Biological Responses to Aliphatic Polyester Scaffolds for
Bone Tissue Engineering Applications

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To my family.
Thank you for making family such a beautiful word
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List of papers

This thesis is based on the following original papers. The papers are referred to in the text by their roman numerals.

Paper I

Paper II

Paper III
Abbreviations

α-MEM  Minimum Essential Medium, alpha modification
ALP    Alkaline phosphatase
BSP    Bone sialoprotein
CBFA1  Core-binding factor a1
cDNA   Complementary Deoxyribonucleic Acid
Col 1  Collagen type 1
C_T    Threshold Cycle
CVS    Crystal Violet Staining
DMEM   Dulbecco’s Modified Eagle’s Medium
DXO    1,5-dioxepan-2-one
D3 [1,25(OH)2D3]  1,25-dihydroxy vitamin D3
ε-CL   ε-caprolactone
ELISA  Enzyme-Linked Immunosorbent Assay
FCS    Fetal Calf Serum
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
HOBs   Human osteoblast-like cells
ISO    International Organization for Standardization
LDH    Lactic Dehydrogenase Based
LLA    L,L-lactide
L929   Mouse fibroblast cell line
2^ΔΔCT  method Comparative C_T method for relative quantification
mRNA   messenger Ribonucleic Acid
MTT    Methylthiazol tetrazolium
OC     Osteocalcin
OD     Optical Density
OP     Osteopontin
PBS    Phosphate buffered saline
PDI    Poly Dispersity Index
PI     Propidium Iodide
P(LLA) Poly(L-lactide)
Poly(LLA-co-CL) Poly(L-lactide-co-ε-caprolactone)
Poly(LLA-co-DXO) Poly(L-lactide-co-1,5-dioxepan-2-one)
qRT-PCR Quantitative Reverse Transcription-Polymerase Chain Reaction
Runx2  Runt related gene 2
SEM    Scanning Electron Microscopy
Sn(Oct) 2 Stannous 2-ethylhexanoate
TCPS   Tissue Culture Polystyrene
Summary

Currently, various strategies are used to stimulate healing of bone defects and restore lost alveolar bone and periodontal support. The concept of tissue engineering has emerged as a valid approach to the current therapies for bone regeneration and is attracting considerable attention. Skeletal tissue engineering requires a biocompatible scaffold conducive to cell attachment and maintenance of cell function, in combination with a rich source of osteoprogenitor cells and osteoinductive growth factors. Selection of the most appropriate material to produce a scaffold is an important step towards the construction of a tissue engineered product. Copolymers of Poly(L-lactide)-co-(e-caprolactone) [Poly(LLA-co-CL)] and Poly(L-lactide)-co-(1,5-dioxepan-2-one) [Poly(LLA-co-DXO)], with better mechanical properties than the resorbable aliphatic polyester Poly(L-lactide) P[LLA], have recently been developed for application as scaffolds in bone regeneration. The influence of these scaffolds on osteogenic potential is unclear and need to be addressed. Further, little is known about how cells respond at a molecular level to tissue engineered scaffold materials.

Thus the overall aims of this series of in vitro studies were to investigate biocompatibility and changes in gene and protein expression profiles caused by these scaffolds and to elicit specific expression profiles and biological pathways at the molecular level. The scaffolds were produced by solvent casting particulate leaching technique.

The aim of Study I was to evaluate the biocompatibility of Poly(LLA-co-CL) and Poly(LLA-co-DXO), using the commonly used material, P[LLA] for reference. Scanning electron microscopy and light microscopy showed that the cells had grown and spread well on the test co-polymers. Indirect contact cytotoxicity tests
(extraction), performed according to ISO requirements, showed that the test scaffolds are not cytotoxic.

The aim of Study II was to assess the growth and differentiation of human osteoblast-like cells (HOBs) seeded onto the two copolymer test scaffolds, Poly(LLA-co-CL) and Poly(LLA-co-DXO) using P(LLA) scaffolding as a control. Cellular response to the scaffold materials was expressed in terms of synthesis of the osteoblast differentiation markers collagen type 1 (Col 1), alkaline phosphatase, bone sialoprotein, osteocalcin (OC), osteopontin and runt related gene 2. Surface analysis disclosed excellent surface attachment and spread of the cells on the test scaffolds compared to the control. Cells grown on the test scaffolds demonstrated higher production of Col 1 and OC and also increased bone marker mRNA expression. Compared to scaffolds of P(LLA), the Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds enhanced attachment and differentiation of HOBs in vitro.

Study II clearly showed that the different scaffold materials had influenced genes involved in differentiation of HOBs. Furthermore, a previous study had shown that Poly(LLA-co-DXO) was significantly more hydrophilic than Poly(LLA-co-CL). Based on these findings, selected scaffolds were loaded with HOBs for 24 hours and 21 days for microarray screening of differential gene expression. Therefore, the aim of Study III was to explore and compare the gene expression profiles of HOBs derived from alveolar bone and to find possible biological pathways involved using cells involved in repair of bone defects following culture on Poly(LLA-co-DXO) or P(LLA). For the cells cultured on Poly(LLA-co-DXO) scaffolds, more genes were found to be differentially expressed (up- or down regulated) at 24 hours than at 21 days, when compared with cells cultured on P(LLA). Most of these genes were related
to cell adhesion, cell cycle, cell division, cytoskeleton, anti-apoptosis, proliferation and bone mineralization. Three main pathways involving integrin signaling, Notch signaling and Ras Pathway were found. For selected candidate genes, the results were confirmed using quantitative real time reverse transcription polymerase chain reaction.

In summary, the results demonstrated that the co-polymers tested in these studies are non-cytotoxic and biocompatible. Compared to scaffolds of P(LLA) and Poly(LLA-co-CL), the Poly(LLA-co-DXO) scaffolds enhanced attachment and differentiation of HOBs in vitro. Microarray analysis disclosed marked differences in global gene expression profiles between HOBs seeded onto Poly(LLA-co-DXO) and P(LLA) scaffolds, especially after 24 hours incubation. Statistical analyses at the chip and probe levels indicated that several genes were differentially expressed as a function of Poly(LLA-co-DXO) scaffolds. Detailed analysis of genes exhibiting differential expression revealed several molecular pathways related to cell adhesion, cell-cell communication and cell proliferation. Therefore, it is concluded that these scaffolds might be appropriate carriers for bone engineering.
1. Introduction

Worldwide, the World Health Organisation has declared 2000 - 2010 as “The Decade of Bone and Joints”. Joint diseases account for half of all chronic conditions in people over 65 years of age and the incidence of fractures due to osteoporosis is increasing. Thus bone disease afflicts over 40% of all women over 50 years of age and treatment of trauma-related skeletal deformities and skeletal disorders accounts for over 25% of health expenditure of developing countries (Tsou & Chng 2002).

Many recent advances in dentistry have been achieved by implementing new technologies, such as high-speed handpieces, modern restorative materials, improvement of prosthetic rehabilitation for replacing missing teeth, implant dentistry and recently tissue engineering (Baum & Mooney 2000, Tyagi & Dhindsa 2009). Tissue engineering is an interdisciplinary field that applies basic engineering principles, with the main objective of developing a biological substitute(s) capable of restoring, maintaining, or improving tissue function, or replacing a whole organ (Langer & Vacanti 1993). A basic requirement of the technique, however, is a fundamental interplay between cells and scaffolds and in some cases growth factors (Howard, et al. 2008).

The technology of tissue engineering has the advantage of bringing together the power of modern biology, chemistry, and physical science with the main objective of helping to solve clinical problems. Of major interest is that tissue engineering can be applied to a number of problems that can be managed by general or specialist dentists. Good examples are cases involving bone or teeth fractures, craniofacial defects, pulp-dentin complex destruction and periodontal diseases. Thus in the field of dentistry, tissue engineering technology holds promise as an improved treatment
approach to intraosseous periodontal defects; enhancement of maxillary and mandibular grafting procedures and the possibility of achieving regrowth of missing teeth (Baum & Mooney 2000, Tyagi & Dhindsa 2009).

In coming decades, tissue engineering technology is expected to have a fundamental impact on the practice of dentistry. However, before potential clinical applications in dentistry can be achieved, major challenges must be addressed. The benefits related to repair and replacement of mineralized tissues, oral wound healing promotion and gene transfer application may be seen during the coming years.

In the oral cavity, management of patient problems related to structure, function, aesthetics and pain constitute major challenges that affect the outcome of treatment, a treatment being often more complex than in other parts of the body. One of the major challenges facing dentistry today is how to regenerate oral and craniofacial defects that demand a combination of basic and clinical science as well as the technology of tissue engineering. However, interest in how to regenerate missing or defective teeth, pathologically affected oral mucosa or salivary glands, bone and periodontium is now attracting research efforts at both national and international levels (Scheller, et al. 2009).

1.1. Grafting materials

Large bone defects caused by tumors, infectious diseases, or trauma result in major medical need for bone regeneration. There are four major characteristics necessary for the ideal bone graft material: i) osteointegration, (ii) osteoconduction, (iii) osteoinduction, and (iv) osteogenesis. Only autogenous bone grafts fulfil all of these requirements (Finkemeier 2002). This is bone transferred from one location to another within the same individual and is considered to be the gold standard for bone grafting.
material. Alternatively, an allogeneic bone graft, which is bone transferred between genetically different members of the same species, can be used. However, allogeneic bone grafts confer risks of transfection and rejection. To avoid this problem, synthetic (alloplastic) materials have been developed as another approach in bone repair mechanisms (Hallman, et al. 2009, Hallman & Thor 2008).

The limited availability of bone grafting materials, together with several other drawbacks restrict their application in clinical practice (Finkemeier 2002). Therefore alternative grafting methods have been developed.

1.2. Bone tissue engineering concepts

It is known that large bone defects caused for example by pathological lesions or trauma have a poor prognosis and reconstruction of these defects constitutes a major clinical challenge. A recent approach to treatment of these conditions is known as bone tissue engineering (BTE) (Figure 1). Although still in its infancy, the literature on BTE contains a considerable accumulation of knowledge about many of the key elements, including scaffold structure and material properties, cell sources and biomolecular activity (Burg, et al. 2000).

The concept underlying BTE is to engineer autographs either by expanding autologous cells in vitro, guided by a scaffold, or by using an acellular scaffold in vivo and allowing the patient’s cells to repair defective tissue, guided by a scaffold. As tissue regeneration proceeds, the scaffold should degrade in time; once the tissue has matured, the scaffold should no longer exist, allowing the newly formed tissue to function. This approach avoids some of the drawbacks related to grafting techniques (Chan & Leong 2008, Howard, et al. 2008).
Figure 1. Schematic representation of bone tissue engineering approach that uses cells and biodegradable scaffolds. Isolated bone cells from patient alveolar bone biopsy are expanded in vitro to achieve large number of cells, and thereafter cells are seeded onto scaffold materials and cultured further for several days with or without growth factors. Obtained tissue engineered construct will be implanted back to the patient to repair or heal bone defect.

In orthopedics, BTE opens new opportunities for bone regeneration and substitution based on an implant composed of a biocompatible, biodegradable scaffold, the recipient’s own cells, and biomolecules that modulate the cells to form new tissue. Such an implant not only creates a local environment for pre-loaded cell proliferation and differentiation in the targeted tissue, but also interacts with the host’s own tissue and integrates into it. Ideally, the only remaining foreign component, the scaffold, will be degraded and excreted. In this process, the implant is eventually transformed into the patient’s own bone (Burg, et al. 2000).
1.3. Overview of bone biology

Bone is a dynamic, highly vascularized tissue with a unique capacity to heal and remodel without leaving any scar (Sommerfeldt & Rubin 2001). Its main role is to provide structural support for the body. The skeleton also serves as a mineral reservoir, supports muscular contraction resulting in motion, withstands load bearing, and protects internal organs (Marks & Odgren 2002). In order to fulfil these functions, bone is therefore continuously broken down and rebuilt.

Bone contains approximately 70% mineral, 8% water, and about 22% collagenous matrix, and the interactions of these constituents play major roles in determining the mechanical behavior of bone (Cullinane & Einhorn 2002). At the macroscopic level, the mature bone skeleton is arranged in two architectural forms: trabecular and cortical (Marks & Odgren 2002). The trabecular bone is commonly found in the metaphyses of long bones, covered by cortical bone, and in the vertebral bodies. The cortical bone on the other hand can be divided into different subgroups: long bones, short bones, and flat bones (Rho, et al. 1998, Salgado, et al. 2004).

1.3.1. Bone cells

The major cells in bone responsible for different functions include osteoblasts, osteocytes, osteoclasts and bone lining cells (Figure 2). Together, these cells play essential roles in bone formation, maintenance, and remodelling.
Osteoblasts are mononuclear, cuboidal shaped, fully differentiated cells that arise from the mesenchymal stem cell lineage or precursor cells, through the process of osteogenesis, and are responsible for production of bone matrix and regulation of mineralization (Aubin 1998). Upon differentiation, these cells are known to synthesize and secrete type I collagen (Col 1), alkaline phosphatase (ALP), and other non-collagenous extracellular bone matrix proteins such as osteonectin, osteocalcin (OC), osteopontin (OP) and bone sialoprotein (BSP). Differential gene expression of osteogenic cells can be defined by three principal biological processes: cellular proliferation, cellular maturation and focal mineralization (Aubin 1998). Col 1 is expressed during the initial period of proliferation and extracellular-matrix biosynthesis, whereas ALP is expressed during the post-proliferative period of extracellular-matrix maturation. The expression of osteopontin, osteocalcin and bone
sialoprotein occurs later, during the third period of extracellular-matrix mineralization (Aubin 1998). Runt related gene 2 (Runx2), which is also known as core-binding factor al (CBFA1), is an important transcriptional determinant of osteoblast differentiation (Franceschi 1999). Deposition of the above mentioned proteins leads to formation of bone matrix, which will undergo the mineralization process that completes bone formation (Marks & Odgren 2002). Osteocytes are mature osteoblasts which have been surrounded by bone matrix. These cells are involved in maintenance of the local bone, and the unique stellate shape with canaliculi connecting adjacent osteocytes makes each of them to function as a hub of cellular communication and nutrient delivery. Osteoclasts are large multi-nucleated cells of hematopoietic origin through the monocyte-macrophage lineage. They resorb bone by dissolving the mineral phase and enzymatically by digesting the organic macromolecules. Osteoclasts reside on the surface of bone and they have a ruffled bordered plasma membrane at their resorbing surface. Bone lining cells are flat, elongated cells that cover bone surfaces where neither bone formation nor resorption is taking place. However, little is known of their functions (Marks & Odgren 2002).

1.3.2. Bone formation

Newly formed bone is formed via either intramembranous ossification or endochondral ossification processes. In the former process, woven bone is formed directly from condensed mesenchymal tissue, without the intermediate formation of a cartilaginous framework of the future bone. It occurs primarily in the embryonic formation of flat bones (e.g. the skull). Endochondral ossification involves an intermediate, cartilaginous phase formed from mesenchymal tissue, and it is this cartilage framework which is then ossified to form the new bone. It occurs in the
embryonic formation of the long bones, in fracture repair, and in the incorporation of bone grafts (Marks & Odgren 2002).

1.3.3. Cells for bone tissue engineering

The outcome of BTE is influenced not only by the scaffolding but also by the type of cell selected for bone regeneration. The ideal cell source should be easily expandable to higher passages, non-immunogenic and have a protein expression pattern similar to the tissue to be regenerated. Use of autologous cells circumvents the risks of immunological incompatibility and transmission of infection.

The stem cells located in the bone marrow, known as Mesenchymal Stem Cells (MSC), have been used in experimental BTE. Besides their differentiation potential, MSCs have other important properties and can be expanded extensively in vitro (Salgado, et al. 2004). During bone formation and as MSC mature into osteoblasts, multiple growth factors are expressed (Wildemann, et al. 2007).

As well as MSC, other osteogenic cells with potential application for bone include periosteal mesenchymal cells and osteoblasts (Coelho, et al. 2000, Oreffo & Triffitt 1999). Experimentally sourcing osteogenic cells from alveolar bone obtained during routine surgery offers two important advantages: first the cells are readily harvested by biopsy and second the procedure causes minimal damage at the donor site.

1.4. Growth factors

Growth factors are cytokines which function as signalling molecules for such events as promotion and/or prevention of cell adhesion, proliferation, migration and differentiation by up- or down-regulating the synthesis of several proteins, growth factors and receptors (Jadlowiec, et al. 2003, Lieberman, et al. 2002). Hence, these
molecules are essential for tissue formation and play an important role in tissue engineering (Hallman & Thor 2008). During bone formation, multiple growth factors are expressed, such as bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), transforming growth factor \(\beta\) (TGF-\(\beta\)) and vascular epithelium growth factors (VEGF). Each has different roles, which may overlap (Jadlowiec, et al. 2003, Lieberman, et al. 2002).

In both angiogenesis and mesenchymal cell mitogenesis, FGF are known to play critical roles. FGF-\(\beta\) for example is expressed by osteoblasts and its activity has been identified during the early stages of fracture-healing (Canalis, et al. 1988). IGF, on the other hand, has been shown to play critical roles in skeletal development (Andrew, et al. 1993). BMPs have been studied under preclinical and clinical conditions (Lieberman, et al. 2002) and are expressed in the early phases of fracture healing (Lind & Bunger 2001). PDGF is known to be produced by osteoblasts, platelets and monocytes/macrophages, and it is believed to have a possible role in migration of MSCs to wound healing sites and in the process of bone regeneration (Rasubala, et al. 2003). TGF-\(\beta\) is expressed in bone, platelets and cartilage. It has been shown that TGF-\(\beta\) can stimulate HOBs to proliferate and thereby promote collagen production \textit{in vitro} (Robey, et al. 1987). VEGF, known as a potent angiogenic factor, is expressed in bone fracture healing sites and is involved in regulating vascularization through recruitment of endothelial cells to the healing site (Furumatsu, et al. 2003, Uchida, et al. 2003).
1.5. Scaffolds for tissue engineering

Central to BTE is the use of three-dimensional structures (3D) (Middleton & Tipton 2000) of large surface area and high porosity with proper pore size (Holy, et al. 2000, Hou, et al. 2003), which function as scaffolds onto which cells with osteogenic properties are seeded. The scaffolds are intended to simulate bone extracellular matrix (ECM) by guiding cell adhesion, migration, proliferation and differentiation and tissue regeneration in three dimensions (Goldberg & Caplan 2004). The scaffold functions as a temporary guide and support during regeneration and formation of new tissue and requires a certain mechanical strength to maintain tissue growth and integration, but is not integrated. Once the new tissue is established, the scaffolding material should be resorbed as new tissue is generated (Hutmacher 2000, Kim & Mooney 1998). There should be complete degradation, producing no toxic degradation products and leaving no residue which could lead to chronic inflammation. Therefore, in both implanted and degraded forms, scaffold materials must be biocompatible and immunologically acceptable (Hutmacher 2000, Middleton & Tipton 2000).

Clearly, the BTE scaffold must meet stringent requirements. Besides the choice of materials that are non-mutagenic, non-antigenic, non-carcinogenic, non-toxic, non-teratogenic, tolerate sterilization and possess high cell/tissue biocompatibility, the macro and micro-structural properties of the materials are of utmost importance (Leong, et al. 2003).

1.5.1. Biomaterials used as bone tissue engineering scaffolds

In the past, different materials have been investigated for applications in BTE. These include metals which provide immediate mechanical support at the defect site, but exhibit poor overall integration with the surrounding tissue and may fail due to
infection or fatigue loading. Ceramics such as hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) have also been tested, but are brittle and have very low tensile strength. Thus their use is contraindicated at sites subject to significant load (Salgado, et al. 2004, Schieker, et al. 2006).

Polymers of natural origin, such as collagen, fibrinogen, chitosan, starch, hyaluronic acid and poly(hydroxybutyrate) as well as of synthetic origin, such as poly(lactic acid), poly(glycolic acid), poly(ε-caprolactone) and copolymers (Griffith 2002, Salgado, et al. 2004) have been investigated for BTE.

More recently, degradable, resorbable polymers belonging to a family of aliphatic polyesters derived from cyclic monomers, *e.g.* lactide, glycolide, and ε-caprolactone and their co-polymers have been proposed as potential scaffolding materials (Nair & Laurencin 2006, Porter, et al. 2009). *In vitro*, these materials degrade hydrolytically, and their degradation rate can be tuned by adjusting the hydrophobicity, molecular weight and crystallinity of the polymer chain (Hakkarainen, et al. 2008, Hakkarainen, et al. 2007). Polylactic Acid (PLA) is a biocompatible, thermoplastic, resorbable aliphatic polyester (FDA approved), which has been used clinically for sutures, bone fracture fixation devices and as drug release systems due to its low degradation rate, better processability, and mechanical properties. PLA is a chiral molecule, existing as l-lactide, d-lactide, and meso-lactide. Thus, four different types of PLA are available: poly(l-lactic acid), poly(d-lactic acid), poly(dl-lactic acid) and meso-poly(lactic acid). Only poly(l-lactic acid) and poly(dl-lactic acid) have been extensively investigated as biomaterials. Biodegradable scaffolds of poly(L-lactide) P(LLA) have been tested as alternatives to ceramic scaffolding (Nair & Laurencin 2006, Porter, et al. 2009). Poly(glycolic acid) (PGA) has high tensile strength with very low solubility in common organic solvents and was...
initially proposed as biodegradable suture material and bone fixation devices. However, due to the high degradation rate and low solubility, coupled with accumulation of acidic degradation products which can lead to inflammatory reactions, use of PGA in biomedical fields is limited (Nair & Laurencin 2006, Porter, et al. 2009). Poly(caprolactone) (PCL) can be obtained from an inexpensive source (caprolactone) and is highly soluble in organic solvents. Due to its low degradation rate, PCL has been investigated as a material for long-term controlled delivery of drugs (Nair & Laurencin 2006, Porter, et al. 2009).

There are several manufacturing methods for producing tissue engineering scaffolds. These methods are required to be accurate and consistent with respect to porosity, pore size, pore distribution and interconnectivity of the scaffolds. Over the years, a series of processing techniques such as solvent casting, phase separation, and rapid prototyping (RP) technologies, also called Solid Free Form methods such as 3-D Printing (3-DP), have been developed with the main objective of producing scaffolds with adequate properties for bone tissue engineering (Hutmacher 2000, Nair & Laurencin 2006, Porter, et al. 2009).

1.6. Cytotoxicity screening of biodegradable scaffolds

The formation of a tissue structure incorporating cells and matrix, involves an in vitro culture step. It is at this stage that cell-scaffold interactions can be evaluated, as an initial screening of potential scaffolding. To date, at the regulatory level, the basic approach is defined in the family of ISO 10993 standards (ISO Standard 10993-5. 2009 & ISO Standard 10993-1. 2003).

In vitro biocompatibility tests simulate the biological reactions to materials intended for clinical dental and medical applications (Hanks, et al. 1996, Schedle, et
Biocompatibility tests offer a less expensive means of preliminary evaluation of newly developed materials, reducing the probability of untoward effects when animal tests or clinical trials are undertaken (Hanks, et al. 1996). One of the most important properties of the proposed biomaterials is their potentially toxic effect on cells. Cytotoxicity testing is therefore the initial phase of evaluation of material biocompatibility (Gomes, et al. 2001, Kirkpatrick 1992, Kirkpatrick & Mittermayer 1990). In vitro studies show a direct correlation between toxicity and cell death, reduced cell proliferation, altered morphology and impaired adhesion (Kirkpatrick & Mittermayer 1990, Pizzoferrato, et al. 1994). Cytotoxicity testing targets mainly substances which leach out of the biomaterials. For example, polymers often have low molecular weight "leachables" such as additives, stabilizer, low molecular weight components and initiator fragments that exhibit varying levels of physiologic activity and cell toxicity (Kirkpatrick 1992, Silva, et al. 2004).

As this brief description shows, the present concept of skeletal tissue engineering requires a biocompatible scaffold conducive to cell attachment and maintenance of cell function, a rich source of osteoprogenitor cells and osteoinductive growth factors. Selection of the most appropriate material for scaffolding is a major determinant of successful outcome.

1.7. Cell/tissue-scaffold interactions

Cell/tissue-implant material interactions constitute the main goals in tissue engineering. The surface of an implant will determine its ultimate ability to integrate into the surrounding tissues. Surface properties such as chemistry, topography, surface energy and roughness often work together to control cell behavior and affect cellular adhesion and proliferation, and consequently tissue formation (Cassinelli, et al. 2003,
Lange, et al. 2002). The topographical properties are of particular interest with respect to osteoconduction. Chemical properties influence the ability of cells to adhere to the material as well as protein interactions with the latter. Although scientific observations on the contribution of chemical properties to tissue growth have been reported, the underlying mechanisms have yet to be clarified (Kieswetter, et al. 1996, Salgado, et al. 2004). Initially, the role of surface energy, as dictated by surface roughness, topography, and composition of the implant, may play a crucial role in determining which proteins, lipids, salts and sugar are adsorbed onto the surface as well as whether or not the cells themselves adhere to the surface (Kieswetter, et al. 1996, Salgado, et al. 2004). It has been previously shown that a “rougher” surface favors osteoblast attachment, facilitates migration of osteogenic cells to the material surface and the ingrowth of bone, and is therefore preferable to a smooth surface (Carlsson, et al. 1988, Gotfredsen, et al. 1992, Mustafa, et al. 2001, Mustafa, et al. 2000). Pore size and interconnectivity are thus important properties, in that they can affect how well cells can penetrate and grow into the scaffold and at the same time what quantity of materials, nutrients, and wastes can be transported into and out of the scaffold. However, the degree of porosity will influence other properties of the scaffold, such as its mechanical stability: thus there must be a balance between porosity and the mechanical requirements of the particular tissue that is to be replaced (Hou, et al. 2003, Salgado, et al. 2004). Tsuruga and coworkers have suggested that the optimal pore size of ceramics for supporting ectopic bone formation is 300-400 µm (Tsuruga, et al. 1997). Holmes similarly suggested that the optimal pore range is 200-400 µm with the average human osteon size of approximately 223 µm (Holmes 1979). Recently, Schander et al. suggested that scaffolds with pore size > 90 µm enhanced human periodontal ligament and alveolar osteoblast-like cell growth
(Schander, et al. 2010). In contrast, Holy et al. (2000) proposed a different concept: that bone reconstruction will only be achieved by having a 3D temporary matrix with a large macroporous interconnected structure with pore size ranging from 1.2–2.0 mm. This approach in fact has evident advantages that will facilitate cell, tissue and blood vessel in-growth. However, this may compromise the mechanical properties, precluding its application in areas requiring mechanical strength (Burg, et al. 2000, Holy, et al. 2000, Hou, et al. 2003).

It is crucial that in vitro testing of the effect on cell proliferation, cell differentiation, and cellular behavior of candidate biomaterials for scaffolding should be conducted at an early stage: firstly because the scaffold materials should have the capacity to stimulate differentiation of osteoprogenitor cells into osteoblasts, and secondly, because proliferation and differentiation of osteoblasts are affected by the chemistry of the substratum (Zreiqat, et al. 1999).

The process of bone regeneration can be followed by monitoring markers associated with the different stages of osteogenesis. Because there is no single specific marker for osteoblasts, cellular differentiation is assessed in terms of cellular expression of a range of non-collagenous and collagenous proteins as well as ALP. Differentiating osteoblasts are known to synthesize and secrete Col 1, ALP, and other non-collagenous extracellular bone matrix proteins, such as osteonectin, OC, OP and BSP (Aubin 1998, Marks & Odgren 2002). While osteoblast activity can be assessed in terms of osteoblast-specific proteins, studying osteoblastic gene expression will provide more accurate, detailed information about cellular activity (Franceschi 1999). A more sensitive indicator of osteoblast activity, however, is the cell’s global pattern of gene expression.
1.8. Microarray gene expression profiling in tissue engineering

The cell’s global pattern of gene expression can be monitored by microarray technology using large numbers of genes, spanning a significant fraction of the human genome, to generate a so-called genetic portrait, revealing up- or down regulated genes involved in the cell system under investigation. Microarray analyses allow monitoring of human cells even at an early stage of material design (Carinci, et al. 2007, Carinci, et al. 2004). With the increasing availability of genetic information, the focus of both biological and molecular studies has begun to shift from characterization of individual components of a biologic system to the holistic behavior of the entire biological system (Iida & Nishimura 2002). Microarray analysis might be relevant to a better understanding of the molecular mechanisms underlying the behavior of cells in tissue regenerative procedure. This might facilitate the design of improved tissue engineering scaffolds.

1.9. Specific background

Biodegradable scaffolds of P(LLA) have been tested as alternatives to ceramic scaffolding. However, the P(LLA) polymer is hydrophobic, with a high molecular weight and melting point, which prolongs its degradation time (Migliaresi, et al. 1994). The weak mechanical properties of the P(LLA) polymer scaffolds are a further disadvantage in structural tissue engineering applications.

Copolymerization of lactide with monomers that have different values of transition temperature (Tg) and crystallinity creates opportunities for designing polymers with widely different properties. One of the interesting monomers is the 1,5-dioxepan-2-one (DXO), synthesized by Albertsson and co-workers (Karlsson, et al. 1994, Mathisen & Albertsson 1989, Mathisen, et al. 1989). The design of this
monomer is part of ongoing studies on the development of new monomers using ring-opening polymerization for creating degradable biomedical materials. DXO co-polymerized with lactide yields materials with an amorphous character and interesting properties for new degradable biomedical polymers (Lofgren, et al. 1994).

To provide a variety of BTE materials with a range of properties more appropriately tailored to specific biomedical applications, co-polymers of L,L-lactide and \(\varepsilon\)-caprolactone (\(\varepsilon\)-CL) [Poly(LLA-co-CL)] or DXO [Poly(LLA-co-DXO)], have been produced which have different hydrophilicity and more appropriate mechanical properties than P(LLA) (Dânmark, et al. 2010, Kallrot, et al. 2007, Kallrot, et al. 2008). Their mechanical properties are more suited to the demands of BTE scaffolding (Odelius, et al. 2005, Odelius, et al. 2008, Plikk, et al. 2006). While the mechanical properties have been well documented, the influence of these scaffolds on the behavior and osteogenic potential of the seeded cells \textit{in vitro} and \textit{in vivo} has yet to be determined. Furthermore, little is known about the response of these cells, at a molecular level, to tissue engineering scaffold materials.

To date, no BTE biomaterial has resulted in an implant with optimum morphology, mechanical properties, biocompatibility, and biodegradability. Lack of an optimum material for BTE has led to increasing interest in the field of biomaterials. Therefore the ultimate goal of this thesis is to test the biological responses to newly synthesized aliphatic polyester scaffolds for BTE applications.
2. Aims of the Study

As the field of tissue engineering progresses, use of biodegradable polymers has become widespread. The manner in which these polymers are processed and the additives used at the time of manufacture allow the final properties of the scaffold to be tailored to specific applications. Aliphatic polyesters synthesized by ring-opening polymerization are well suited for use in bone tissue engineering. This is due to their biocompatibility and resorbability, and the possibility of copolymerizing monomers to tailor their chemical, physical and mechanical properties. The need for novel scaffold structures and reproducible fabrication techniques has become of paramount importance. Toward this end, a series of experimental in vitro studies was designed to investigate Poly(LLA-co-DXO), Poly(LLA-co-CL) and P(LLA), three biodegradable polymer scaffolds with different hydrophobicity and mechanical properties, currently under appraisal by our research group. Therefore, the overall aims of this thesis were as follows:

**General Aim**

To investigate the effect of recently designed and developed co-polymer scaffolds for bone tissue engineering on the initial cellular responses (Papers I-III).

**Specific Aims**

- To evaluate the cytotoxicity and cytocompatibility of Poly(LLA-co-DXO) and Poly(LLA-co-CL) scaffolds (Paper I).
- To study the differentiation of HOBs seeded onto Poly(LLA-co-DXO) and Poly(LLA-co-CL), compared with P(LLA), by examining expression and synthesis of osteoblast differentiation markers Col 1, ALP, BSP, OC, OP and Runx2 (Paper II).
To define the most appropriate scaffold for bone tissue engineering which is aimed from this thesis by identifying and comparing gene expression profiles of HOBs following culture on biodegradable polymer scaffolds of Poly(LLL-co-DXO) and P(LLL) (Paper III).
3. **Materials and Methods**

This section provides an overview of the materials and methods used in the three studies (Figures 3 and 4). Full details are presented in the original papers (I-III) included in this thesis.

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**Figure 3.** Flow chart describing the different kinds of materials and methods used in the present thesis (Papers I-III). **HOBs:** Human osteoblast-like cells, **L929s:** Mouse fibroblast cells line, **CVS:** Crystal violet staining, **SEM:** scanning electron microscopy, **MTT:** Methylthiazol tetrazolium, **LDH:** Lactate dehydrogenase assay, **qRT-PCR:** Real-time quantitative reverse transcriptase-PCR and **ELISA:** Enzyme-linked immunosorbent assay.
3.1. Scaffold preparation (Papers I- III)

The copolymerization of L,L-lactide (LLA), 1,5-dioxepan-2-one (DXO) or ε-caprolactone (CL) was performed in bulk using ethylene glycol and a low amount of Stannous 2-ethylhexanoate, at a ratio to monomer of approximately 1:10 000 (Figure 5) (Stjerndahl, et al. 2008). Polymerizations were carried out at 110 °C for 72 h under nitrogen atmosphere. The resulting copolymers consisted of 75 mol % L-lactide and 25 mol % of the corresponding comonomer, verified by 1H-nuclear magnetic resonance. The molecular weights were found to be ~100,000 g/mol for all polymers, confirmed by Size exclusion chromatography calibrated against Poly(styrene) standards (Odelius, et al. 2005).

Porous 3D scaffolds of Poly(LLA-co-CL), Poly(LLA-co-DXO) and a 3D control of P(LLA) were prepared by a previously described solvent casting particulate leaching method (Dánmark, et al. 2010, Idris, et al. 2010, Idris, et al. 2010, Odelius, et al. 2005). Mortared sodium chloride particles (NaCl) were used as the pore-creating
additive and added to polymer in a ratio of 10:1 by weight, yielding a wide range of particle sizes with no discriminating dimensions. Total porosities of approximately 90 vol % (Odelius, et al. 2005) were achieved for all polymer scaffolds. The chloroform was allowed to evaporate slowly to leave a solid composite. Scaffolds, for multi-well cell-culture plates, were punched out from the composites. The salt particles were leached from the composites by repeated soaking in deionized water and the salt-free scaffolds were dried under vacuum. The samples were thereafter subjected to irradiation under an inert nitrogen atmosphere (Odelius, et al. 2008, Plikk, et al. 2006) by electron beam using a 6.5 MeV pulsed electron accelerator (Microtron, Accelerator teknik, The Royal Institute of Technology, Stockholm) with a radiation dose of 2.5 MRad.

![Figure 5](image_url)

**Figure 5.** Images of P(LLA) scaffold (left), Poly(LLA-co-CL) scaffold (centre) and Poly(LLA-co-DXO) scaffold (right). The scaffolds have been formed as discs of 12 mm diameter and 1.5 mm thickness.

### 3.2. Cell cultures (Papers I-III)

In order to study the cellular reaction on each scaffold surface, two cell culture types were used: HOBs and the mouse fibroblast L929. HOBs were isolated from human mandibular bone specimens, free of any clinical or radiographic evidence of pathology, and were obtained from patients undergoing routine oral surgery at the
Department of Maxillofacial Surgery, Haukeland University Hospital and Department of Clinical Dentistry, Faculty of Medicine and Dentistry, University of Bergen, Bergen, Norway. All the donors were healthy subjects with no known systemic diseases. The specimens were taken from the molar region and harvested and maintained by a modification of the method described by Beresford et al. (1984). The cells were characterized using different assays (Mustafa, et al. 2001, 2000).

An established cell line, mouse fibroblast L929 (American Type Culture Collection CCL 1) was cultured in Dulbecco’s modified Eagle’s medium containing 2 mM L-glutamine (DMEM; PPA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal calf serum (FCS) and 1% antibiotics at 37 °C in a humid atmosphere containing 5% CO₂. Twenty-four hours before starting each cytotoxicity test, cells were trypsinized and counted by a Countess™ Automated Cell Counter (Invitrogen™, Carlsbad, California) prior to further use.

In papers I-III, scaffolds measuring 12.5mm in diameter and approximately 1.5mm in thickness were placed into 48-well culture plates and soaked with the culture medium [Minimum Essential Medium, alpha modification (α-MEM; Gibco, Grand Island, NY, USA), supplemented with 1% antibiotics (penicillin/streptomycin solution, L-glutamine and 10% FCS. After 24 hours, HOBs that strongly expressed ALP and OC were seeded onto the scaffolds and allowed to incubate in 5% CO₂ at 37 °C for 1, 7, 14, and 21 days. The medium was changed every 2 days. Tissue Culture Polystyrene (TCPS) served as two dimensional (2D) controls.

The study protocol for the use of HOBs was approved by The National Committee for Research Ethics, Western Norway (225.05, dated 07.11.05).
3.3. Extract preparation (Paper I)

Scaffolds (1.25 cm\(^2\)/ml) were incubated in culture medium at 37 °C with constant shaking (60 rpm) in order to simulate closely the effect of degradation products in a dynamic environment. Extract was then filtered (0.2 \(\mu\)m pore size) to eliminate any solid material particles and maintained at -20°C. For the extraction tests, complete culture medium in TCPS served as a negative control; 20 mg/ml phenol 99+ % (Sigma-Aldrich, USA) in the media served as a positive control.

3.4. Assessment of cell viability and proliferation

3.4.1. Methylthiazol tetrazolium (MTT) assay (Papers I and II)

Cell viability and proliferation were analyzed using MTT mitochondrial reaction. This is a colorimetric assay, based on the ability of live cells to reduce yellow MTT reagent (Sigma, St Louis, MO, USA) to a purple formazan product (Mosmann 1983). In paper I, L929 cells were seeded for 24 hours with 24 hour and 7 day extracts. Negative and positive controls were also included.

In paper II, HOBs and scaffolds were washed with PBS (phosphate buffered saline) and transferred into new well plates after 1 and 7 days. MTT reagents were added to each sample and incubated in the dark for 4 hours at 37 °C, under a CO\(_2\) (5%) atmosphere. MTT was aspirated and the formazan product was solubilized in 0.5 ml DMSO containing 6.25% (v/v) 0.1 M NaOH and the end product was quantified by microplate spectrophotometry (BMG LABTECH, GmbH, Germany) at a wavelength of 570 nm and expressed as optical density units (OD) after blank subtraction.
3.4. 2. Lactate dehydrogenase assay (LDH) (Paper I)

Cell viability and cytotoxicity were also evaluated by measuring the viability of L929 cells on the newly developed scaffolds via total cytoplasmic lactate dehydrogenase, using the commercially available kit TOX-7 (In vitro Toxicology Assay Kit, lactic dehydrogenase based, Sigma-Aldrich, USA) in accordance with the manufacturer’s instructions (Weyermann, et al. 2005). OD at 450 nm was recorded and taken as a measure for the quantity of cells.

3.5. Cell morphology

3.5.1. Crystal violet staining (CVS) (Paper I)

HOBs were cultivated for 24 hours and incubated with extract from the various scaffolds or with control culture medium for another 24 hours. Briefly, cultured cells were fixed in 10% formalin buffer at room temperature for another 2 min and stained with crystal violet for 2 min. The attached cells were washed gently with distilled water and allowed to dry overnight. The samples were photographed under light microscopy with an inverted optical microscope (NIKON ECLIPSE TS100, Invitrogen, Japan).

3.5.2. Scanning electron microscopy (SEM) (Papers I and II)

The surface topography of the scaffolds and cellular morphology were documented by SEM (Jeol JSM-7400F, Tokyo, Japan). The scaffolds were seeded with HOBs, and the samples were fixed in glutaraldehyde, dehydrated, critical point dried and sputter-coated with a layer of gold-platinum for observation by SEM.
3.5.3. Scaffold histology (Paper III)

Poly(LLA-co-DXO) scaffold seeded with HOBs were prepared for histological analysis. The presence of cells within the scaffolds was demonstrated by sectioning the construct and staining the cell nuclei with propidium iodide (PI). Sections (8 µm) were cut by using a microtome (Cryostat, Leica CM 30505, Leica Microsystems Nussloch GmbI, Germany) and analysed by fluorescence microscopy for PI-stained nuclei to identify the location of cells within the scaffolds.

3.6. Determination of cell death (Paper I)

3.6.1. Caspase 3/7 activity assay

Apoptosis was measured via active caspases 3/7 in whole living, intact cells using Magic Red Caspases 3/7 Detection kit (ImmunoChemistry Technologies, Bloomington, USA), following the manufacturer’s instructions. Cells on the scaffolds were incubated with staurosporine to induce apoptosis and used as positive control. The intensity of fluorescence was measured with a fluorescence plate reader at 540-590 nm excitation and >610 nm emission.

3.6.2. Bcl-2 and Bax gene expression levels

The effects of test scaffolds on the gene expression levels of Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) in HOBs were studied using real time qRT-PCR.

3.7. Enzyme-linked immunosorbent assay (ELISA) (Paper II)

Col 1 and OC, expressed into the medium by the HOBs that had grown on the scaffolds for 7, 14 and 21 days, were measured by a human-specific ELISA using the commercially available kits Metra™ CICP Enzyme Immunoassay Kit.
(QUIDEL/METRA, San Diego, USA) and Immunoassay Kit Human Osteocalcin (BioSource™ Europe S.A., Nivelles-Belgium), in accordance with the manufacturer’s instructions.

3.8. RNA isolation (Papers I-III)

In papers I and II, the scaffold and the cells were washed in PBS and total RNA was isolated at different culture times, using combined TRIzol® reagent (Gibco BRL, Carlsbad, CA, USA) and E.Z.N.A.™ Tissue RNA kit (Omega Bio-Tek, Doraville, USA) protocols, following the manufacturer’s instructions. The quantity and quality of the extracted RNA were checked by spectrophotometry and 300 ng of the total RNA was reverse transcribed using High-Capacity cDNA Archive Kit system for cDNA synthesis.

In paper III, total RNA was extracted (from six independent biological replicas) using lysis buffer with β- Mercaptoethanol and E.Z.N.A.™ Tissue RNA kit protocols, following the manufacturer’s instructions. Only RNA samples with an A260/A280 ratio of 1.9–2.1 were selected for further analyses after further quality and quantity control by The Norwegian Microarray Consortium facilities (NMC).

3.9. Real-time quantitative reverse transcriptase- PCR (qRT-PCR) (Papers I-III)

Quantitative real-time PCR assays were performed using ABI StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, USA). PCR amplification of the selected markers was done in triplicate with 10 µl reaction volume each. PCR reactions contained 0.5 µl of TaqMan probes [for apoptotic markers (Paper I), bone markers (Paper II), probes for genes selected for validation of microarray findings (Paper III)], 3.5 µl of nuclease free water, 5 µl of TaqMan universal fast PCR master mix (Applied Biosystems) and 1 µl cDNA. Thermocycling conditions were 95 °C for
20 s, followed by 40 cycles at 95 °C for 1 s and 60°C for 20 s. Gene expression levels were calculated according to the comparative $2^{\Delta \Delta Ct}$ method (Livak & Schmittgen 2001) and normalized relative to GAPDH as the reference housekeeping gene, in each of the RNA samples ($\Delta C_T = C_{T,\text{target}} - C_{T,\text{GAPDH}}$).

3.10. Gene expression profiling and data preprocessing (Paper III)

3.10.1. Experimental design

Microarray technology enables simultaneous detection of expression of thousands of genes in the same sample. For Study III, the microarray platform Illumina was used. Illumina is a one-channel system which uses HumanWG-6 v3.0 Expression BeadChips. The one channel nature of the platform yields the simple design of one sample to be hybridized to one array on a microarray slide. The particular chip used in the present study has 6 arrays on each slide.

3.10.2. RNA quality control

To evaluate RNA quality, the Agilent 2100 Bioanalyzer chips (Agilent Technologies, Wilmington, DE, US) and the NanoDrop ND-1000 were used. RIN values of 7.5 or more were chosen for microarray experiments (Figure 6).
Figure 6. Examples of electropherograms of RNA of different quality and RNA Integrity Numbers (RIN). The RIN value ranges from 2 (degraded RNA) to 10 (perfect RNA).

3.10.3. cDNA synthesis, labelling and microarray hybridization

Microarray experiments were performed using the Illumina iScan Reader, which is based on fluorescence detection of biotin labelled cRNA. Of each sample, 250 ng of the total RNA was reversely transcribed, amplified and Biotin-16-UTP–labelled, using the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems/Ambion, USA). Then 1500 ng of the biotin labelled cRNA was hybridized at 58°C for 16 hours and 50 minutes to the HumanWG-6 v3.0 Expression BeadChip (targeting >48 000 probes derived from human genes in the NCBI RefSeq database and UniGene database), according to the manufacturer’s instructions (Figure 7).
Figure 7. Biotin labelling of total RNA using the Illumina TotalPrep Amplification Kit from Ambion. Total RNA from each sample was reversely transcribed, amplified and Biotin-16-UTP–labelled. Biotin labelled cRNA was hybridized to the HumanWG-6 v3.0 Expression BeadChip. The data from the scanning of arrays on Illumina iScan Reader was investigated in GenomeStudio and J-Express 2009.

Data obtained from scanning of the arrays on the Illumina iScan Reader were thereafter subjected to further analysis using GenomeStudio and J-Express 2009 software programs for quality and control measures.

3.10.4. Quality control and preprocessing of the data

All images from the scans were inspected visually. The raw data from the scans were controlled in the GenomeStudio software where internal controls on the array were checked, and control plots were constructed to search for outliers. SampleProbeProfile was loaded into J-Express software (Dysvik & Jonassen 2001) and further quality control of the data was performed using Log2-transposed data for quality control.

For detection of outliers, *i.e.* samples that behave differently from other samples regardless of the biology, both hierarchical clustering (*Figure 8*) and
correspondence analysis (Figure 9) were used. No outliers were found: samples from the same donor were clustered. This indicated that the quality of the data set is acceptable, and further analysis could proceed with all samples included in the data sets.

Figure 8. Global hierarchical clustering of samples after 24 hours (8A) and 21 days (8B), un-normalized data to the left and quantile normalized data to the right. We generally see samples from the same donor clustering together. No outliers are observed.
Figure 9. Correspondence analysis plot after 24 hours (9A) and 21 days (8B), un-normalized data to the left and quantile normalized data to the right. Samples from the same donor are clustered together. No outliers are observed.

3.11. Statistical analysis (Papers I-III)

In papers I-III, statistical analysis was performed using SigmaStat version 3.1 package software and One Way Analysis of Variance (ANOVA) with All Pairwise Multiple Comparison tests, followed by Tukey Test. Differences between the means were considered statistically significant when P<0.05. For microarray data (paper III), gene expression data were analyzed using J-Express 2009. Rank Product (RP) test was used to determine differences in gene expression levels of HOBs grown on two types of scaffolds (Breitling, et al. 2004). Changes in gene expression profiles between pairs of groups were identified using RP tests at an estimated false discovery rate (FDR) of less than 10%. The differentially expressed genes were mapped to gene ontology
(GO) directed acrylic graph (DAG) in the J-Express 2009 and compared with the total number of probes (>48 000) to determine over-representation of the GO terms (Draghici, et al. 2003). A similar analysis was performed against a reference list of genes (NCBI: H. sapiens gene) in the protein analysis through evolutionary relationships (PANTHER) classification system (http://www.pantherdb.org/) using the Gene Symbol for each gene to identify over-represented key pathways. Bonferroni correction for multiple testing was applied.
4. **Results and Discussion**

4.1. **Biocompatibility of polyester co-polymer scaffolds**

In paper I, extracts following soaking of scaffolds with medium, were used to assay cytotoxicity, i.e. by applying indirect contact methods. The MTT results revealed that the extracts from P(LLA), Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds soaked for 24 hours and 7 days did not affect the viability or proliferation activity of the L929 cell line. The proliferation corresponded well with that in the control medium and no inter-scaffold differences were observed (P >0.05). The direct contact method, using LDH assay for the analysis, showed that the cells were able to grow and proliferate quite well. A noncytotoxic effect was noted for all three scaffolds. Comparison with the negative control disclosed no statistically significant differences after 24 hours. Cell proliferation in all the 3D scaffolds showed a continual increase from 24 hours to 7 days. The cell proliferation values for the Poly(LLA-co-DXO) scaffolds were significantly higher than those for the P(LLA) scaffold and TCPS (p <0.05). This might be attributable to greater hydrophilicity of the Poly(LLA-co-DXO), as previously demonstrated for Poly(LLA-co-DXO) copolymer films compared to P(LLA) polymers (Dânmark, et al. 2010). These results are consistent with those of previous studies, in which PLA/glass foams, P(LLA) films with apatite or apatite/collagen composite coating and PLLA films modified with poly(ethylene imine), respectively, markedly improved the adhesion, viability, proliferation and function of HOBs compared with PLA/glass foams and P( LL A) films, respectively (Chen, et al. 2008, Liu, et al. 2009, Navarro, et al. 2004).
HOBs were cultivated in extracts from P(LLA), Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds, stained and observed by inverted phase contrast microscopy. Cell viability staining indicated that HOBs showed good viability after incubation for 24 hours. No obvious differences in cell morphology were observed between cells grown on TCPS with scaffold extracts, compared to TCPS with α-MEM. The results confirmed the in vitro biocompatibility of both Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds with the two cell types, promoting cell proliferation without a cytotoxic effect. SEM is a valuable technique for visualising cell morphology and distribution along the scaffold surface and its inner pores. The SEM micrographs showed good spread of the cells on the test scaffolds. Moreover, the cells adhered to the scaffold surface, with some of the cells attaching to pore rims.

Apoptosis, or programmed cell death, is a physiological process of cell death and is thought to be involved in every homeostatic and pathological process in the body (Gastman 2001). In the present study, apoptotic activity within HOBs on the three scaffolds was investigated using a fluorogenic substrate for caspases 3/7 and real time RT-PCR. The results for cell death showed that at 24 hours, the enzymatic activity of caspases 3 and 7 on the scaffolds was similar to that on the negative control ($P > 0.05$). mRNA expression of $Bcl-2$ and $Bax$ was assessed by qRT-PCR 24 hours and 7 days post-seeding of HOBs onto P(LLA), Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds. After 24 hours, no statistically significant differences were found in HOBs seeded onto the test scaffolds in comparison with the control. However, after 7 days, significant changes were observed: an increase in $Bcl-2$ and a decrease in $Bax$ expression ($P < 0.05$). At this time-point, better cell viability and fewer cell deaths were noted for the Poly(LLA-co-DXO) scaffold.
Bcl-2 and Bax belong to a gene family involved in the regulation of cellular apoptosis. In a viable cell, proapoptotic members such as Bax have an antagonist antiapoptotic member such as Bcl-2 (Adams & Cory 1998). Downstream from Bcl proteins in the apoptotic cascade are the caspases, a family of intracellular cysteine aspartic specific proteases which play a pivotal role in apoptosis. Moreover, the increase in caspase-3 activity is synchronized with increased Bax and decreased Bcl-2 expression. Bcl-2 inhibits apoptosis and contributes to cell survival and resistance of cells to damage: cell survival is enhanced under conditions of relatively high expression of Bcl2 and low expression of Bax (Chinnaiyan & Dixit 1996, Chinnaiyan, et al. 1996, Gillardon, et al. 1995). Thus the present results confirmed that apoptotic activity, measured in terms of Bc12 and Bax expression and caspase 3 and 7 activity, was not increased in cells seeded onto the test scaffolds. The enhancement of proliferation of HOBs on the test scaffolds compared to PLLA, as shown by MTT, LDH and SEM, are in accordance with the data reported by Dånmark et al. (2010) and with the result presented in paper II (Idris, et al. 2010). The observed caspase activity is supported by the qRT-PCR results, confirming that the newly developed scaffolds do not induce death of HOBs.

4.2. Polyester co-polymer scaffolds enhance expression of bone markers

In Paper II, scaffolds without HOBs were examined by SEM, to document the surface texture of the three types of scaffolds used. SEM micrographs showed that the scaffolds contain pores ranging from a few micrometers up to several hundred micrometers. A preceding study on scaffolds fabricated using the same wide range of salt particles showed that most pores were in the range of ~20-150 µm (Odelius, et al. 2005). To assess spread and morphology of the HOBs grown on the scaffolds, the
surface of each scaffold was examined by SEM. On day 1 of culture, there was a pronounced difference between the test and control scaffolds: more cells had attached to the test scaffolds, the cells had spread well and the pseudopodia were elongated. Furthermore, on both test scaffolds the cells had started to form sheets, indicating good attachment. After 1 and 7 days of culture, the viability and proliferation of HOBs grown on the Poly (LLA-co-CL) and the Poly (LLA-co-DXO) scaffolds were significantly higher ($P < 0.05$) than on the P(LLA) scaffold. On day 14, a thick layer of cells and ECM had formed on both test scaffolds. On the control scaffold, fewer and more widely separated cells were observed. For all three scaffolds, MTT activity increased with culture time, indicating adequate cell proliferation.

SEM and MTT analysis disclosed that the HOBs had responded well to the Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds. In contrast, the surface of the P(LLA) scaffold showed poor cellular attachment and proliferation. The Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds also showed higher MTT activity than the PLLA scaffold. The physical and chemical properties of biomaterial surfaces, such as topography, chemical composition, and hydrophilicity, could affect cell-biomaterial interactions (Chen, et al. 2008). Thus, the present results suggest that the test scaffolds had a positive influence on cellular viability and proliferation. A previous study on different triblock copolymers of DXO and LLA, where DXO was the middle block, showed that the hydrophilicity increased with the amount of DXO and that both keratinocytes and fibroblasts adhered best to the most hydrophilic material (Mattioli-Belmonte, et al. 2005). Another recent study reported the more hydrophilic nature of the Poly (LLA-co-DXO) scaffold with a contact angle of $75^\circ \pm 5^\circ$, compared to the Poly(LLA-co-CL) scaffold with a contact angle of $85^\circ \pm 3^\circ$ and the P(LLA) scaffold with a contact angle of $86^\circ \pm 2^\circ$ (Dånmark, et al. 2010). Furthermore, Chen et al.
(Chen, et al. 2008) reported high efficiency of attachment, proliferation, and viability of HOBs on a more hydrophilic apatite/collagen composite coated P(LLL) film compared with P(LLL) control film.

After culturing of HOBs on the three scaffolds, concentrations of Col 1 and OC in the culture media were quantified by ELISA. Col 1 production was significantly higher ($P<0.05$) on the Poly (LLA-co-DXO) test scaffold than on the control P(LLL) throughout the culture period, whereas OC production increased over time in the test scaffolds and was significantly higher ($p<0.05$) on the Poly (LLA-co-DXO) scaffold than on the control scaffold throughout.

The qRT-PCR results showed significantly higher ($p<0.05$) mRNA levels of the bone markers ALP, Col 1, BSP, OC, OP, and Runx2 in HOBs grown on the test scaffolds [Poly(LLL-co-CL) and the Poly(LLL-co-DXO)] than on the control scaffold P(LLL). Other studies have indicated that the hydrophobic/hydrophilic nature of biomaterials could affect differentiation of mesenchymal stem cells (Curran, et al. 2006).

Runx2 is involved in direct regulation of Col 1, BSP and OC expressions (Bjerre, et al. 2008). In the present study, Runx2 expression by HOBs grown on the Poly (LLA-co-CL) and the Poly(LLL-co-DXO) scaffolds on day 14 increased more than 3.0-fold, compared with 1.1-fold for the control scaffolds. This suggests that the test scaffolds stimulated bone cells to constant expression of Runx2, which promotes osteoblast maturation. The reduced expression of Runx2 on day 21 suggests the presence of a negative feedback mechanism that controls Runx2 expression after maximum expression on day 14. These results are in accordance with those of previous studies of expression of Runx2/Cbfa1 by human mesenchymal stem cell (hMSC) and HOB cell lines grown on different types of scaffolds of silicate-
substituted tricalcium phosphate (Si-TCP) (Bjerre, et al. 2008), coral (Foo, et al. 2008) and coralline hydroxyapatite (Mygind, et al. 2007).

ALP is a known marker for osteoblastic phenotype characterization of HOBs cultured in vitro (Aubin 1998) and is one of the most commonly used markers for osteogenic differentiation (Kasten, et al. 2008). In this study, ALP expression by the cells grown on the Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds peaked on day 7 and decreased on days 14 and 21 compared to the control scaffold. Similarly, Bjerre et al. reported an increase in ALP expression by hMSCs seeded for up to 21 days on Si-TCP scaffolds on day 7 but a decrease on days 14 and 21 (Bjerre et al. 2008). In contrast, Malicev et al. investigated the expression of several bone markers by alveolar bone cells seeded onto constructs made of a fibrinogen solution (fibrin glue) and high porosity hydroxyapatite granules for up to 21 days and reported an increase in ALP expression on day 14 (Malicev, et al. 2008). Such discrepancies in results may be attributable to differences in culture method and scaffold type. In the present studies, the cells were statically cultured and the pore size range was similar for test and control scaffolds. The results confirm that the test scaffolds have the properties required to promote osteogenic differentiation.

Col 1 is the most abundant protein of ECM, comprising about 90% of the organic phase of bone. It is expressed during the initial period of proliferation and extracellular-matrix biosynthesis (Aubin 1998). In paper II, expression of Col 1 by HOBs grown on the Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds, was measured by qRT-PCR, and was found to be greater than that of HOBs from the control P(LLA) scaffolds. In a study of two types of coralline hydroxyapatite scaffolds which differed in porosity and pore size, Mygind et al. reported an increase in Col 1 by hMSCs retrieved from the scaffolds from day 7 to day 21 (Mygind, et al. 2007).
The porosity of the scaffold with a mean pore size of 200 µm was 75%, while that of the scaffold with a mean pore size of 500 µm was 88%. The only significant difference in Col 1 expression between the two types of scaffolds occurred on day 21, and the highest expression was found for the scaffold with a mean pore size of 500 µm.

Analysis of the present results revealed no pronounced differences between the test and the control scaffolds with respect to the porous structure. This suggests that the material properties may be responsible for the differences in gene expression. Moreover, ELISA results confirmed the qRT-PCR finding. Thus at 7 days, HOBs seeded onto Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds exhibit greater synthesis of Col 1 protein than those seeded onto P(LLA) scaffolds.

BSP is an indicator of cellular maturation (Mygind, et al. 2007). In paper II, expression of BSP by cells grown on the Poly(LLA-co-CL) and the Poly(LLA-co-DXO) scaffolds was relatively greater than the control on day 7 and day 14 and greatest on day 21. The genetic expression of this marker revealed an increase in the level of cellular maturation. In a study of statically cultivated coralline hydroxyapatite scaffolds with a mean pore size of 200 µm, Mygind et al. reported a decrease in BSP expression by MSCs on day 14 and increases on days 7 and 21 (Mygind, et al. 2007). However, Ma et al. demonstrated that MC3T3-E1 osteoblasts, cultured in vitro on highly porous P(LLA)/hydroxyapatite composite scaffolds, showed increased BSP expression on day 14 compared with P(LLA) scaffold (Ma, et al. 2001). Hydroxyapatite has osteoconductive properties and closely resembles the mineral phase of bone: its incorporation into P(LLA) scaffolds clearly enhanced expression of BSP. The inconsistencies in BSP expression reported in the literature might be attributable to differences in scaffold materials, cell types, porosities, and pore sizes.
OC is the most specific protein marker of mature osteoblasts and can be detected during the earlier phases of proliferation and matrix maturation (Aubin 1998). In the present study, qRT-PCR results disclosed higher levels of expression of OC mRNA by the cells grown on the Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds than on the control scaffolds throughout the culture period, with greatest expression on day 21. Malicev et al. reported an increase in OC expression by alveolar bone cells cultured in tissue-engineered hydroxyapatite granule constructs on day 7, followed by a decrease on days 14 and 21 (Malicev, et al. 2008). In contrast, a study of osteoblasts seeded onto coral scaffolds in static culture showed an increase in OC expression at 7 and 18 days (Foo, et al. 2008). In the present study, ELISA disclosed greater OC expression by HOBs grown on the Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds than on the P(LLA) scaffold, progressively increasing towards day 21. This suggests that the test scaffolds might induce earlier cell maturation. The HOBs grown on the test scaffolds showed the same tendency with respect to osteogenic mRNA and the translated proteins for Col 1 and OC at a given time point. This indicates a correlation between levels of mRNA and protein expression for the same scaffolds.

OP is one of the predominant noncollagenous proteins in bone tissue (Aubin 1998) and is produced by osteoblasts (Foo, et al. 2008). Expression of OP by the osteoblasts seeded onto the Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds was also relatively higher than from the control P(LLA) scaffolds. The consistent osteoblastic expression of OP indicates that the Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds might promote mineralization by expressing organic protein, which aids in bone regeneration. These results are consistent with those of previous studies of expression of OP by HOB cell lines and hMSC grown on different types of coral scaffolds (Foo, et al. 2008) and coralline hydroxyapatite (Mygind, et al. 2007).
4.3. Gene expression profile of cells attached to polyester co-polymer scaffolds

The results of study II clearly showed that the test scaffolds had influenced genes involved in differentiation of HOBs. On the basis of these findings, microarray analysis was applied in Paper III, in order to better understand the differential impact of the Poly(LLA-co-DXO) and P(LLL) scaffolds on the osteogenic activity of seeded HOBs and to determine whether these scaffolds alter the gene expression profile of the HOBs. For this, we used the Illumina microarray platform containing more than 48,000 probes derived from human genes in the National Center for Biotechnology Information (NCBI) representing the whole human genome.

In paper III, we identified several genes whose expression profile was up- or down-regulated at 24 hours. In contrast, very few genes were differentially expressed at 21 days. This finding may be related to the good adaptation of the HOBs to the Poly(LLA-co-DXO) and P(LLL) scaffolds following a longer incubation period. Among the genes that were found as differentially expressed at 24 hours, many were related to cell adhesion (including *i.a.* integrin alpha 3 [ITGA3], immune response (interleukin 8 [IL8]), skeletal system development (insulin-like growth factor 2 [IGF2]), apoptosis regulation (heme oxygenase 1 [HMOX1], Growth arrest and DNA-damage-inducible protein GADD45 alpha [GADD45A]), proliferation (Notch homolog 1, translocation-associated [NOTCH1], jagged 1 [JAG1]) and skeletal system development (ALP). Gene related to bone mineralization (ALP) was also differentially expressed at day 21, in addition to genes involved in ossification (stanniocalcin 1 [STC1], matrix Gla protein [MGP]) and apoptosis (serum/glucocorticoid regulated kinase 1 [SGK]).

PANTHER analyses provided valuable mechanistic information about three key biological pathways involving integrin, Notch and Ras. Integrins constitute a
widely expressed family of cell surface receptors that are involved in cell-extracellular matrix (ECM), cell-cell adhesion, cell-cell interaction and in stimulation of specific signal transduction pathways (Danen & Sonnenberg 2003, Giancotti & Ruoslahti 1999, Shattil, et al. 2010). Moreover, integrins are important regulators of cellular and host responses to implanted devices, biological integration of biomaterials and tissue-engineered constructs (Anderson 2001, Anderson 2006, Lutolf & Hubbell 2005). In the present study, HOBs cultured onto Poly(LLA-co-DXO) scaffold expressed higher levels of ITGA3, ITGA5 and ITGB4 genes than HOBs cultured onto P(LLA) scaffold construct, at 24 hours. Ras is a key regulator of cell growth, differentiation and survival (Vojtek & Der 1998). In the present study HOBs cultured onto Poly(LLA-co-DXO) scaffold which has high hydrophilicity expressed higher levels of genes related to Ras pathway than HOBs cultured onto P(LLA) scaffolds. Our findings are also in agreement with other studies comparing behavior of osteoblastic cells on hydrophilic and hydrophobic surfaces (Chang, et al. 2005). Taken together, we suggest that Ras pathway is an essential target that warrants careful consideration when surface modifications of hydrophobic biomaterials are going to be tested in order to induce adequate cell responses in the bone tissue. Notch is a transmembrane receptor that mediates local cell-cell communication and coordinates a signaling cascade present in all animal species studied to date. The major biological role of Notch signaling is to control the developmental fates of cells, and cells become distinguished from one another according to whether they predominantly send or receive Notch signals (Lai 2004, Mumm & Kopan 2000). Calvi et al (2003) reported an increase in the trabecular bone mass and overexpression of the Notch ligand, JAG1 in the osteoblasts. Our results showed up-regulation of JAG1 in HOBs grown into Poly(LLA-co-DXO)
scaffold at 24 hours and the PANTHER search also revealed a Notch signaling pathway.

In study III, many genes which regulate osteoblastic differentiation and bone formation - e.g. insulin-like growth factor 2 (IGF2) and platelet derived growth factor (PDGFA) - were significantly up-regulated in HOBs seeded onto Poly(LLA-co-DXO) scaffolds for 24 hours. The microarray data revealed the presence of osteoblast marker genes (COL I, OC, OP, osteonectin, and Runx2) in both test and control scaffolds. These findings are consistent with those of previous studies carried out by our group on Poly(LLA-co-DXO) and P(LLA) scaffolds, and demonstrated that mRNA expression and protein levels for the above genes from different types of bone cells increased with Poly(LLA-co-DXO) scaffold compared to the P(LLA) scaffold (Dänmark, et al. 2010, Idris, et al. 2010, Schander, et al. 2010, Xue, et al. 2010).

The genetic profiles described in different studies may vary depending on the time point selected in the experimental design. In Paper III, the time points of 24 hours and 21 days of stimulation were selected in order to gain information about cellular activity during the early and late stages of cell stimulation. The genes elaborated on represent only a limited number of those found to be differentially expressed. Microarray data for some of the genes with known functions were further validated by qRT-PCR. In order to gain a global comprehension of the molecular events related to Poly(LLA-co-DXO) scaffold/cell interaction, however, further studies are necessary using a variety of scaffolding surfaces, osteoblast-like cell lines and different time-points.
5. **Concluding Remarks**

Tissue engineering concepts combine multidisciplinary knowledge with cutting-edge technologies, in order to achieve regeneration of damaged or lost tissue. Biomaterials suitable for tissue engineering must meet stringent requirements with respect to biocompatibility, biodegradability and biomechanical properties.

The present studies concern a new generation of biodegradable biomaterials, Poly(LLA-co-DXO), Poly(LLA-co-CL) and copolymers of P(LLA), with great potential for tissue engineering, particularly in orthopedic applications. In particular, Poly(LLA-co-DXO) has emerged as a good candidate for bone tissue engineering applications, exhibiting many desirable properties such as biocompatibility, biodegradability, tunable mechanical stability, flexible surface properties, and wide availability. The studies on which this thesis is based showed that the copolymer scaffolds are cytocompatible with HOBs, and do not impair cell adhesion and proliferation. The scaffolds are not cytotoxic and cells adhered well to scaffold surfaces during the first 24 h. Cell viability, cytotoxicity and apoptosis assays reflected good cell growth and proliferation. In vitro, the test scaffolds met cytocompatibility requirements and therefore warrant further investigation as promising constructs for application in bone tissue engineering.

The results of these studies also support the hypothesis that co-polymer scaffolds might be appropriate cell carriers, superior to P(LLA) and suitable for osseous tissue engineering. Initially, more cells attached to the test scaffolds than to control scaffolds of P(LLA), and after 14 days more extensive ECM formation and cell growth were observed. Enhanced osteoblast proliferation and differentiation were demonstrated by increased mRNA expression of the *ALP*, *COL 1*, *BSP*, *OC*, *OP*, and
Runx2 genes. Compared to control scaffolds, the test materials supported early osteoblast maturation, increasing the secretion of bone matrix, which aids in bone regeneration. In vivo studies are now warranted to compare the performance of the HOB/scaffold constructs in a clinical bone tissue engineering context.

The results demonstrated a profound difference in the global gene expression profiles of HOBs grown on Poly(LLA-co-DXO) and P(LLA) scaffolds, especially after initial incubation of 24 hours. Statistical analyses at the chip and probe levels indicated that several genes were differentially expressed as a function of Poly(LLA-co-DXO) scaffolds. More detailed examination of the differentially expressed genes revealed several interesting molecular pathways related to cell adhesion, cell-cell communication and cell proliferation. The study presented a model for gathering data about cell/biomaterial interactions at the molecular level. The various biological pathways found to be associated with Poly(LLA-co-DXO) scaffold interactions might be explored individually in greater detail. In future, proteomic studies might be applied to reveal the relationship between transcript and protein levels. Further studies are warranted to examine the cellular response, in terms of specific HOB markers or signalling pathways, stimulated by interaction with scaffolding.

6. Future Research Directions and Challenges

The results presented in this thesis not only indicate potential directions for future research, but also raise several important questions which need to be addressed if Poly(LLA-co-CL) and Poly(LLA-co-DXO) scaffolds are to be tested or applied for orthopedics and in particular, in the broader field of tissue engineering. Among the most challenging issues to be addressed are the mechanical properties, large-scale production and controllable degradation.
7. References


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8. Original Papers