogenesis. In a recent *in vitro* investigation it was reported that the osteoclast activity was suppressed on TCP substrates, in comparison to HA (Nakamura *et al.* 2013). The reason for this was likely related to TCP's excessive high solubility, which also increases the pH. The osteoclast activity is strongly inhibited at pH levels above 7.3, while their maximum stimulation occurs at pH 6.9 (Arnett 2003). However, if the released particles are small enough they can be phagocytosed and be eventually eliminated (Wenisch *et al.* 2003). When the elimination is not possible, or when too many particles are released, inflammatory responses may be initiated, which sometimes is a disadvantage (Ghanaati *et al.* 2012). What role osteoclasts

may play in remodeling BG once osteogenesis begins is still an unanswered question (Jones 2013).

In summary, bioactive ceramics are promising as bone substitute materials, although their clinical applications have been limited by their brittleness, high density and a porous structure that cannot sustain the mechanical loading needed for bone remodeling (Ramakrishna *et al.* 2001).

2.3.2 Biopolymers

Scaffolds for TE are commonly constructed from biodegradable polymeric materials (biopolymers), which can be categorized as naturally derived polymers or synthetic polymers. Naturally-derived polymers, such as hyaluronic acid (Collins & Birkinshaw 2013), collagen (Mostafa *et al.* 2014, Ueda *et al.* 2002), fibrin (Gao *et al.* 2014), chitosan (Di Martino *et al.* 2005) and silk fibroin (Almeida *et al*. 2013) have the advantage of biological recognition and potential bioactive behavior. However, there are several disadvantages associated with the use of naturally-derived polymers, which include limited control over the mechanical properties, biodegradability and batch-to-batch consistency (Liu & Ma 2004). There is also a potential immunogenicity issue, and they may contain pathogenic impurities (Stevens 2008). As a result of these limitations, synthetic biodegradable polymers have been extensively studied for the use in bone TE applications. Synthetic polymers are more beneficial because their mechanical properties, microstructure and degradation rate can be controlled to a great extent by their composition and fabrication technique (Naveena *et al*. 2012).

Poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(caprolactone) (PCL) and their co-polymers belong to the family of aliphatic polyesters, and are the most frequently used synthetic biodegradable polymeric materials in bone TE (Liu & Ma 2004). The reason for this is primarily the fact that they are already used in medical devices approved by the US Food and Drug Administration (FDA), which likely will accelerate the transition of the product into a clinical setting (Poh *et al*. 2013). The aliphatic polyesters have similar chemical structures as shown in Fig 2. In addition, lactic acid is a chiral molecule, which exists as two enantiomers, L and D-lactic acid. The different types of PLA are represented as copolymers of the two forms poly(D, L-lactic acid) (PDLLA), or in pure form, i.e., all L-forms and all D-forms, poly(L-lactic acid) (PLLA) and poly(D-lactic acid) (PDLA), respectively (Lim *et al*. 2008, Suesat *et al*. 2003). The polymers degrade *in vivo* through hydrolysis of the ester bond in the polymer backbone and

through bulk erosion. The degradation products are nontoxic components, natural metabolites, which then are incorporated into the tricarboxylic acid cycle and are extracted. (Catıker *et al*. 2000) The change in the chemical structure highly influences the degradation rate. As a result, PLA is more hydrophobic compared to PGA, because of the extra methyl group in the repeating PLA unit. This slows down the hydrolysis rate. Likewise, PCL is known to exhibit a higher hydrophobic character than PLA (Gomes *et al.* 2008, Sung *et al*. 2004). Nevertheless, another parameter that strongly influences the degradation rate is the degree of crystallinity, which is directly related to the polymers initial molecular weight (M_w) and process used. The amorphous domains are easily attacked by water molecules and tend to degrade before the crystalline domains. (Codari *et al.* 2012, Woodruff & Hutmacher 2010) When the crystalline domains start to degrade, the mechanical properties of the material drastically decrease. Taking these parameters into account, together with the size of the implant and the location site, the degradation time for these polymers do range from days to years *in vivo.* (Ambrose & Clanton 2004, Stähelin *et al*. 1997)

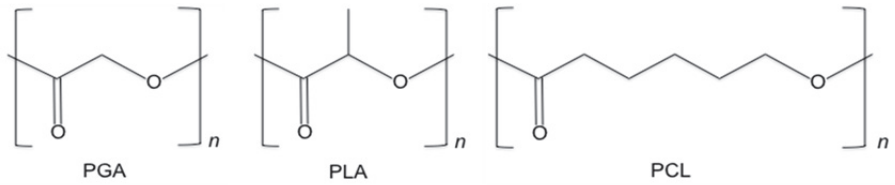

Fig. 2. Chemical structures of aliphatic polyesters commonly used in tissue engineering, poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and poly(caprolactone) (PCL).

In spite of the good biodegradability and biocompatibility of these polymers, there are some controversies around the use of these polymers for orthopedic applications. First, the polymers do not offer biological adhesion sites, hence the cell response to the materials surface is poor (Li *et al*. 2012, Sui *et al*. 2007, Woodruff & Hutmacher 2010). Second, due to the acid release during degradation, the surrounding pH is reduced. The reduced pH in the implant site has been reported to elicit an adverse tissue response and in a few cases also clinically significant local inflammatory tissue reactions (Böstman & Pihlajamäki 2000). However, Sung *et al.* (2004) have reported that the degree of inflammation caused by acidification is related to the level of vascularization of implants

through angiogenesis. In addition, it is known that osteoblast activity is impaired by low pH (Arnett 2003). In order to overcome these disadvantages, a promising approach has been to incorporate bioactive ceramics into the polymer i.e., to develop composites.

2.3.3 Composites

Composite materials are made of two or more components with significantly different physical and chemical properties that, when combined, result in a material with entirely different properties (Fowler *et al*. 2006). As mentioned previously, no single polymer or bioceramic can meet all the requirements for bone TE scaffolds. Therefore, the area of composites made from synthetic biodegradable polymers incorporated with bioceramics for bone TE has recently emerged as a huge field. The benefits with these composites are numerous. Typically improved strength, stiffness and toughness are reported, compared to their individual components (Boccaccini & Maquet 2003, Jeong *et al*. 2008, Puppi *et al*. 2012). The incorporation of bioactive ceramics in the polymer also changed the degradation profile (Danoux *et al*. 2014, Verheyen *et al*. 1993). Sue *et al.* (2007) have demonstrated that the introduction of HA into PLA slows down the degradation rate owing to OH^- release, which also helps to maintain the pH. In contrast, another study concluded that a greater weight loss was found for PLA/HA composites as compared to neat PLA upon 12 weeks immersion in simulated physiological saline (SPS) and in simulated body fluid (SBF), but PLA/HA degradation was significantly lower *in vivo* (Danoux *et al*. 2014). In addition, the bioactivity was increased in the presence of HA, which enhanced the cell attachment, proliferation, differentiation and tissue regeneration (Asli *et al*. 2012, Wu & Wang 2012). As a matter of fact, natural bone is an organic/inorganic material and, therefore, composites of polymers and bioceramics have found widespread application in bone TE applications (Liu & Ma 2004). There are considerable scientific challenges involved in the incorporation of bioceramics in polymers, and to process the composite into a desirable scaffold. It is important to consider each component's intrinsic property, since they have different degrees of processability and processing requirements. Bioceramics are in general thermally very stable, whereas biodegradable polymers are highly sensitive to elevated temperatures. In addition, the polymer's solubility in organic solvents depends on its chemical composition, molecular weight and degree of crystallization. A literature survey revealed that a homogeneous distribution of the bioceramics in

the polymer is of significant importance in order to achieve good mechanical properties (Kothapalli *et al*. 2005). Likewise, the bioceramics need to be exposed on the scaffold's surface in order to maintain the bioactive effect (Jung *et al*. 2005, Kim *et al*. 2006)

2.4 Scaffold fabrication

While the choice of material is very significant, the method by which the scaffold is fabricated is of equal importance. A number of fabrication technologies have been applied with different levels of success. Among these processing techniques for fabrication of bioresorbable polymer/bioceramics scaffolds are methods such as solvent casting and particulate leaching, gas foaming, emulsion freeze-drying, electrospinning, rapid prototyping and thermally induced phase separation. Each process technique has its advantages and disadvantages. (Liu *et al.* 2007, Salgado *et al*. 2004) Table 1 summarizes the key characteristics and parameters of these techniques. However, most of these techniques utilize organic solvents in the fabrication process, and residual solvent may be present in the scaffold, which can be harmful to the cells or the host tissue (Jung *et al*. 2005, Lee *et al*. 2004a). Another major challenge for most of the techniques is to simultaneously optimize the mechanical properties with an adequate porosity, since the mechanical properties usually decrease with increasing porosity (Chen *et al.* 2008). In addition, many of the conventional processing routes still present low reproducibility. For these reasons, researchers still seek new and better methods to produce 3D porous scaffolds. One solution may be to utilize the textile manufacturing technology to produce fiber-based scaffolds.

Table 1. Key characteristics of scaffold techniques currently used

Method	Advantages	Disadvantages	References
Thermal induced phase separation	Good mechanical properties, high porosity with interconnected pores	Use of organic solvent, limited pore size	Boccaccini & Maquet 2003, Ciapetti *et* al. 2012, Maquet *et al*. 2004, Nam & Park 1999, Wei & Ma 2004
Solvent casting and particle leaching	Simple operation, controlled porosity, controlled interconnectivity (if the particles are sintered)	Pore shape is limited to the size of the salt, limited to thickness ranging from 0.2 to 5 mm, poor mechanical properties, use of organic solvent	Kothapalli *et al.* 2005
Gas-Foaming	High porosity, relatively large pore size	Closed-pore structure with only 10-30% interconnected pores, poor mechanical properties	Montjovent *et al*. 2005, Thein-Han & Xu 2013
Emulsion Freeze Drying	High porosity, large pore size	Closed-pore structure, use of organic solvent	Sultana & Wang 2008, Whang *et al*. 1995
Rapid-Prototyping	Able to produce complex 3D structures, reproducible, anisotropic properties	Difficult to fabricate scaffold with fine microstructure, costly, time consuming	Bose *et al*. 2013, Poh *et al*. 2013
Melt moulding	Shape and geometry easily controlled, controlled pore size and porosity	Difficult to control pore distribution, requires melting of the polymer	Gomes *et al*. 2001
Electrospinning	Resemble the ECM, large surface area to volume ratio	Shape limited to sheets or cylinders, small pore size,	Chuenjitkuntaworn *et al.* 2010, Sun *et al*. 2007

2.4.1 Fiber-based scaffolds

Fiber-based structures represent a wide range of morphological properties and geometric possibilities that can be tailored and used in a variety of medical applications (Tuzlakoglu & Reis 2008). There are numerous examples of textile structures that have been successfully used in complex biological environment as

part of devices, such as heart valve sewing rings, vascular grafts, hernia repair meshes and percutaneous access devices (Almeida *et al.* 2013, King *et al*. 2001).

The main advantage of textile structures is their mechanical properties, their flexibility and elasticity, as well as their superior control over the design, manufacturing precision and reproducibility (Barber *et al.* 2011). With the developments in textile technology, it is also possible to produce porous 3D textile structures that can serve as a scaffold to engineer different biological tissues. The properties of textile structures depend on the characteristics of the constituent yarns or fibers, as well as the geometry of the formed structure. Fibers can be produced in different diameters and shapes (round, grooved, star-shaped), as well as with different strengths (Chung *et al*. 2011). The textile bonding further influences medically important geometric parameters, such as porosity and pore size. In general, a braided structure is dimensionally very stable, but less elastic and porous compared to knitted and woven structures. The yarn density and braiding angles can regulate the pore size of the braided structure (Cooper *et al*. 2005). In comparison with braided structures, knitted structures are highly porous and have better potential to support tissue ingrowth. Knitted structures are also known to exhibit better extensibility or flexibility (Yeoman *et al*. 2010), whereas woven structures normally show nearly no elasticity. In addition, woven structures are generally stronger and stiffer, thus, they have a greater potential to maintain structural stability during mechanical load (Shikinami *et al*. 2004). Very dense structures can be obtained using nonwoven technologies.

Clearly, the uses of fiber-based scaffolds have tremendous potential in TE applications, which is also evidenced by the growing number of publications in the field. Cooper *et al.* (2004) designed a 3D braided scaffold from polylactide-*co*-glycolide for anterior cruciate ligament (ACL) replacement. The study showed that the tissue-engineered ACL scaffold had mechanical properties of native ligament and adequate porosity for tissue ingrowth. Recently, Samberg *et al.* (2014) used electrospinning to fabricate a nonwoven scaffold, which contained silver nanoparticles. The poly(L-lactide-*co*-epsilon-caprolactone) scaffold containing silver nanoparticles between 0.5-1.0 mg (silver) g(scaffold)$^{-1}$ was both biocompatible and antibacterial and suitable as a skin tissue engineering graft scaffold (Samberg *et al*. 2014). Honkanen *et al.* (2003) used a 3D knitted structure made from poly(L/D)lactide 96/4, and reported that this was a new successful approach for the reconstruction of damaged metacarpophalangeal joints. Shikinami *et al*. (2010) utilized 3D woven technologies with a triaxial fiber alignment to develop an artificial intervertebral disc (AID). The discs had high

biocompatibility and superior mechanical properties when compared to conventional implants and they were also demonstrated to have potential clinical use if applied correctly by surgeons (Shikinami *et al*. 2010). Moutos *et al* (2007) also utilized 3D weaving for the design of functional TE cartilage. This study was highlighted as a breakthrough in the design of biomimetic cartilage scaffolds and several additional studies have been done using the same scaffold (Brunger *et al*. 2014, Glass *et al*. 2014, Liao *et al*. 2013, Moutos & Guilak 2009, Moutos *et al*. 2010, Ousema *et al*. 2012). However, to date, no textile structure with outstanding potential as scaffold for bone TE has been reported and economically the treatment of injured/fractured bone is so far the most important activity. In this regard, a 3D woven scaffold would have a great potential, considering the structure stability during mechanical load as previously mentioned.

2.4.2 3D weaving

Traditionally woven structures consist of two sets of yarns in two mutually perpendicular directions, warp (y-direction) and weft (x-direction). The manner in which the two sets of yarn are interlaced determines the weave. The weaves are considered to have a two-dimensional (2D) sheet form. By using various combinations of the three basic weaves (plain, twill and satin), it is possible to produce almost an unlimited variety of constructions. The type of weave in combination with the yarn used defines the structure's stability, strength, flexibility, drapeability, permeability and shape. (Hu 2008a, Islam 2012) However, to extend the use and value of woven structures into more advanced industrial applications, such as aerospace, automobile and marine industries, which typically require strength in more than one direction, 3D weaving has been introduced. (Hu 2008a, Hu 2008b, Mouritz *et al*. 1999) Following the growing medical and healthcare industry, this technology has also recently been applied in the construction of scaffolds for TE applications (Brunger *et al*. 2014, Glass *et al*. 2014, Liao *et al*. 2013, Moutos *et al*. 2007, Moutos & Guilak 2009, Moutos *et al*. 2010, Ousema *et al*. 2012, Shikinami *et al*. 2010) There are many different views on what 3D woven structures are because researchers and scientists differ in their opinions (Beheraa & Mishra 2008, Khokar 2001). One common understanding is, however, that 3D structures must have substantial dimension in the thickness direction formed by layers of fabric or yarns (Chen *et al*. 2011, Kamiya *et al*. 2000). In this context, a weave that takes the shape of three dimensions are considered to be a 3D structure.

3D woven structures are constructed either by using a specially built weaving machine or on a conventional weaving machine. The advantage of using a specially built weaving machine is that the structure can be made much thicker, and that crimp in the structure is completely eliminated, which thereby increases the Young's modulus of the structure (Beheraa & Mishra 2008, Moutos *et al*. 2007). Crimp refers to the amount of bending that occurs in a fiber as it interlaces with the fibers that are perpendicular to the fiber. Regardless of the types of machine used, it is fair to say that a variety of 3D structures with different geometrical shapes can be accomplished (Gokarneshan & Alagirusamy 2009). Chen has classified these 3D geometrics into solid, hollow, shell and nodal structures, and the differences between them are listed in Table 2 (Chen *et al*. 2011).

Table 2. 3D textile structures and weave architecture

Structure	Architecture	Shape
Solid	Multilayer	Compound structure, with regular or tapered
	Orthogonal	geometry
	Angle interlock	
Hollow	Multilayer	Uneven surfaces, even surfaces, and tunnels on
		different levels in multi-directions
Shell	Single layer	Spherical shells and open box shells
	Multilayer	
Nodal	Multilayer	Tubular nodes and solid nodes
	Orthogonal	
	Angle interlock	

The 3D solid woven structures are the most common and characterized by the presence of several layers of yarn in both weft and warp as well as in the through-the-thickness direction (y-direction). The through-the-thickness yarn can be incorporated at varying levels and angles within orthogonal, multilayer and angle-interlock woven principles to obtain desirable mechanical properties. However, the orthogonal have yarns in the three principle directions, which give a stiffer and stronger structure against tensile loading. (Chen *et al*. 2011) In this 3D orthogonal structure, the weft and warp yarns are arranged at right angles in many layers, and they are then bound together by another set of warp yarn, the binding warp, which introduces straight yarns in a through-the-thickness direction (Gokarneshan & Alagirusamy 2009, Pastore 2008). The warp and weft yarns go

straight in different layers, among them the binding yarns go up and down to bind layers of warp and weft together, as illustrated in Fig 3.

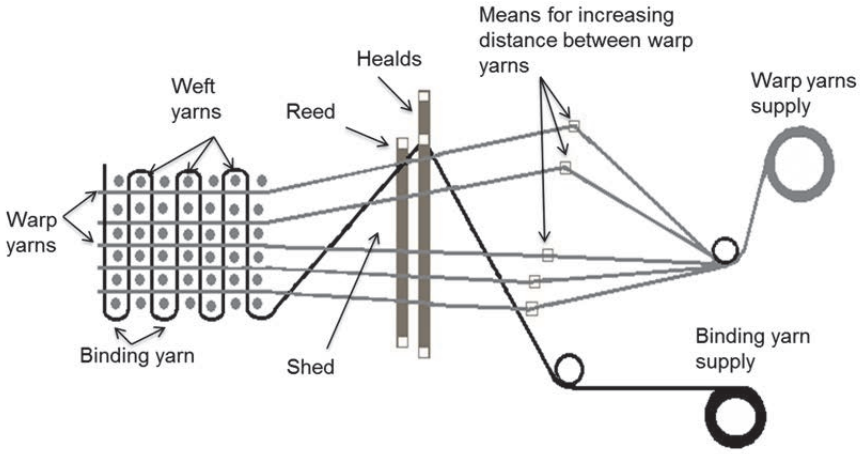

Fig. 3. Schematic view of 3D weaving. Modified from Hu 2008b

In addition, the binding yarn can be separated into two types: the ordinary and the enhanced. In the ordinary type, the binding yarn has the same number of binding yarns as the warp or weft yarns, whereas for the enhanced type, the number of binding yarns is double of the number of warp or weft yarn. (Chen & Potiyaraj 1999) The enhanced type generates a tighter weave than the ordinary type, due to the couple-binding yarns. Owing to the high porosity required in scaffolds for bone TE application, the ordinary structure is a better alternative. In addition, the interconnected internal pore dimensions (space between the fibers) can be adjusted by varying the number of yarns per centimeter (Moutos *et al*. 2007).

2.5 Fiber formation of biomaterials

The most distinctive characteristic feature of synthetic fiber materials, which separates them from other polymeric products, is the strong anisotropic material structure. Geometrically this is described by a very high aspect ratio of diameter to length. (Steinmann *et al*. 2013) On a structural level, the molecular chains tend to be aligned in the fiber axis direction, which can be separated into regions of

orders (crystals) in combination with regions of partial or complete disorder (amorphous domains). Molecular orientation in a fiber is very important, because it gives rise to its mechanical strength, affects its thermal properties, chemical adsorption/reactivity, diffusivity and optical refractive index. (Hatch 1993) Since practically all textile applications of fibers depend to some degree on their mechanical properties, the most important of these is the tensile properties. Regarding fibers used for a medical purpose, it is also important to classify them as absorbable or nonabsorbable and define their hydrolytic stability (Chu 2013).

The building block of a synthetic fiber is a polymer, which consists of long chain molecules, made up of monomer units linked by permanent covalent bonds. A chain may contain from about a hundred to several thousand monomer units, which determine the chain length and its M_w. A fiber-forming polymer should have a high M_w and long molecular chain length, i.e., sufficient degree of polymerization (DP). It is difficult to predict exactly in which range the DP should be, since different polymers are built up from different monomers that differ in chemical structure, size, shape and functionality. In general, the lower the interchain cohesive force, the higher is the minimum M_w required. In addition, a fiber forming polymer should be linear, and have small or polar pendent groups in order to be easily crystallizable, as well as meltable and dissolvable. (Hill & Walker 1948) A polymer that meets these criteria can be extruded into fibers using either melt spinning or solution spinning (i.e. dry or wet spinning). (Chu 2013, Hatch 1993)

In both melt spinning and solution spinning, the diameters of the fibers are controlled by the size of the hole in the spinneret and the diameters range from about 10 μm for multifilament to 500 μm for monofilament. It is the number of holes in the spinneret that defines the number of filaments in the fiber being produced. The number, shape and size of holes in the spinneret can vary considerably (Chung *et al*. 2011). Usually, the filament dimension is expressed in terms of yarn linear density such as tex, which is defined as the mass in grams of 1,000 m of yarn. However, to obtain finer diameters it is necessary to use an alternative spinning technique such as electrospinning, where fibers with the diameters in the range of 1 μm down to 100 nm or less have been reported. (Chen *et al*. 2006, Jeong *et al*. 2008, Sill & von Recum 2008) Finer fiber diameters are an advantage in TE applications since they resemble the natural ECM and provide a larger surface area for cell attachment (Chuenjitkuntaworn *et al*. 2010). There are, however, several limitations with this technique of electrospinning. Such limitations include the challenge of producing the fiber in significant quantities,

and the difficulty of controlling the mechanical properties, as well as the fact that the material is limited to a nonwoven sheet. Therefore, in order to obtain a fiber that can be used for weaving and knitting it is better to use either melt spinning or solution spinning, which yield fibers with an aspect ratio higher than 100. An aspect ratio of at least 100 is the minimum requirement for a fiber to be processed on regular fabrication equipment (Gajjar & King 2014). From an economical and environmental point of view, melt spinning is preferable since there is no need for solvent removal and the production speed is normally higher (Gupta *et al*. 2007). A further important advantage of the melt spinning route is the large quantity of fine fibers that can be produced (John *et al*. 2013).

2.5.1 Melt spinning

A basic design of a melt spinning process is illustrated in Fig 4. In melt spinning, the polymer resin is heated above its melting temperature and extruded through a spinneret into an air stream, which cools the melt and solidifies the filament. The cooling of the filament from melt to ambient temperature takes place under stretching stress, i.e., spin-line stress. The spin-line stress is referred to as the melt draw ratio (MDR) and is characterized by the ratio between draw down and melt extrusion speed. After solidification, the filaments usually undergo a consecutive stretching, which is known as the solid-stage drawing (SSD). Herein, the filament is commonly drawn out between godets having different speeds, where the second godets run at a higher speed than the first one (Cicero & Dorgan 2001). The speed ratio between the godets is referred to as the solid-state draw ratio (SSDR). (Steinmann *et al*. 2013) Usually, the SSD takes place at a temperature between the polymers glass transition temperature (T_g) and the crystal melting temperature (T_m), by heating the godets. The drawing can be done in a continuous process, i.e., directly after extruding, or in a separate two-step process. In a two-step process the fibers are wound up after the melt drawing (referred to as as-spun fiber), and then the as-spun fibers undergo the SSD. In both cases, the fibers are drawn down to a thinner section, which causes the disordered microfibrils to become more oriented in the direction of the fiber axis. Regions of order, crystals, are formed within the microfibrils through individual molecular chains bonding together along the length, in combination with regions of partial or complete disorder. The disorder regions, amorphous regions, are between and at the surface of the microfibrils. (Gupta 2013, Rawal & Mukhopadhyay 2014)

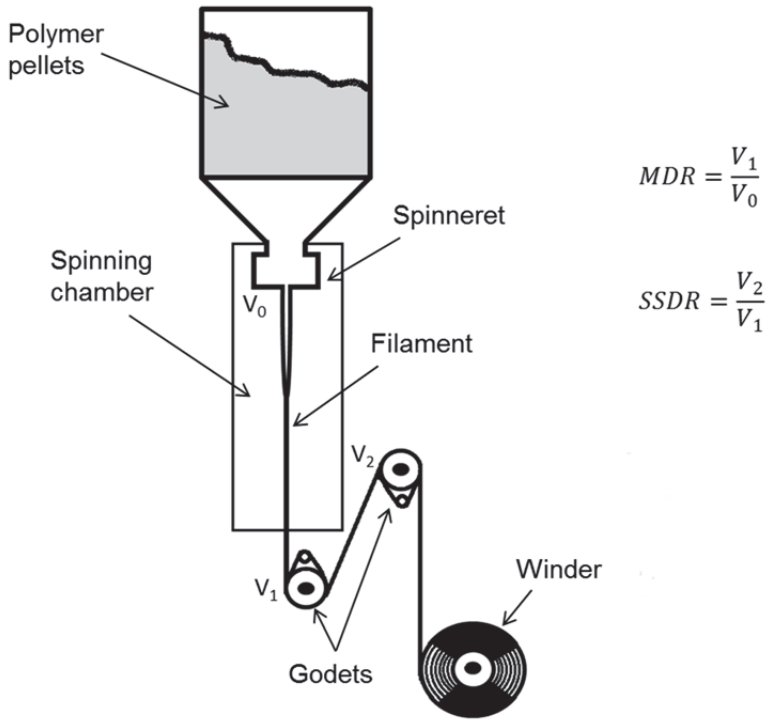

Fig. 4. Schematic view of melt spinning process. The molten polymer is extruded into an air stream, which cools the melt and solidifies the filament under melt draw ratio (MDR), where V_0 is the filament speed at the spinneret exit and V_1 is the filament speed at the first godet. The filament is further undergoing solid-state drawing (SSD) under temperature controlled godets, where v_2 is the speed at the second godet.

Melt spinning requires thermoplastic polymers and preferable polymer that are not affected by the elevated temperatures, which are necessary in the melt spinning process (Hatch 1993). The melting temperature of the polymer should be higher than its T_m but lower than its decomposition temperature.

Synthetic degradable polymers derived from the three monomers, lactide, glycolide and caprolactone are commonly used clinically as suture thread. All three polymers have also been studied using melt spinning, although the process is complicated due to their thermal instability (Pal J *et al.* 2013, Yang *et al*. 2007, Yuan *et al*. 2000). During processing, parameters, such as melt temperature and initial $M_{w,}$ are known to influence the degradation rate. Regarding PLA, which is the most widely studied bioresorbable polymer by melt spinning, it is reported

that there is a direct correlation with initial M_w and thermal stability, which decreases with increased M_w (Yuan *et al*. 2001). One solution to overcome this problem is to add additives like tris (nonylphenyl) phosphate prior to processing (Cicero *et al*. 2002a), but this is not attractive when the intended applications are in the medical field (Gupta *et al*. 2006b).

2.6 Cell-material interaction

In TE cell adhesion to the biomaterial's surface is critical because it appears that the quality and quantity is a determining step in any further cell function, such as cell spreading, migration, differentiation and tissue regeneration (Costa *et al*. 2013, Deligianni *et al*. 2000, Rizzi *et al*. 2001). Since the growth and function of many primary cells require attachment and spreading on a solid substrate, the events surrounding cell adhesion are fundamentally important. Cell adhesion and its performance have been reported to depend on the characteristics of the biomaterial surface, including chemical composition (Li *et al*. 2012, Wang *et al*. 2004), surface charge (Keselowsky *et al*. 2004), water wettability (Cassidy *et al*. 2014, Keselowsky *et al*. 2003) and roughness (Costa *et al*. 2013, Deligianni *et al*. 2000, Rechendorff *et al*. 2006, Webster *et al*. 1999, Yanagida *et al*. 2009). Understanding the mechanisms whereby cells sense and respond to a surface has facilitated the development of biomaterials with controlled cell behavior (Okada *et al*. 2010). On the other hand, to evaluate the cells actual response to a biomaterials' surface, *in vitro* cell culture has to be performed because no universal basic rules are applicable to predict cell behavior just by knowing certain material surface properties (Ma *et al*. 2007).

The attachment of cells to a biomaterial surface is closely related to proteins adsorbed on the surface, which is dependent on the materials' chemistry and morphology (Chaudhary *et al*. 2012, Tamada & Ikada 1993, Van Wachem *et al*. 1987). Protein adsorption is the first event upon introduction of a material into a biological milieu, and it is through the adsorbed protein layer that cell membrane-associated receptors interact with the material.

The ECM adhesion receptors are called integrins, which are heterodimeric transmembrane receptors composed of an α- and β-subunit, which are connected to the cytoskeleton (Mitra *et al*. 2013, Ruoslahti & Pierschbacher 1987). For instance, in MSCs, osteoprogenitors and osteoblasts, which are anchorage-dependent cells, the attachment is mediated by specific structures called focal adhesions (FAs). FAs are large protein complexes, assembled by intra and

extracellular proteins, which are coupled to each other through transmembrane integrins (Kim & Wirtz 2013). The cell surface integrin receptors anchor cells to the adsorbed adhesion proteins, via the specific tri-peptide sequence, the RGD (arginine-glycine-aspartic acid) motif (Cavalcanti-Adam *et al*. 2006, Elbert & Hubbell 1996, Huang *et al*. 2009). Important cell-adhesion proteins that have these chemotactic RGD motifs are fibronectin and vitronectin (Anselme 2000, Moursi *et al*. 1996). In the absence of such proteins in the cell surroundings (i.e. in serum free medium) the cells secrete these proteins (Van Wachem *et al*. 1987). Nonetheless, upon anchoring to the cell-adhesion protein, the integrins at the cell surfaces cluster, which leads to the assembly of actin filaments. This clustering and rearrangement of the cytoskeleton is evident in the formation of FAs (Cassidy *et al*. 2014). Vinculin, which is a component of the cell's FA plaque, can easily be stained by immunocytochemistry for FA visualization (Cassidy *et al*. 2014, Cavalcanti-Adam *et al*. 2006, Humphries *et al*. 2007, Hunter *et al*. 1995). Moreover, the cell surface integrins also activate signal transduction pathways, which regulate gene expression and phenotypical responses, such as proliferation or differentiation (Anselme 2000, Wu & Wang 2012). However, the differentiation of progenitor cells is dependent on more than biochemical signaling. The behavior of cultured cells on surfaces with edges, grooves, or other textures is different from the behavior on smooth surfaces. If grooves are present on the surfaces, it is known that cells orient and migrate along these, a phenomenon called contact guidance (Boyan *et al*. 1996). In a recent study by Cassidy *et al* (2014) it was demonstrated that grooved surfaces increased the number of FAs formed but decreased the average FAs' length in relation to planar controls on human osteoprogenitor cells. As a result, the expression of osteogenic markers (RUNX2 and BMPR2) was down regulated by grooved surfaces. In addition, it has been demonstrated that the spatial organization of cell recognition motifs influence cell spreading and FA assembly. The size and density of integrin clusters are decreased in cells plated on RGD surfaces at large distances, i.e., >58 nm, and the cell adhesion is "turned-off" if the RGD patterns are ordered (Cavalcanti-Adam *et al*. 2006, Huang *et al*. 2009). Furthermore, the elastic modulus of the substrate on which cells reside, also affect the cell differentiation. Cells use the FAs for anchoring, but also for recognition of force regimes and on rigid materials that mimic bone cells differentiation into osteoblasts (Charras *et al*. 2001, Ko & McCulloch 2001).

2.7 Cell sources for bone tissue engineering

A key aspect in TE approaches is the choice of a reliable source of cells that allows isolation, is easily expandable to higher passages and permits control of cell differentiation *in vitro* (Salgado *et al*. 2004). Primary cells derived from the patient's healthy tissue (i.e. autogenic cells) and used in conjunction with scaffolds to regenerate tissues have been a successful strategy (Howard *et al*. 2008). The main advantage of using primary autogenic cells in TE applications is that problems associated with immune rejection of foreign tissues are avoided. This strategy, however, has limitations. The cell yields and proliferation rates tend to be low and harvesting a tissue sample from the patient to isolate cells is not always an option. (Heath 2000) In addition, the procedure of harvesting is associated with donor site morbidity and in some cases, limited tissue availability. The advantage of using primary cells derived from a donor (allogeneic cells) or from a different species (xenogeneic cells) are the good availability, but rejection by the host's immune system and possible pathogen transmissions are serious risks to be considered. These limitations have lead to the development of alternative cell sources. Stem cells are at the moment the center of attention in TE strategies (Griffith & Naughton 2002).

By definition, stem cells are precursor cells capable of both self-renewal and differentiation into more specialized cells (Blau *et al*. 2001). In addition, there are various sources for stem cells including embryonic tissue, bone marrow, adipose tissue and the brain that can be used for tissue regeneration. Each stem cell type has been shown to have a capacity for differentiation to cell types of multiple lineages. Stem cells are classified as totipotent, pluripotent, multipotent or unipotent depending on their capacity to differentiate into other cell types (Gomillion & Burg 2006) Unipotent cells differentiate into one type of progenitor, while embryonic stem cells (ESCs) derived from early embryos (1 to 3 days old) are totipotent cells, and can differentiate into all types of cell lineages. (Choumerianou *et al*. 2008)

2.7.1 Pluripotent stem cells

ESCs derived from the inner cell mass (ICM) of the blastocyst are considered pluripotent stem cells (PSCs), which have the capacity to develop into all three germ layers of the embryo: ectoderm, mesoderm and endoderm (Choumerianou *et al.* 2008, Poulsom *et al.* 2002, Rao & Mattson 2001). This differentiation

potential of the ICM derived ESCs makes them a valuable source for TE approaches. Another important feature of these cells is their ability to proliferate in long-term culture, while maintaining their pluripotent nature (Choumerianou *et al*. 2008). However, the use of ESCs comes with ethical dilemmas and concerns over immunological rejection of tissue leading to the need for lifelong immunosuppression and risks of tumor formation mainly due to their unlimited proliferation capability (Poulsom *et al.* 2002). Nevertheless, alternative approaches to generate PSCs by reprogramming mature cells, such as fibroblasts, have been demonstrated by Takahashi and Yamanaka (Takahashi & Yamanaka 2006). These cells known as induced pluripotent stem cells (iPSCs) have been considered in the field of TE and regenerative medicine as the optimal cell source (Harrison *et al*. 2014, Zou *et al*. 2013), since they have similar favorable features as ESCs. In addition and most importantly, the use of iPSCs will circumvent the ethical issues surrounding ESCs. Furthermore, studies have shown that iPSCs have the possibility to de-differentiate into populations of osteogenic precursors (Bilousova *et al*. 2012), which makes them to a potential promising cell source for use in bone repair and regeneration. However, the differentiation of iPSCs towards an osteoblastic phenotype presents numerous of problems, including time-consuming methods, poor reproducibility and low efficiency (Kanke *et al*. 2014, Ochiai-Shino *et al*. 2014). The differentiation of these cells also often results in a heterogeneous mixture of multiple or only partially differentiated cells (Grskovic *et al*. 2011). In addition, their clinical safety in terms of tumorigenicity has not been established. (Yamashita *et al*. 2013).

2.7.2 Mesenchymal stem cells

Adult multipotent stem cells, which have a limited differentiation capacity, have already proven their versatility in a number of human clinical trials for certain applications, including bone TE (Lee *et al*. 2004a, Mesimäki *et al*. 2009, Pittenger 2001). The use of adult stem cells, for instance MSCs, as a cell line is advantageous for several reasons. Bone marrow aspirates can be taken under local anesthesia and in combination with *in vitro* cell culture it is possible to obtain large numbers of them. In addition, it has been suggested that these are an "immune privileged" type of cells and, therefore, do not stimulate an immune response *in vivo*, which would make them suitable for allogeneic and xenogeneic transplantation (Devine 2002, Raicevic *et al*. 2011). MSCs have been widely used in TE and their application in the regeneration of bone and cartilage tissues has

rapidly progressed toward clinical practice (Haleem *et al*. 2010, Wakitani *et al*. 1994). Friedenstein was the first to isolate these multipotent cells from the bone marrow, and he described them as clonal and fibroblastic with a strong osteogenic potential (Friedenstein *et al*. 1970, Friedenstein *et al*. 1968, Friedenstein *et al*. 1966). Almost 20 years later, Caplan (Caplan 1991), termed them 'mesenchymal stem cells'. Their classical multilineage differentiation potential into bone, cartilage, fat, tendon, muscle and marrow stroma tissue types was demonstrated by Pittenger *et al*. (Pittenger *et al*. 1999, Salgado *et al*. 2004). MSCs are mainly isolated from the bone marrow but can also be isolated from other tissues, such as adipose tissue (Zuk *et al*. 2002), umbilical cord blood (Lee *et al*. 2004b), skin dermis (Dominici *et al.* 2006), and placenta (Miao *et al*. 2006). In order to distinguish MSCs from other cells in the mesenchymal tissues the International Society for Cellular Therapy (ISCT) has established three minimal criteria they must demonstrate *in vitro:* (i) adherence to plastic in standard culture conditions (ii) specific surface antigen expression, and (iii) multipoint differentiation along osteogenic, chondrogenic, and adipogenic lineages under standard *in vitro* differentiating conditions (Dominici *et al*. 2006). The MSC surface markers are measured with a flow cytometer and according to ISCT, the MSC population must express greater than 95% of CD105 (endoglin), CD73 (ecto 5´nucleotidase), and CD90 (Thy-1), and lack expression (≤ 2% positive) of CD45 (pan-leukocyte marker), CD34 (hematopoietic progenitor and endothelial cell marker), CD14 or CD11b (monocyte and macrophage markers), CD79α or CD19 (B-cell markers) and HLA-DR (marker of stimulated MSCs) (Dominici *et al*. 2006). On the basis of current knowledge there is no specific single marker that can be used to identify a MSC population.

Differentiation of MSCs into osteoblastic lineages *in vitro*, usually revealed by their capacity to express alkaline phosphatase (ALP) and ability to form mineralized nodules, are normally induced by the presence of bioactive agents in the cell culture medium (Ramier *et al*. 2014). Osteogenesis inducing medium for MSCs isolated from the bone marrow includes ascorbic acid, β-glycerophosphate and dexamethasone. Ascorbic acid (vitamin-c) is important to promote collagen synthesis and secretion, while β-glycerophosphate is required for the formation and mineralization of bone-like extracellular matrix (Jaiswal *et al*. 1997). Dexamethasone, which is an anti-inflammatory steroid, has been reported to stimulate the osteogenic differentiation demonstrated by up-regulated ALP activity, although its mechanisms in the early stages of osteogenic differentiation are not very well understood (Hamidouche *et al*. 2008, Naveena *et al*. 2012). In

fact, osteogenic differentiation of MSCs is also possible without dexamethasone (Hoch *et al.* 2012). In addition, MSC differentiation can be mediated by the cell-matrix interaction cultured on various scaffold matrices, as mentioned before. (Gigante *et al*. 2009) These authors reported that MSC culture on type I + II collagen differentiated into cells expressing chondrocyte markers, while those cultured on type I collagen + HA differentiated into osteoblast-like cells.(Gigante *et al*. 2009)

As stated before, there are several advantages of using MSCs in TE repair, but there are some issues regarding the proliferation and differentiation capability of donors from different ages as well as between individuals of the same age. It has been demonstrated that the MSCs isolated from elderly patients exhibit a decreased proliferation capacity and differentiation potential compare to younger donors (D'Ippolito *et al*. 1999, Mendes *et al*. 2002, Stenderup *et al*. 2003). Contradictory to that, it has also been demonstrated that the osteogenic potential of MSCs does not diminished with age at late adulthood (Leskelä *et al*. 2003). Nevertheless, their ability to adhere to cell culture plastic, which makes them easy to culture and their accessibility endorse their utility for bone TE approaches, at least until some of the issues regarding PSCs are solved.

3 Aims of the study

Biodegradable materials in the form of fibers and yarns have attracted increasing attention in the field of TE since they provide a large surface area to volume ratio and thus are desirable as a scaffold matrix material. Using textile technology, fibers can also be easily processed into a variety of shapes and sizes with high precision. For load-bearing tissue, such as bone, the design requires a stiff polymeric scaffold with high mechanical strength. Due to the developments in textile manufacturing technology, it is now possible to produce 3D woven structures, which could be ideal material for resorbable porous scaffolds to meet such requirements. Therefore, the overall goal of this study was to develop a fiber based textile scaffold utilizing 3D weaving that can direct a specific cellular response, so that damaged bone tissue can be regenerated with full recovery of its biological function.

The specific purposes of the study were as follows:

1. To produce PLA fibers by melt spinning and study the influence of process parameters on the fibers' mechanical, thermal and morphology properties.
2. To develop a methodology suitable for melt-spinning of composite fiber composed of PLA and HA and to optimize the loading concentration of HA so that the fiber still maintains the mechanical properties needed for further processing by weaving into a textile structure.
3. To evaluate the impact of different loading concentrations of HA in PLA composites on the initial cell attachment of preosteoblastic cells in order to examine the underlying biocompatibility of the material *in vitro.*
4. To fabricate a textile scaffold utilizing 3D weaving and investigate the impact of 3D architecture compared with 2D control surfaces on the osteogenic capacity of hMCSs.

4 Materials and methods

The methods used in the thesis are summarized in Table 3 below. Detailed information with references is described in the original papers I-IV. The human bone marrow derived MSCs used in paper IV were collected with the approval from the Ethical Committee of The Northern Ostrobothnia Hospital Districts.

Table 3. Methods used in the original publications (I-IV).

Methods	Used in
Material preparation	
Master batch preparation of PLA and HA composites	II, III, IV
Melt extrusion	II, III, IV
Melt spinning	I, II, IV
Scaffold construction	IV
Material characterization	
Intrinsic viscosity measurements	I, II
Fourier transform infrared spectroscopy	I, II, III
Thermal gravimetric analysis	I, II, III
Differential scanning calorimetry	I, II
Dynamic mechanical thermal analysis	I
Tensile testing	I, II
Gas chromatography	II
Micro-computed tomography	IV
Microscopic techniques	
Optical microscopy	I, II
Atomic force microscopy	II, III
Scanning electron microscopy	I, II, III, IV
Confocal laser scanning microscopy	III, IV
Protein analysis	
Protein assay	III
SDS-Page - farmer's reducer sensitized silver staining	III
Western blotting	III
Cell cultures	
Murine calvarial preosteoblasts (MC3T3-E1)	III
Human bone marrow derived MSCs	IV
Cytochemical staining	
Immunofluorescence staining	III, IV
Fluorescence – activated cell sorting	IV
Cell proliferation and osteoblastic differentiation analysis	
Cell viability- *MTT* assay	IV
Alkaline phosphate activity	IV

Alkaline phosphate staining	IV
von Kossa staining	IV
Statistical analysis	II, III, IV

5 Results

5.1 Important process parameter of melt-spun PLA fibers

The most important process parameters affecting the physical and mechanical properties of PLA fibers prepared by melt spinning in this study were: the extrusion temperature, extrusion time, initial M_w, the cross sectional diameter of the hole in the spinneret, the MDR and the SSD.

5.1.1 Influence of melt draw ratio

For our set up of the experiment the volume throughput was controlled by an adjustable piston speed and was kept constant at 1.33 cm^3/min. The winding speed was set to 8.9 m/min, which correlated to a MDR of 5.3 for the monofilament fibers and 14.2 for the multifilament fibers. Due to this MDR, polymer chains were oriented and orientation-induced crystallization took place. Higher MDRs induced a higher degree of crystallinity, which subsequently was reflected in the thermal properties of the fibers (Table 4). However, the MDR was rather low in this experiment, thus, the as-spun fibers were characterized to be brittle with low tenacity (Table 4).

Table 4. Thermal and mechanical properties of as-spun PLA and PLLA mono-and multifilament fibers

Parameters	PLA		PLLA	
	Monofilament	Multifilament	Monofilament	Multifilament
Thermal Properties[1]				
T_g (°C)	60.9 ± 0.5	63.6 ± 0.3	57.4 ± 0.3	61.4 ± 0.7
T_c (°C)	124.0 ± 2.1	114.5 ± 0.6	109.4 ± 0.2	100.9 ± 0.4
T_m (°C)	167.7 ± 0.4	167.2 ± 0.3	178.7 ± 0.4	177.8 ± 0.0
Crystallinity (%)	3.8 ± 0.4	24.2 ± 1.6	7.9 ± 1.8	39.5 ± 2.5
Mechanical properties				
Initial modulus (cN/Tex)	82.0 ± 12	161.2 ± 15.8	71.0 ± 10.0	198.1 ± 15.9
Tenacity at max force (cN/Tex)	4.6 ± 0.3	3.7 ± 0.8	4.3 ± 0.6	6.1 ± 1.0
Elongation at break (%)	11 ± 2	31 ± 16	8 ± 1	4 ± 1

[1]Thermal properties; T_g = glass transition temperature, T_c = cold crystallization temperature, T_m =crystal melting temperature

5.1.2 Influence of solid-state drawing process

The total draw ratio (i.e., MDR + SSDR) plays an important role in the structural development of the fiber. In order to improve the tensile strength, the as-spun fibers were subjected to SSD. The SSD temperature had a pronounced influence on the fibers' properties. The optimum SSD temperature was observed to be 70 °C for PLA and 90 °C for PLLA, independent of filament formation. With increasing SSDRs, a steady trend towards higher molecular orientation and crystallinity was observed, accompanied by improved tenacity. In addition, the SSD was dependent on the as-spun fibers initial crystallinity and cross section diameter. The lower the MDR of the as-spun fiber, the higher maximum attainable SSDR could be used. In addition, an increase in the fibers opacity and density was observed with increasing SSDR, which was attributed to enhancement of the molecular orientation and effective packing between chains in the filaments (Fig 5). The highest tenacity values were found for the PLA polymer with the highest M_v (i.e. 545 600 compared with 178 300). The monofilament fiber showed a maximum tenacity of 28.9 cN/Tex at SSDR 6 and 18.7 cN/Tex at SSDR 3.5 for the multifilament fiber, respectively.

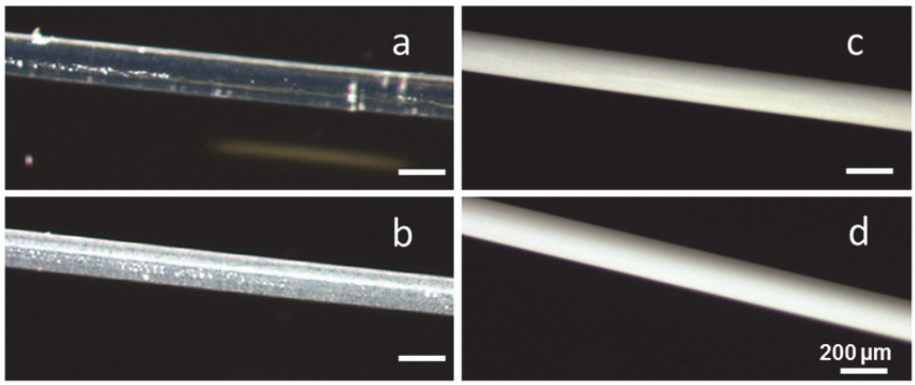

Fig. 5. Optical micrographs of PLA monofilament fibers showing that they become opaque with increasing SSDR, a) SSDR 3, b) SSDR 4, c) SSDR 5 and d) SSDR 6. Scale bar = 200 µm. (Paper I)

5.1.3 Influence of extrusion time

The duration of the melt extrusion significantly influenced the thermal degradation of the fibers, which was strongly dependent on the initial *Mv* of the polymer. For the PLLA fibers with higher M_V (i.e. 545 600 vs 178 300) the extent of decrease was almost 58% after 60 min heat exposure, while the lower M_v PLA polymer showed an insignificant degradation during the melt spinning. However, when the low M_v PLA polymer was subjected to two consecutive thermal processing steps (i.e., compounding and melt spinning), as in paper II, the extent of decrease in M_v dropped sharply by 89.7%, but without significant effects on the fibers mechanical properties.

5.2 Incorporation of HA in PLA matrix

Incorporation of HA particles in the PLA matrix was successfully achieved by combining a solution-based method followed by compounding extrusion. In the solution process, the HA particles were dispersed in 1,4-dioxane and mixed with the PLA granulates under magnetic stirring until the solution became homogeneous. Then, PLA/HA microspheres were obtained by freezing droplets of the solvent in liquid nitrogen before the solvent was allowed to evaporate. The HA particles where thermally imbedded in the PLA matrix using a twin-screw extruder. Films could then be formed by drawing the sample directly from the extruder, or granulates were made to be further processed into fibers. This technique enables homogeneous dispersion of HA in the PLA matrix and prevented the HA particles from sticking to the walls of the twin-screw extruder. GC-analysis also confirmed that the 1,4-dioxane was successfully removed from the sample.

5.2.1 Influence of spinning process

The introduction of HA at various loading concentrations (i.e. 5, 10, 15, 20 wt%) in the PLA matrix did not limit the spin-ability or the SSD of the fibers. However, in order to homogenously distribute the HA particles in the PLA matrix, and to prevent unexpected polymer degradation it was necessary to prepare the fibers in a three step process, as previously described. From the study (II) it was concluded that the processing was feasible to prepare PLA/HA composite fibers with excellent mechanical strength and thermal stability to be useful in a textile

process. In addition, it was demonstrated that the particles, even aggregates, were evenly distributed in the polymer matrix and they were also located on the surface of the fibers as demonstrated with scanning electron microscopy (SEM), atomic force microscopy (AFM), Fourier transform infrared spectroscopy (FT IR) and thermal gravimetric analysis (TGA), using this experimental set-up (Fig 6). However, in the case of mechanical reinforcement, the addition of HA had an insignificant effect, although the highest tenacity was achieved with the sample containing 10 wt% HA (i.e., 16.7 cN/Tex) at SSDR 5.

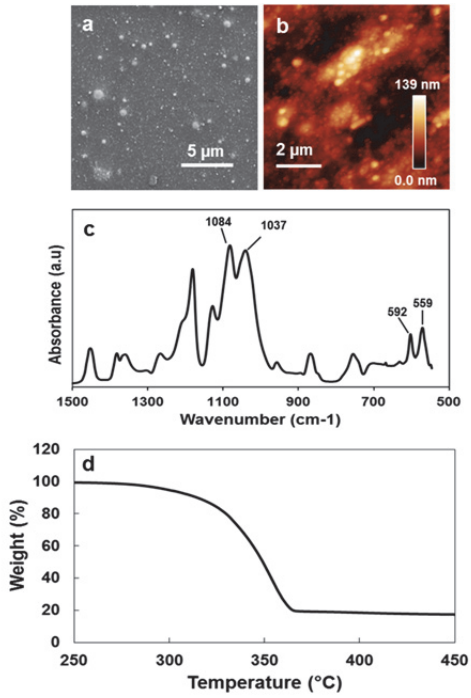

Fig. 6. Composite fibers from PLA and 20 wt% HA particles were prepared using melt-spinning and investigated in terms of their morphological properties. a) SEM demonstrate that the particles are present at the surface (seen as whitish features), b) AMF topography images show that the HA particles have a granular morphology and that agglomerates are present, c) FT IR spectra demonstrate chemical evidence of the presence of HA particles on the surfaces and d) TGA plots show that the original amount (i.e., 20 wt%) of HA particles are successfully incorporated in the PLA composite fibers. (Paper II)

5.2.2 Effect on surface properties

The addition of HA particles resulted in increased surface roughness with increasing loading concentration as confirmed with AFM. In addition, it was revealed that that the HA density (particles/surface area) increased with loading concentration, and SEM and FT IR provided evidence that the HA particles were exposed on the surface. Moreover, it was observed that the melt-spun PLA/HA fibers surface characteristics were similar to the PLA/HA composite films.

5.3 Textile scaffold construction

Four different 3D orthogonal woven structures were made from the PLA mono- and multifilament fibers as described in paper I. The mono- and multifilament fibers were combined either in the weft or the warp direction as well as in all perpendicular directions (Fig 7) in order to study the impact of the filaments on the geometrical architecture, as evaluated using micro-computed tomography (microCT).

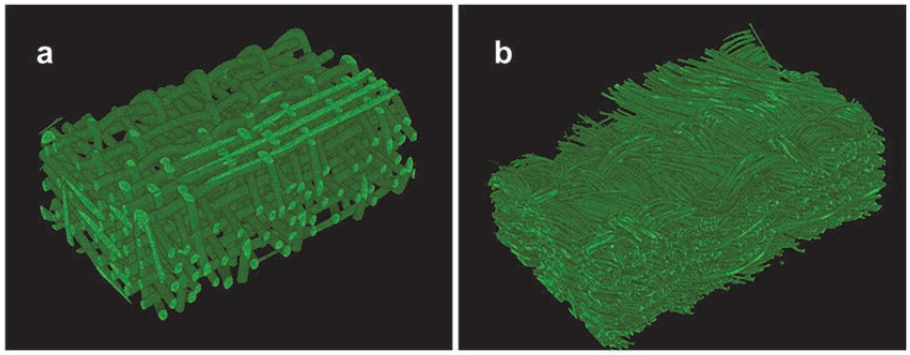

Fig. 7. MicroCT reconstruction of PLA textile structures, a) monofilament fibers and, b) multifilament fibers in all three directions

All obtained woven structures presented a regular, well-controlled architecture with 100 % interconnectivity. As expected, the size of the pores and porosity depended significantly on the type of filament (i.e. mono- or multifilament) and its directions (i.e. warp or weft) in the woven structure, as shown in Table 5.

Structures woven with multifilaments in the warp (y-) and through the thickness (z-) directions showed a smaller pore-size and less total porosity, which resulted in a denser structure compared to structures with a monofilament in the same directions. These differences were related to the differences in the respective filament thickness. However, as a result of the small fiber diameter in the multifilament yarn an increase in the surface-to-volume ratio was observed. Considering scaffolds for bone tissue regeneration, large pores are preferable and structures woven with monofilament fiber in all three principle directions resulted in a pore size of 224 micrometer and yield a total porosity of 64.2 %. Therefore this monofilament structure was selected for further studies.

Table 5. Geometrical parameters for 3D orthogonal woven structures made from PLA mono-and multifilament fibers

Geometrical parameter	Monofilament, all directions*	Multifilament, all directions	Monofilament- warp Monofilament - weft	Multifilament – warp Monofilament - weft
Total porosity (%)	64,2	69.6	73.9	67.2
Pore size (um)	224	133	220	137
Surface-to-volume ratio (mm^1)	35,8	74.6	47.6	61.3
Thickness (um)	2,0	1.03	0.99	1.20

5.4 Cell-material interaction

In this part of the work, both murine calvarial preosteoblasts (MC3TC-EI, Paper III) and hMSCs (Paper IV) were used to evaluate the responses of the cells to the polymer substrates, as well as to the textile scaffold as an important aspect in TE. It was clearly demonstrated that the materials used in the study (i.e., PLA and PLA/HA composite) were non-

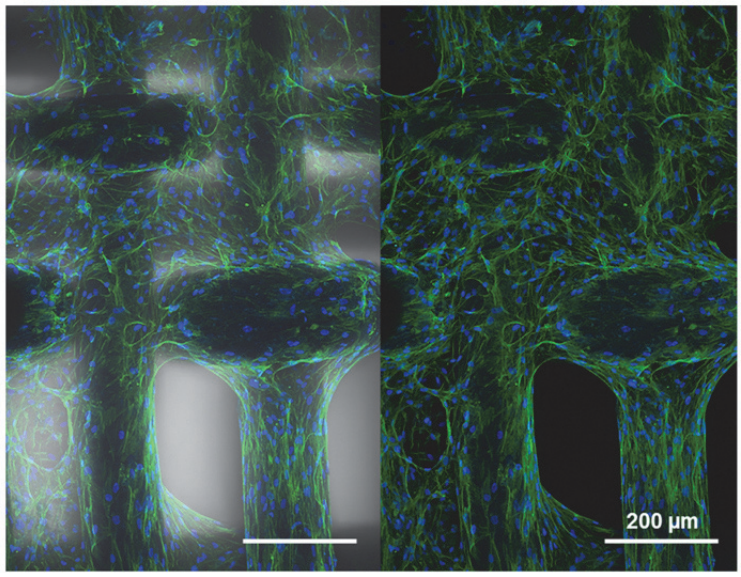

Fig. 8. Morphology of hMSCs cultured in osteogenic induction medium on a 3D HA composite woven scaffold after 21 days as visualized by fluorescent staining of actin filaments (green) and nuclei (blue). The images reveal that the cell sheet had begun to bridge across the scaffolds' fibers. Bar= 200 µm (Paper IV)

cytotoxic, and able to support cell attachment, cell proliferation and differentiation. In both cell studies (Paper III and IV), it was demonstrated that the presence of HA accelerated the initial cell attachment and proliferation, as confirmed from confocal images and MTT test (data not shown). The hMSCs were also able to form a cell sheet that bridged across the scaffold's fibers, which indicates that the pore-size distribution in the scaffold was sufficient (Fig. 8).

5.4.1 Protein adsorption

Cell adhesion to a material is closely related to the proteins adsorbed to the surface. In this study (Paper III), it was found that serum proteins adsorbed slightly more to the composite films containing higher amount of HA (i.e., > 10 wt %) than to neat PLA (Fig. 9), although the difference was not statistically significant. In addition, silver-stained SDS-PAGE gels indicated that the adsorbed protein was mainly albumin, since a species with molecular weight around 65-70 KDa was detected on all surfaces. Fibronectin, an important cell-adhesion protein

known to be present in the serum, was further studied but was not detected to be absorbed, as confirmed from the Western blot.

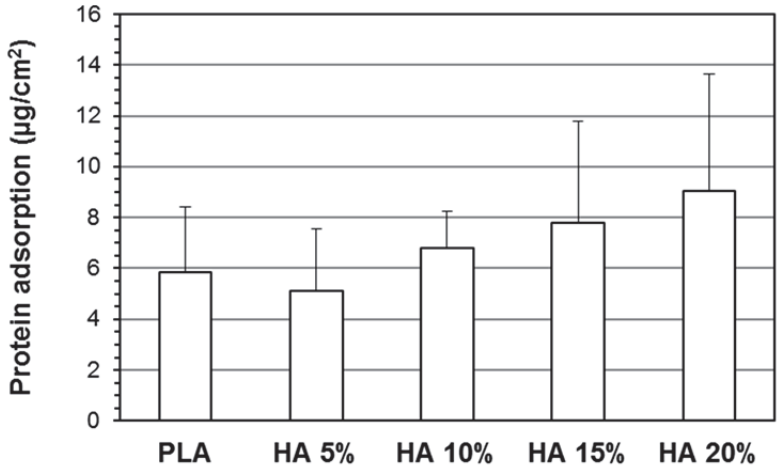

Fig. 9. Serum protein adsorption on PLA and PLA/HA composite surfaces at different HA loading concentrations (i.e. 5 wt%, 10 wt%, 15 wt% and 20 wt%) after 4 h incubation. Bars = mean ± SD (Paper III).

5.4.2 Cell attachment and FAs formation

The quality of the initial cell attachment to PLA and PLA/HA composite surfaces was evaluated after 4, 24 and 48 h culture using MC3T3-EI cells. After the initial 4h culture, it was evident that cells responded to the materials' composition in a similar manner. At the later culture times (i.e., 24 and 48 h) composite substrates containing HA supported the initial cell attachment better then neat PLA substrates, as indicated by cell proliferation and spreading. The formation of vinculin-containing FAs was also enhanced by the presence of HA (Fig. 10), and quantitative measurement (i.e. number, length and proportional area) of the FAs increased with increasing loading concentration of HA. There was also a direct correlation (R= 0.9213) between the proportional areas of FAs with the loading concentration of HA on composite films.

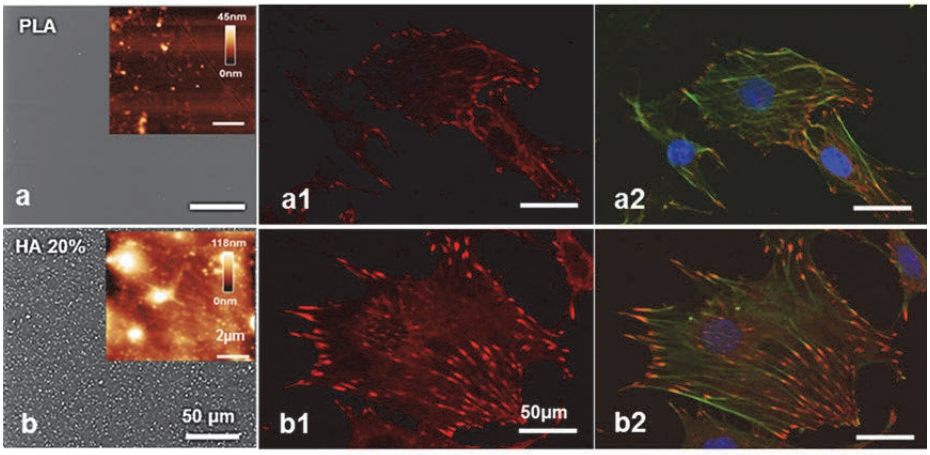

Fig. 10. Morphological characterization of PLA and PLA containing 20 wt% HA composite films surfaces using SEM and inset AFM topography image (a-b). The attachment of MC3T3-E1 cells was visualized by fluorescent staining of the vinculin formation (red) of the cells at the substrates after 48 h. Vinculin formation was diminished on PLA substrates compared to HA composite samples (a1-b1). In the merged image (a2-b2), actin filaments are seen as green and nuclei as blue, and actin and vinculin colocalization as yellow. Bar = 50 μm in SEM and confocal images, 2 μm in AFM inset (Paper III).

5.4.3 Cell differentiation and matrix mineralization

In paper IV, it was demonstrated that the presence of HA in the PLA composite promoted cell differentiation of the hMSCs into osteoblasts, as confirmed from the increased ALP activity as well as procollagen I N-terminal propeptide (PINP) secretion (data not shown). In addition, it was confirmed that osteoblast differentiation was enhanced when the hMSCs were cultured in the presence of bioactive agents such as ascorbic acid, β-glycerophosphate and dexamethasone. However, the single most striking observation to emerge from the comparison between the 3D woven scaffolds against the 2D control surfaces was the hMSCs' ability to mineralize. From the study (Paper IV), it was concluded that the use of a 3D scaffold is essential for the hMSCs to differentiate into mature osteoblasts and for cell mineralization as confirmed from SEM images, (Fig. 11 a-d). In addition, it was demonstrated from microCT analysis that the cell matrix mineralization was through-the-thickness of the scaffold (Fig. 11 e), indicating that the pore-size distribution was optimal for the cells to be able to infiltrate the scaffold.

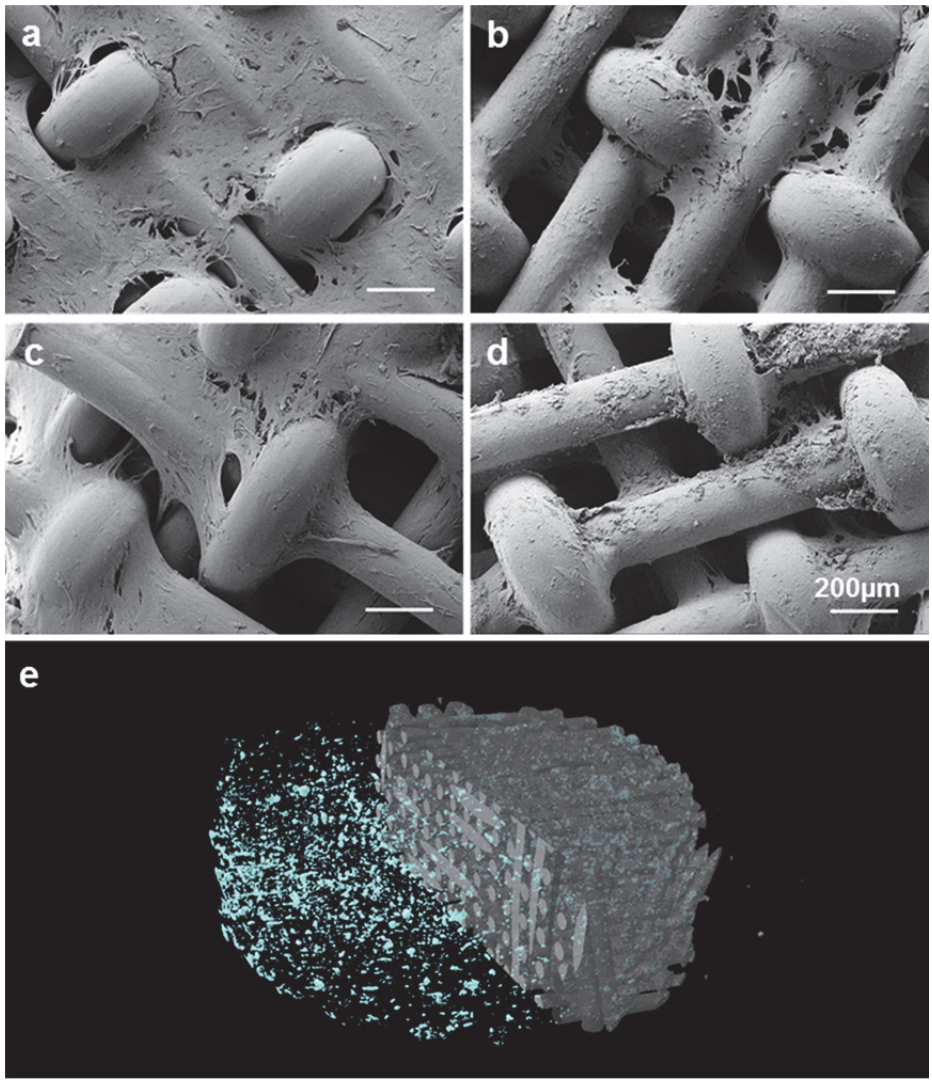

Fig. 11. FE-SEM images of 3D woven scaffolds after 35 days of culture in basic cell culture medium (a-c) and with the presence of bioactive agents, such as ascorbic acid, β-glycerophosphate and dexamethasone (b-d) on PLA (a-b) and PLA/HA composites (c-d). Cell mineralization was observed on the PLA/HA composite scaffolds' surface (d) and was also present in the through-the-thickness, as confirmed from microCT analysis (e). Scale bar in SEM images: 200 µm. (Paper IV)

6 Discussion

6.1 Melt spinning of PLA fibers

The first aim of this study was to determine the important process parameters, which influence the physical properties of melt-spun PLA fibers manufactured from two different PLA grades (i.e., clinically approved vs. fiber grade resin). Although melt spinning is a very well-known method and was one of the first methods used to produce PLA fibers (Gupta *et al*. 2007), it was considered relevant to gain a deeper knowledge in this field for further studies in this project. The important process parameters established from this study, such as extrusion temperature, extrusion time and total draw ratio, confirm the previous findings regarding melt spinning of PLA (Cicero & Dorgan 2001, Furuhashi *et al*. 2006, Grijpma *et al*. 1994, Kim *et al*. 2008, Nishimura *et al*. 2005, Paakinaho *et al*. 2009, Schmack *et al*. 2004, Suesat *et al*. 2003, Taubner & Shishoo 2001, Yuan *et al*. 2001, Zhang *et al*. 2009). In addition this study demonstrated that the MDR should be low, since higher SSDR was preferable in order to increase both the degree of crystallinity and the tenacity of the fiber. In fact, every step during the fiber preparation affects the fibers' properties, especially the molecular weight, if the polymer degrades by hydrolysis. As stated in the literature, polymers that degrade through hydrolysis are also sensitive to elevated temperatures, a feature that is a disadvantage in melt-spinning (Kim *et al*. 2008, Yuan *et al*. 2001). From that point of view, it was decided that the best method to produce the PLA fibers was by a two-step melt spinning-process. The maximum pressure of the piston could then be used, which resulted in a reduced manufacturing time, i.e., the time during which the polymer was exposed to heat was reduced. A two-step melt spinning process also enabled detailed studies of each process parameter's influence on the fibers' physical and mechanical properties. A single step process is preferred when all machine settings have been clarified.

It has been observed that there is a direct correlation between used extrusion temperature used and thermal degradation, especially for high molecular weight PLA, thus this temperature should be as low as possible (Furuhashi *et al*. 2006, Nishimura *et al*. 2005, Yuan *et al*. 2001). Therefore, the extrusion temperature in this study (Paper I) was selected based on the pristine material's T_m determined by DSC, as well as from a trial experiment using a microcompounder. Although the effort in minimizing the extrusion time and use the lowest possible extrusion

temperature, extendable thermal degradation of the higher molecular weight PLLA could not be diminished in our experimental set-up. It was still 57.9 % after 60 minutes processing. In previous studies, this molecular weight drop has been reported to be related to random chain scission reactions, intramolecular and/or intermolecular transesterification (Carrasco *et al*. 2010, Cicero & Dorgan 2001, Garlotta 2001, Lim *et al*. 2008). In addition, it is important to dry the polymer material well prior to processing to avoid a hydrolysis reaction during extrusion. As confirmed from FT IR (data not shown) the drying conditions were optimal (i.e. 80˚C for 4 h), since no new hydroxyl groups were formed from carboxylic acid and alcohols, which would be the product of ester hydrolysis.

An important step in melt-spinning is the subsequent SSD for the as-spun fibers, since the molecular chains orient during SSD and, as a result, the mechanical properties are improved. The SSD drawing temperature used has been identified as a major contributing factor affecting the molecular orientation in as-spun PLA fibers (Cicero *et al*. 2002b). In addition, it is important to use a temperature above the polymers T_g in order to increase the mobility of the molecular chain (Gupta *et al*. 2006a). Therefore, the impact of various drawing temperatures was investigated using DSC and tensile test. Based on these results, it was confirmed that for both the low molecular weight PLA and the high molecule weight PLLA optimal degree of crystallinity and thus best mechanical properties were obtained at 70 ˚C and 90˚C, respectively. These temperatures were then further used to investigate the impact of SSDR on the fibers properties. Due to the different throughput for the multifilament and monofilament fibers the MDR achieved was higher for the multifilament fibers. As the MDR increased, the molecular orientation was enhanced within the as-spun fiber and as a result, the maxima in attainable SSDRs were reduced for the multifilament fibers as compared with the monofilament ones. As expected, the maximum SSDRs achieved were higher for the high molecular weight PLLA, independent of fiber formation compared with the PLA fibers. Likewise, the mechanical properties and thermal transition temperatures were higher for the PLLA fibers. It is important to note that T_g is influenced by the degree of crystallinity in PLA (Gupta *et al*. 2007) and interestingly, no clear *Tg* could be estimated from the DSC data for the PLLA fibers. The results, therefore, indicated that the PLLA fibers crystallized sufficiently during the SSDR. In addition, differences were observed in the mechanical properties in relation to the maximum achievable SSDR between the monofilament and multifilament fibers. For the monofilament fiber, independent of molecular weight, the highest fiber tenacity was not observed at the highest

SSDR. An increased SSDR of more than 5 and 6 for PLA and PLLA, respectively, resulted in an overdrawn fiber, a phenomenon that has also been reported in previous studies. However, contradictory to previous studies (Cicero & Dorgan 2001, Nishimura *et al*. 2005), it was demonstrated that the opacity of the melt-spun PLA fibers was directly related to the degree of crystallinity of the PLA, which turned from opaque to white as the degree of crystallinity increased.

6.2 Melt spinning of PLA/HA composite fibers

The second approach of this study was to develop a methodology in order to obtain a bioactive composite fiber from PLA and HA utilizing melt-spinning. Incorporation of additives and fillers in fibers, even at low levels (i.e., 5%), is a challenging process, since it changes the spinnability, and the risk for spin-line failures increases (Lee & Youn 2008, Solarski *et al*. 2008). Nanosize materials are defined as materials in which at least one dimensions is less than 100 nm. Although nanosize additives have been shown to overcome the spinnability problem, the addition of additives is known to restrict the drawability of the fibers, which decreases for higher concentrations (Lee & Youn 2008). As stated before, drawing is an important step in the fiber process, because it gives the fiber its strength. In addition, the key elements to improve the mechanical properties are the features of the additive, such as its size and dispersion, as well as its interaction with the matrix polymer.

In order to obtain a homogenous dispersion of the additive, the dispersion technique used is crucial (Hooshmand *et al*. 2014). Therefore, in this study the composite PLA/HA fibers where prepared in a three-step process, as previously described. The three-step process allowed high incorporation of HA (i.e. 20 wt. %) in the PLA matrix, which still maintained the mechanical properties needed for further processing by weaving into textile structures. It was also demonstrated that the HA particles did not influence the SSD or the spinnability of the fiber. However, due to the two consecutive thermal processing steps (i.e. compounding and melt spinning) it was not possible to use high molecular weight PLLA in this study (Paper II), because of the prominent thermal degradation observed in the previous study (Paper I). As noted in the literature review, the use of organic solvent is a disadvantage when preparing materials intended to be used in medical applications, since there might be a risk that residues of solvent are present after processing. However, due to multiple drying steps and melting of the

material, no trace of 1,4-dioxane was detected in the final composite fiber, as confirmed from the GC analysis.

In this study (Paper II), the purpose of the composite fibers fabricated was to improve the osteoconductivity of PLA by incorporating HA into the PLA matrix. It is well known that HA confers bioactivity to PLA composite materials, but in order to maintain the bioactive properties of PLA, it is important that the particles are exposed on the surface, as well as in the bulk of the polymer. SEM, AFM and FT IR showed that the HA particles manufactured in this study were present at the surface of the fiber, and that the density of the particles (particles/area) increased with increasing loading concentration. In addition, it was demonstrated that HA agglomerates were also increased with increasing loading concentration. The aggregation occurs because PLA has no chemical affinity for the HA particles. One way to overcome this problem would be the use of surfactant (Kim *et al*. 2006), but the use of surfactants may be a disadvantage since in later contact with cells, there is an increased risk for protein unfolding.

On the other hand, the agglomerated HA particles gave rise to an increase in the surface roughness, which has been proposed to enhance cell adhesion since it will expose more surface area for possible interactions with both proteins and cells (Cui *et al*. 2009, Yanagida *et al*. 2009). Besides information on the surface roughness from the AFM topography images, the viscoelastic properties of the surface were acquired with AFM phase images. AFM phase images record the phase shift signal in tapping mode, and the contrast in the image is often interpreted as variations in the viscoelastic properties of the surface. This information is of high importance, since the matrix stiffness plays an important role in many cellular processes ranging from motility to phagocytosis and differentiation (Schneider *et al*. 2006). As a result, the obtained data showed homogenous phase images, indicating that the incorporation of HA particles or agglomerates did not affect the surface viscoelastic properties. Although the literature has reported that agglomerations of additives can influence the mechanical properties of the fibers, the composite fibers produced in this study maintained their tenacity even at high loading concentrations. However, it should be noted that there was a statistically significant difference at level 0.05 between neat PLA fibers and 20 wt% HA composite fibers in terms of initial modulus and tenacity. Based on the phase transition analysis from DSC, the observed decrease in mechanical properties could be attributed to the restricted macromolecular orientation caused by the presence of HA during the SSD. Nonetheless, similar to

the previous study (Paper I), the SSDR had a pronounced effect on all fibers' mechanical properties, which increased with increasing SSDR.

6.3 Initial cell attachment

The purpose of the third study was to analyze the effect of the incorporated HA particles at different loading concentrations (i.e. 5, 10, 15 and 20 wt. %) into the PLA matrix with respect to the surface properties on the initial cell attachment, in order to examine the underlying biocompatibility of the materials *in vitro*. The quality and quantity of the initial cell attachment is a determining step in any further cell function, implant/biomaterial biocompatibility and activity. In order to study the cell-polymer interactions without the complexities associated with 3D structures, such as porosity, pore-size distribution and their interconnections, 2D composite films were studied. Furthermore, to be able to extrapolate the results obtained from this study to the PLA/HA composite fibers, the same PLA and HA quality analyses and fabrication technique used to prepare the fibers were applied in this study. TGA measurements provided evidence of successful incorporation at all HA loading concentrations, and the presence of HA on the surface, was determined by SEM, AFM and FT IR.

An important first step in cell attachment is the adsorption of surrounding protein since it is through adsorbed proteins that cells interact with the material and HA has an excellent ability to promote protein adsorption (Kilpadi *et al*. 2001). Cell adhesion has also been demonstrated to increase when nanosize particles such as HA, have been used to compared to particles in the microsize range (Guo *et al*. 2007, Shi *et al*. 2009). As expected, a slight increase in the adsorption of serum proteins (although not statistically significant) from the culture medium was detected with increasing HA loading concentration. It was further confirmed that the surface roughness had an impact on protein adsorption, since there was no difference in the protein concentration adsorption between neat PLA and composite films containing 5 wt% HA. These samples had comparable surface roughness, as confirmed by AFM analysis. It was further revealed that there was no difference in wettability (contact angle ~ 80˚ for all surfaces), which is another indication that the surface topography is a dominant factor for protein adsorption. These results are consistent with previous studies, which also stated that the surface roughness increase the protein adsorption (Kilpadi *et al*. 2001, Rechendorff *et al*. 2006). This enhanced biological effect is attributed to the increased surface area in contact with tissue fluid and cells. Due to the presence

of agglomerated HA particles, which was especially pronounced at higher HA loading concentrations (i.e., < 10 wt%), of the PLA/HA composites, these samples had an increased surface roughness compared to neat PLA, which was also shown to improve the cell attachment. However, it should be mentioned that the serum contains many different proteins at different concentrations, of which albumin and γ-globulin (IgG) have the highest concentrations. Both of these proteins are known to inhibit and delay cell adhesion, and are more competitive adsorbents compared to cell-adhesive proteins, such as fibronectin (Tamada & Ikada 1993, Van Wachem *et al*. 1987). The findings in this study are consistent with those, since albumin adsorption was detected on the samples, but not fibronectin after 4 h immersion in the cell culture medium.

Regarding the cell attachment, it is well known that cells respond differentially to variations in surface properties. MC3T3-E1 is a cell line that is commonly used to investigate the cell-material interaction (Kokkonen *et al.* 2008, Kokkonen *et al*. 2007, Kokkonen *et al.* 2012, Lipski *et al*. 2008, Webb *et al*. 2000). The time points used to investigate the initial cell attachment were 4 h, 24 h and 48 h. In good correlation with the protein adsorption at the initial 4 h time point, no significant differences in the cell morphology and number of cells were observed between the samples. At the later time points (i.e., 24 and 48 h), the MC3T3-E1 consistently proliferated and spread better on surfaces containing HA compared to neat PLA. These results suggest that the initial cell attachment is enhanced in the presence of HA. An explanation for this could be that the presence of HA on the composite surfaces formed a more stable interaction with the adsorbed proteins, either adsorbed from the serum-proteins in the cell culture medium or from proteins secreted by the cells. In a previous study, it has been reported that the proteins secreted by the cells themselves play a more important role in cell attachment and may replace the preadsorbed proteins from the serum (Van Wachem *et al*. 1987). Furthermore, it was revealed that the cells spread more homogenously on the entire surface of PLA/HA composite films and increased the formation of actin stress fibers and FAs, as identified by the existence of vinculin. The vinculin-complex formation was also shown to be significantly improved with increasing HA loading concentration.

6.4 Textile scaffold for 3D cell cultures of hMSCs

The fourth part of study aimed to fabricate a textile scaffold utilizing 3D weaving from the melt-spun PLA and PLA/HA composite fibers from paper II and address

its usefulness as a scaffold for bone TE *in vitro.* The key to successful bone TE includes the use of complex scaffolds and advanced materials as well as the choice of cell source. Therefore, in order to gain a deeper understanding of these critical features the study was also designed to determine the impact of the 3D architecture against 2D control surfaces on the osteogenic capacity of hMSCs. Such an approach enables the study of the importance of 3D structure vs. importance of the material itself.

As stated in the literature review, the scaffold's pore-size distribution and porosity is of significant importance for sufficient cell penetration, tissue-in-growth and vascularization. Low porosity and small pore-size prevents cell infiltration and limits cell migration through the thickness, which likely results in implant loosening and resurgery over long-term implantation (Poh *et al*. 2013). However, if the pores get too large the surface area is decreased, which limits the cell adhesion and it might not be possible for the cells to bridge over the pores (Sun *et al*. 2007). Taking this into account and further considering that the average size of a human osteon is approximately 223 μm (Holmes 1979) it was decided to use monofilament fibers in all directions in the 3D woven structure since they resemble a pore-size distribution near this value (i.e., 224 μm and 249 μm for the PLA and PLA/HA composite scaffold, respectively) as determined with microCT. From the confocal images and SEM, it was confirmed that the cells were able to bridge over and to infiltrate the scaffold through the thickness.

In order to evaluate the geometrical influence and the stimulatory potential of the HA particles on osteogenesis, the ability of hMSCs to differentiate into osteoblasts was investigated. The expression of osteogenic markers, such as ALP and type 1 procollagen, are commonly revealed from *in vitro* cell culture studies (Aho *et al*. 2013). Type 1 collagen is expressed during the initial period of proliferation and ECM synthesis, whereas ALP is expressed during the post proliferative period of ECM maturation. After cellular maturation, matrix mineralization can be visualized, for instance, through von Kossa staining and SEM. Previous studies have demonstrated that composite materials from PLA and HA showed high osteogenic potential (Kim *et al*. 2013, Zong *et al*. 2014) and that the osteoconductivity of the composite was dose-dependent on HA (Danoux *et al*. 2014). In addition, it has been shown that calcium ions influence and enhance the proliferation, morphology and osteogenic differentiation of hMSCs (Barradas *et al*. 2012), whereas osteoblast-like cells treated with high levels of inorganic phosphate are shown to undergo apoptosis (Meleti *et al*. 2000). In this study (Paper IV), composite containing 20 wt% HA was used, which was shown to be

optimal for initial cell attachment (based on the formation of FAs) from paper III as well as neat PLA as a control. As evidenced from the MTT test the material used was non-cytotoxic to the hMSCs, since the cells proliferated well, and their metabolic activity steadily increased during the time tested.

Regarding the hMSCs' ability to differentiate into osteoblasts, it appeared that the geometry of the material (i.e., 3D or 2D) had a significant impact. The 3D woven scaffolds enhanced the cell differentiation, and the process was accelerated in the presence of HA. It was further observed that the presence of bioactive agents (i.e., ascorbic acid, β-glycerophosphate and dexamethasone) in the cell culture medium induced the differentiation, as expected. However, one of the more significant findings from this study was the matrix mineralization in the 3D HA composite scaffold. Compared with the 2D samples, it was concluded that the use of a 3D scaffold is essential for the hMSCs to differentiate into osteoblasts and to start to mineralize. The relevance of a 3D structure for hMSCs for the initiation of bone formation *in vitro* was clearly supported since the 2D control films used did not differ from those in the 3D scaffolds, as confirmed from paper II and III. Morphological changes in the material substrate should, therefore, not be a factor in the hMSCs' response to the material in this study.

6.5 Study limitations and future aspects

The present study makes several noteworthy contributions to the cell-material interactions *in vitro,* but to fully confirm the applications of the 3D woven scaffolds as a bone substitute material it is necessary to evaluate the material *in vivo.* In the preclinical tests, the material will face a different environment, much more complex, and probably more aggressive, which is difficult to stimulate *in vitro.* Additionally, the foreign body reaction following implantation of biomaterials may impact the biocompatibility of the material, as well as the short and long-term tissue responses (Anderson *et al*. 2008, Franz *et al*. 2011). As stated before, the degradation profile of a biodegradable material is also different *in vitro* compared to *in vivo* studies. A reason for this could be related to the macrophages and foreign body giant cells' possible release of oxygen free radicals, enzymes and acids during the foreign body reaction (Andersson *et al.* 2008) which also could result in device failure. The effect of sterilization techniques must also be considered, since it may lead to modifications in the surface and bulk properties. Therefore, a more comprehensive study including

long term *in vivo* experiment should be done to establish the safety and useful of the material in a biological milieu.

7 Conclusions

Returning to the aim of the study posed earlier, it is now possible to state that 3D woven scaffolds could be ideal for use in bone TE applications. However, on the basis of these studies, the main conclusions from papers 1-IV, are as follows:

Paper I: The feasibility and the influence of process parameters on the formation of melt-spun mono-and multifilament fibers from a clinically approved PLA grade resin and PLA fiber-grade resin were investigated. From the study, it is concluded that the important process parameters affecting the physical and mechanical properties are extrusion temperature, extrusion time, density of the melt, cross sectional diameter of the hole in the spinneret, MDR and SSDR

Paper II: In order to improve the osteogenic properties of the PLA fibers, HA particles were incorporated in the polymer matrix. Following a three-step process of master batch preparation, melt compounding and melt-spinning, it was possible to prepare PLA/HA composite fiber even at high loading concentrations (i.e. 20 wt.%). According to the results the HA particles can be homogeneously distributed in the PLA matrix, as well as exposed on the surface. In addition, the composite fibers possessed adequate tenacity to be useful in textile processes.

Paper III: The initial cell attachment was investigated on the PLA and PLA/HA composite films by screening preosteoblastic MC3T3-E1 cells at different time points (i.e. 4 h, 24 h and 48 h). It was found that the PLA/HA composite surfaces are superior to neat PLA in the promotion of protein adsorption and cell attachment, as well as the induction of actin stress fibers and vinculin expression.

Paper IV: 3D woven scaffolds using either PLA or PLA/HA composite fibers were prepared and their architecture was evaluated against 2D control surfaces made from the same material *in vitro* using hMSCs. The results clearly demonstrate that the 3D woven scaffolds enhance cell differentiation and that the HA composite containing scaffolds promote mineralized bone formation *in vitro*, whereas 2D samples impede matrix mineralization of hMSCs.

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