3D Differentiation of Adipose-derived Stem Cells in a Thermosensitive Poly-Caprolactone Scaffold

Doctoral thesis at the Medical University of Vienna for obtaining the academic degree

Doctor of Philosophy

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**Declaration**

All work described in this thesis was performed at the

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Histological processing was done at the

Bernhard Gottlieb University School of Dentistry, Vienna, Austria

The scaffold material used in the described experiments was developed and produced at the

University of Nottingham, Division of Drug Delivery and Tissue Engineering,
Nottingham, United Kingdom

Heinz Redl as well as Susanne Wolbank consulted during the whole project. Except chapter 2.3, all parts of this thesis were written by the author. The coauthors of the University of Nottingham contributed to chapter 2.1, 2.2 as well as 2.3 by providing the thermoresponsive scaffold. Chapter 2.1 has been accepted for publication by “Biomedical Materials”. Alexandra Meinl assisted in the development of the histological procedures. All authors proof-read the article prior to publication.

In chapter 2.2, Georg Feichtinger assisted in the design of the qRT-PCR primers used. Chapter 2.3 have been written by Paul Slezak, except the description of the material and its background in the introduction, the preparation of the material in the materials & methods section, and by contributing to the discussion. The author prepared the thermoresponsive scaffold for its in vivo use and for the analysis using SEM. The author conducted the SEM analysis.
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Summary

For tissue engineering (TE) purposes, scaffolds of various components and designs are combined with cells to form a construct that can be implanted in a defect site. A new approach involves the usage of injectable scaffolds, which offer additional options compared to solid matrices, such as the possibility to homogenously distribute cells or growth factors within the construct, the reduced invasiveness or the potential to fill and adjust to irregular shapes. One class of injectable scaffolds are thermoresponsive scaffolds, in which the materials are cross-linked via physical interactions. In this study, a fully bioresorbable biomaterial, poly(ε-caprolactone) (PCL) was combined with the polymer poly(PEG methacrylate ethyl ether) (polyPEGMA-EE_{246}) to form a thermoresponsive construct that is liquid at 4°C and solid at 37°C.

As a first step, a method was established that allows the histological analysis of thermoresponsive scaffolds. Using gelatin embedding, the scaffold can be stabilised in its solid form and can be subsequently transferred, histologically prepared, sectioned and stained.

We were interested in the suitability of the scaffold to support bone and soft TE. Therefore, the PCL- polyPEGMA-EE_{246} scaffold was combined with cells featuring stem cell characteristics. The C2C12 cell line has myogenic as well as osteogenic differentiation potential and serves therefore as ideal characterisation cell line. Human adipose tissue-derived stromal cells (ASCs) were used as primary cell source to proof the relevance of the PCL- polyPEGMA-EE_{246} for TE. ASCs have the potential to differentiate towards cell types of mesodermal origin, including adipocytes and osteoblasts. The suitability of the scaffold to support 3D cellular attachment, survival, distribution, persistence as well as differentiation was characterised in comparison to 2D cultures on regular cell-culture dishes. C2C12 cells as well as ASCs were viable and found evenly distributed within the scaffold. C2C12 cells showed a limited potential to differentiate towards the osteogenic lineage in 3D conditions and improved myogenic differentiation in 3D compared to 2D. Increased adipogenic differentiation in 3D compared to 2D was found when differentiating ASCs, whereas also ASCs showed no improved osteogenic differentiation in 3D compared to 2D.
The performance of a scaffold *in vivo* is dependent on the formation of new vessels in the constructs for cellular nutrient-supply. We evaluated whether the scaffold was able to support vascularisation in an *in vivo* model by sheathing a vessel bundle with a silicon tube that can be filled with the biomaterial of interest. An increase in newly formed vessels was detected during the first two weeks followed by a decline in total vascularisation at week four. No signs of inflammation were visible.

To conclude, a thermoresponsive PCL-based scaffold was evaluated for its suitability for TE purposes. *In vitro*, the scaffold was able to support the differentiation of C2C12 cells towards the myogenic lineage as well as adipogenic differentiation of ASCs. *In vivo*, cellular infiltration and vessel formation was observed in an angiogenesis model. This suggests that the scaffold supports the formation of soft tissues and is therefore a promising candidate for further TE approaches.
Zusammenfassung

Im Bereich der Gewebezüchtung werden Materialien aus verschiedenen Komponenten und mit verschiedenen Designs mit Zellen kombiniert um ein Zellgerüst zu formen, welches in einen Defekt implantiert werden kann. Ein neuer Ansatz involviert die Verwendung on injizierbaren Trägermaterialien. Im Vergleich zu festen Materialien bieten diese zusätzliche Optionen, unter anderem die Möglichkeit Zellen oder Wachstumsfaktoren mit dem Konstrukt zu vermengen, eine reduzierte Invasivität oder die Möglichkeit ungleichmäßige Formen zu füllen. Eine Kategorie von injizierbaren Matrizen sind thermosensitive Träger, bei denen die Materialien mittels physikalischer Interaktionen verbunden werden. In dieser Studie wurde ein komplett bioresorbables Biomaterial, poly(ε-caprolactone) (PCL), mit dem Polymer poly(PEG methacrylate ethyl ether) (polyPEGMA-EE_{246}) kombiniert um ein thermosensitives Konstrukt zu formen, welches bei 4°C flüssig und bei 37°C fest ist.

Zuerst wurde eine Methode etabliert, welche die histologische Analyse von thermosensitiven Biokonstrukten erlaubt. Durch die Einbettung in Gelatine kann das Konstrukt in seiner festen Form stabilisiert werden und dadurch anschließend transferiert, histologisch präpariert, geschnitten und gefärbt werden.

gesteigertes adipogenes Differenzierungspotential wurde beobachtet wenn Fettstammzellen in 3D differenziert wurden, dagegen wurde keine Verbesserung im osteogenen Differenzierungspotential von Fettstammzellen gefunden, wenn sie in 3D differenziert wurden.


Publications arising from this thesis

Parts of the study have been presented as poster or oral presentation at following conferences or meetings:

Summer school on Biomaterials and Regenerative Medicine
Riva del Garda, 19.-23.9.2011 (Oral Presentation)

Tissue Engineering and Regenerative Medicine International Society (TERMIS)
Granada, 7.-10.6.2011 (Poster)

Transeuro, Optistem, Plasticise, Endostem, Angioscaff (TOPEA) summerschool
Barcelona, 29.6.-1.7.2011 (Poster)

7th YSA-PhD-Symposium
Vienna, 15.-16.6.2011 (Poster)

European Cooperation in Science and Technology (COST) Meeting
Vienna, 4.-5.9.2012 (Oral Poster)

Tissue Engineering and Regenerative Medicine International Society (TERMIS)
World Congress
Vienna, 5.-8.9.2012 (Poster)

“TERM STEM 2012”
Guimaraes, 9.-13.10.2012 (Oral Presentation)

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Vienna, 19.-21.11.2012 (Oral Presentation)

Following article has been accepted for publication:
“Gelatin embedding for the preparation of thermoreversible or delicate scaffolds for histological analysis“

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ASC</td>
<td>adipose tissue-derived stromal cells</td>
</tr>
<tr>
<td>aSC</td>
<td>adult stem cell</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BMSC</td>
<td>bone-marrow derived mesenchymal stem cells</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-Diaminobenzidintetra-hydrochlorid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GVHD</td>
<td>graft-versus-host disease</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cells</td>
</tr>
<tr>
<td>iPS</td>
<td>induced pluripotent stem cells</td>
</tr>
<tr>
<td>LCST</td>
<td>lower critical solution temperature</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>NIH</td>
<td>national institute of health</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>PCL</td>
<td>poly(epsilon-caprolactone)</td>
</tr>
<tr>
<td>PECE</td>
<td>PEG-PCL-PEG</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PGA</td>
<td>poly(glycolic acid)</td>
</tr>
<tr>
<td>PLA</td>
<td>poly(lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(D,L-lactide-co-glycolide)</td>
</tr>
<tr>
<td>PNIPAm</td>
<td>Poly(N-isopropyl acryl amide)</td>
</tr>
<tr>
<td>PEGMA-EE&lt;sub&gt;246&lt;/sub&gt;</td>
<td>poly(PEG methacrylate ethyl ether)</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet rich plasma</td>
</tr>
<tr>
<td>SVF</td>
<td>stromal vascular fraction</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>tissue engineering</td>
</tr>
<tr>
<td>THA</td>
<td>total hip arthroplasty</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
</tr>
<tr>
<td>WOMAC</td>
<td>Western Ontario and McMaster Universities Osteoarthritis Index</td>
</tr>
<tr>
<td>βTCP</td>
<td>β-Tricalcium phosphate</td>
</tr>
</tbody>
</table>
Danksagung

Nur durch die viele Unterstützung konnte ich diese Arbeit fertig stellen und ich würde gerne diese Möglichkeit nützen um mich bei einigen davon zu bedanken.


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CHAPTER ONE: INTRODUCTION

1. Background

1.1. Strategy of tissue engineering

The term „tissue engineering“ was first used by Wolter and Meyer in 1984 while observing the growth of epithelial-like cells on the surface of a keratoprosthesis (Wolter and Meyer, 1984). In 1993, tissue engineering (TE) was defined as “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function” (Langer and Vacanti, 1993). The objective of TE is to direct cells with the help of a scaffold and molecular signalling, such as growth factors, to form a living tissue that should be indistinguishable from the surrounding tissue found in nature both in a structural but also functional way. Ideally, autologous cells serve as cell source to avoid immunogenic reactions and are principally obtained from the bone marrow, blood or fat. Cells that exhibit stem cell features can be used to replace tissue from other origins, such as cells derived from adipose-tissue, which are able to differentiate towards osteocytes and can be used to stimulate bone regeneration. The formation of blood vessels to provide sufficient nutrient supply within a newly formed tissue plays a crucial role in TE.
CHAPTER ONE: INTRODUCTION

Figure 1: The strategy of tissue engineering: Cells isolated from the patient (a), cultured \textit{in vitro} (b) and seeded 3D in a scaffold combined with growth factors, small molecules (such as adhesion proteins including RGD amino acid sequences) and micro- and/or nanoparticles (c). The cell-scaffold construct is further cultivated under optimal conditions for organization into a functional tissue (d) and the successfully engineered tissue can be then transplanted into the defect site (e). Figure taken from Dvir et al., 2010.

Some of the most prominent approaches for tissue regeneration include bone and soft TE.
1.2. Bone tissue engineering

Bone tissue has two major functions: First of all it gives mechanical support, allowing protection and movement (in combination with muscles, tendons, ligaments and joints). Second, it is involved in the regulation of phosphate and calcium levels in the body. Comparing the structure of bone tissue, two types have been identified: Cancellous bone, which is also called trabecular or spongy bone, and compact bone, also referred to as dense or cortical bone. The mechanically strong compact bone is found on the outer layer of the bone, while the inner mass consists of cancellous bone (Clarke, 2008; Gaalen et al., 2008). Osteoprogenitor cells can differentiate to osteoblasts, osteocytes and lining cells. Osteoblasts are bone forming cells as they produce uncalcified matrix that is further calcified by calcium salt deposition (Civitelli, 2008; Gaalen et al., 2008). Osteocytes, which are found in the osteoid, have two major functions: The regulation of mineralization and the formation of dendritic processes (Bonewald, 2011). Very little is known about lining cells, which are thought to be quiescent osteoblasts. Another hypothesis suggests that lining cells are major mechanosensory cells in the adult skeleton (Bonewald, 2011; Cowin, 2002). Osteoclasts are derived from mononuclear monocyte-macrophage precursor cells and are the only cells known to be able to resorb bone tissue (Clarke, 2008).

Bone formation occurs by intramembranous or endochondral ossification. Condensations of mesenchymal cells, that contain osteogenic cells, can form bone in association with adequate vascularisation. This process of intramembranous ossification, which does not require an intermediate step, leads to the formation of flat bones (such as cranial bones). Several small bone masses align and form trabeculae. Layered osteoid formation occurs on the trabecular surfaces by osteoblast zones. This appositional bone formation takes place until sufficient bone is obtained. Shape and density are subsequently adapted by bone remodelling, in which bone resorption by osteoclasts and bone formation by osteoblasts happen simultaneously.

Long bone formation is achieved by endochondral ossification. In the first step, mesenchymal stem cells (MSCs) differentiate into chondroblasts, which produce cartilage matrix. In the centre of this cartilaginous tissue, cells become hypertrophic and form a calcified extracellular matrix (ECM). The ECM is the secreted product of resident cells within a tissue and has a structural as well as functional task (Gaalen et
al., 2008). The invasion of capillaries deliver osteoblast progenitors, which start ossification centres and further differentiate into mature osteoblasts. Cartilaginous ECM is replaced by bone ECM that is secreted from osteoblasts. Ossification takes place in almost the entire bone, except the growth plate, a narrow zone at both ends of the bone responsible for bone elongation until adolescence, and the central canal (Gaalen et al., 2008; Pechak et al., 1986; Petite and Quarto, 2005).

For decades, the golden standard for bone grafts has been autologous bone harvested from the iliac crest or by resection of a rib. The major drawbacks of this method include the prolonged operation time, limited availability and surgical complications, such as wound infections or chronic donor site pain. Therefore allografts and xenografts have been considered as replacement for autografts. However, the risk of transmitting diseases and immunological responses limits its usage in vivo (Gaalen et al., 2008). Bone TE attempts to stimulate bone formation using implants that are preferably created according to the principles of scaffold design criteria (see chapter 5 “Key Elements in Tissue Engineering”). Bone TE ideally replaces autologous bone grafts and should lead to osteoinduction (the process by which osteogenesis is induced), osteoconduction (bone growth on a surface) and osteointegration (direct contact between bone and implant) (Albrektsson and Johansson, 2001; Albrektsson et al., 1981). Standard scaffold materials used for bone TE include β-tricalcium phosphate (βTCP), hydroxyapatite, bicalcium phosphate, octacalcium phosphate, calcium sulfate, poly(D,L-lactide-co-glycolide (PLGA), Poly(ε-caprolactone) (PCL) (Szpalski et al., 2012a). Adult stem cell types frequently used for bone TE include bone-marrow derived mesenchymal stem cells (BMSCs), umbilical vein mesenchymal stem cells, adipose tissue-derived stromal cells (ASCs, also known as adipose-derived stem cells), human umbilical vein endothelial cells and dental pulp-derived stem cells (Bourin et al., 2013; Seong et al., 2010; Szpalski et al., 2012b). For in vitro predifferentiation, osteogenic differentiation can be stimulated via factors, such as of ascorbic acid, β-glycerophosphate (βGP) or dexamethasone, bone morphogenetic proteins (BMPs), fibroblast growth factors (FGF) and Vitamin D, depending on the cell type, (Park, 2012; Seong et al., 2010).
1.3. **Soft tissue engineering**

Soft tissue is defined as supporting extraskeletal tissues of the body, including muscle, fat, fibrous tissue and blood vessels (Weiss, 1974).

**Muscle tissue**

Vertebrates contain three types of muscle: skeletal muscle, smooth muscle and cardiac muscle. The primary function of skeletal muscles, longitudinal force, is achieved by uniaxially directed bundles, consisting of up to 12 myofibrils. Myofibres, the smallest unit of a myofibril, are terminally differentiated multinucleated muscle cells and are surrounded by connective tissue (Stern-Straeter et al., 2007). A number of factors were isolated and showed to activate myogenesis in skeletal muscles, including MyoD and myogenin (Braun et al., 1989). Several reasons can lead to a decrease or loss in muscle tissue, such as traumatic injuries, extensive surgical tumour ablation or muscle fibre atrophy. The most common method to restore skeletal muscle tissue is autologous grafting, but it is associated with several disadvantages, such as donor-site morbidity, functional loss or volume deficiency (Rossi et al., 2010; Stern-Straeter et al., 2007). Smooth muscles are critical components of the gastrointestinal, cardiovascular and urological tissues (Kim et al., 1999a). The major intermediate-sized filament constituent of smooth muscle cells originating from the gastrointestinal, respiratory and urogenital tract is desmin. In contrast to vascular smooth muscle cells, these cell types also show a predominance of γ-type smooth muscle actin. Vascular smooth muscle cells contain abundant amount of vimentin, while minimal amounts of desmin are present (Gabbiani et al., 1981; Lazarides and Hubbard, 1976).

The majority of cardiomyocytes are mono- or binucleated cells. A minimal fraction consists of tri- and tetranucleated cells (Olivetti, 1996; Schneider and Pfitzer, 1973). Smooth muscle cells and cardiac muscle are also known as “involuntary” muscle, which contract due to impulses by the body. Skeletal muscle is called “voluntary” muscle and is normally consciously controlled by the individual. In opposition to skeletal muscle, cardiac muscle cells are connected via intercalated discs, which are responsible for mechanical and electrical communication among cardiomyocytes (Wang et al., 2012). Indications for smooth muscle TE include gastrointestinal diseases, such as the short bowel syndrome, vascular grafting, and applications in
the urogenital tract (Atala et al., 2006; Edelman, 1999; Kim et al., 1999b; Spurrier and Grikscheit, 2013)

Similar to bone TE, adult stem cells are the main source of cells for muscle TE. Cell sources for skeletal muscle TE include muscle satellite cells, skeletal myogenic progenitors, muscle stem cells, mesoangioblasts, pericytes, and MSCs (Rossi et al., 2010). A variety of biomaterials is explored as 3D scaffold for muscle TE, such as collagen, fibrin gel, poly(glycolic acid) (PGA), PCL and alginate (Eschenhagen et al., 1997; Guex et al., 2012; Rossi et al., 2010; Yeong et al., 2010).

**Adipose tissue**

On a macroscopical level, five types of adipose tissue have been described: bone marrow, brown, mammary, mechanical and white adipose tissue. Bone marrow adipose tissue fills space no longer required for haematopoiesis, serves as energy deposit and as cytokine source for osteogenesis and haematopoiesis. Brown adipose tissue is responsible for nonshivering thermogenesis and is located around the major organs in new born infants (Cannon and Nedergaard, 2004; Choi et al., 2010; Gimble et al., 2007). Pregnancy-associated hormones regulate mammary adipose tissue, which supplies nutrients and energy during lactation. Mechanical adipose tissue provides support for critical structures, including the eye or the hand. White adipose tissue forms the energy reservoir and insulation of the body. The secretion of factors, such as adiponectin, leptin, resistin and other adipokines causes systemic physiological and pathological effects. In humans, white adipose tissue is mainly found intra-abdominally and subcutaneously. Each depot shows distinct biological properties and therefore ASCs demonstrate depot-specific differences. For example, ASCs isolated from visceral white adipose tissue (WAT) show an increased osteogenic differentiation potential than those from subcutaneous WAT (Cawthorn et al., 2012a). Several other factors, such as age and body mass index might be able to influence ASC cell number, viability and function (Cawthorn et al., 2012b; Gealekman et al., 2011; Madonna et al., 2011; Tchkonia et al., 2007; Zhu et al., 2009). White adipose tissue is the only tissue that is able to change its mass in adulthood. The fat mass in females usually varies between 14-28%, while men show values between 9-18%. Adipose tissue consists of mature adipocytes, stromal-vascular cells, blood vessels, lymph nodes and nerves. Growth of adipose tissue occurs by cellular
hypertrophy (increase in cell size) and hyperplasia (increase in cell number), also called adipogenesis. Adipogenesis is the result of proliferation and differentiation of preadipocytes (Hausman et al., 2001). It has been shown that adipose tissue contains a multipotent cell population that has similar properties to BMSCs (Fraser et al., 2006; Zuk, 2002; Zuk et al., 2001). Attempts to replace adipose tissue in reconstructive surgery include ECM/Tissue matrix (such as fibrin or hyaluronic acid gels), silicone, minerals/vegetable oils, paraffin and polymers (including PLA or PLGA) as augmentation materials (Choi et al., 2010; Patrick, 2001; Patrick et al., 1999). TE strategies involve the transplantation of adipocytes and preadipocytes to restore damaged sites. Transplantation of mature adipocytes has been shown to be largely unsuccessful most likely due to insufficient angiogenesis of the transplant. Preadipocytes, a subpopulation of the stromal vascular fraction (SVF), represent a promising cell source, as they can be easily obtained, cultured, expanded and differentiated (Gomillion and Burg, 2006).
1.4. Angiogenesis

Efficient vascularization depicts a key challenge in TE to allow long-term survival and function. Two vascularization strategies are currently applied. The first strategy, angiogenesis, focuses on the ingrowth of newly formed blood vessel from the host tissue into the implant. Angiogenic growth factors, such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (FGF), can stimulate the activation of the host microvasculature. VEGF and basic FGF can be produced by host cells due to tissue damage or in consequence to an inflammatory response to the implant. Other approaches combine these factors with the implanted biomaterial or use cell types capable of the production of angiogenic factors (Laschke and Menger, 2012; Laschke et al., 2008; Schumann et al., 2009). Implants can be also optimised for endothelial ingrowth by modifying the scaffold architecture. Scaffolds with slight proinflammatory properties might be able to stimulate angiogenesis at the implantation site. As the growth rate of a vessel is estimated to be around 5µm/hour, cell death cannot be avoided in the centre of large implants. The time demand- until the vessel is able to support the centre of an implant with nutrients- represents a major drawback in this strategy (Butler and Sefton, 2007; Laschke and Menger, 2012; Rücker et al., 2008). The second strategy, inosculation, describes the fast blood supply to the tissue implant by using generating preformed microvascular networks prior to implantation. After implantation, the host tissue has to develop interconnections with the preformed vessels (Laschke and Menger, 2012; Orr et al., 2003).
1.5. Key elements in tissue engineering

Scaffold design criteria

Scaffolds for TE purposes ideally implement several design criteria. First of all the scaffold should support cell adhesion and proliferation and it should allow the perpetuation of cellular functions. The scaffold should be biodegradable, but neither the scaffold nor its degradation products should provoke inflammatory responses or even toxicity. The porosity of the scaffold should permit nutrient supply within the whole construct, leading to homogenous tissue formation. Sufficient space should be available to support cell adhesion and extracellular matrix formation. The engineered tissue should be reproducible and should match the required mechanical properties in situ (Chen et al., 2002).

Surface adaption

Surface treatments of scaffolds allow an improved cellular attachment and proliferation. The surface alteration of titanium implants, such as used in dentistry, can improve the contact between the titanium implant and surrounding bone and thereby improving the patients’ quality of life (Pinholt, 2003). Surface roughness influences the adhesion as well as the morphology of cells and was positively correlated with the extent of bone-implant interface and bone formation (Anselme and Bigerelle, 2005; Buser et al., 1991; Gottfredsen et al., 2000; Larsson et al., 1996; Schwartz et al., 1997). Other treatments include alterations of the chemical and energy surface of materials (Amaral et al., 2007; Guo et al., 2012). One of the most prominent functionalization techniques is sodium hydroxide (NaOH) treatment. It is used for the modification of various polymers, including PLA, PGA and PCL. The main advantage of NaOH treatment is that no specialized equipment is required and therefore this method can be easily carried out in any laboratory. Surface treatment with NaOH improves cellular attachment and proliferation by removing oils and waxes from the scaffold surface and therefore increasing the rugosity of the material (Regis et al., 2012; Le Troedec et al., 2008). NaOH alters at the same time the surface energy by increasing the hydrophilicity, which has been shown to play a role in the performance of cell attachment, of polymer materials (Griesser et al., 1994; Yang et al., 2003; Yeo et al., 2010a).
Biocompatibility

Biomaterials should be biocompatible, which means that neither the material, nor any of its degradation products should cause inflammatory or toxic reactions. Therefore materials for TE should be non-toxic, non-immunogenic, non-thrombogenic, non-carcinogenic and non-irritant. As some applications require material degradation or tissue reaction, such as in case of an inert material, the term biocompatibility was defined as “the ability of a material to perform with an appropriate host response in as specific situation” (Williams, 1999).

Biodegradability

The meaning and definitions of the words biodegradable, bioresorbable, bioerodible and bioabsorbable are often used misleading in literature. Therefore the terms were defined by Vert et al. as described in table 1 (Hutmacher, 2000; Vert et al., 1992).

**Biodegradable** are solid polymeric materials and devices which break down due to macromolecular degradation with dispersion *in vivo* but no proof for the elimination from the body (this definition excludes environmental, fungi or bacterial degradation).

**Bioresorbable** are solid polymeric materials and devices which show bulk degradation and further resorb *in vivo*; i.e. polymers which are eliminated through natural pathways either because of simple filtration of degradation by-products or after their metabolisation. Bioresorption is thus a concept which reflects total elimination of the initial foreign material and of bulk degradation by-products (low molecular weight compounds) with no residual side effects.

**Bioerodible** are solid polymeric materials or devices, which show surface degradation and further, resorb *in vivo*. Bioerosion is thus a concept, too, which reflects total elimination of the initial foreign material and of surface degradation by-products (low molecular weight compounds) with no residual side effects.

**Bioabsorbable** are solid polymeric materials or devices, which can dissolve in body fluids without any polymer chain cleavage or molecular mass decrease. For example, it is the case of slow dissolution of water-soluble implants in body fluids. A bioabsorbable polymer can be bioresorbable if the dispersed macromolecules are excreted.

**Table 1: Definitions given by Vert et al. (table taken from Hutmacher et al., 2000)**
Nondegradable implant materials often cause inflammation, corrosion, fatigue or failure, requiring subsequent implant removal or replacement (Temenoff and Mikos, 2000). In contrast, degradable scaffolds temporarily fill bone defects allowing the bone to replace the defect by own tissue. Ideally, the degradation rate is altered to fit specific orthopaedic applications (Timmer, 2003).

**Porosity**

The material should mimic the structure of the tissue that shall be replaced. For TE purposes the scaffold shall imitate tissue morphology, structure and function. Several tissues consist of different layers and have therefore different properties. Bone, for example, is composed of trabecular bone, which has 50 – 90% porosity, and cortical bone with 3 – 12% porosity (Cooper et al., 2004; Kaplan et al., 1994). The two most common parameters that have been surveyed, when studying the morphology and architecture of biomaterials, are pore size and porosity. A pore can be defined as void space within a scaffold, whereas porosity represents the percentage of void space in a material (Gaalen et al., 2008; Karageorgiou and Kaplan, 2005). Ideally, the pores of a scaffold show a high interconnectivity to allow homogenous cell infiltration.

**3D structure**

A major requirement for a scaffold is to provide mechanical support for the tissue. Several fabrication techniques are used to build 3D scaffolds, including phase-separation, gas foaming, electrospinning or 3D printing. Depending on the requirements of the defect the adequate fabrication process should be chosen.
1.6. Fabrication and construction of 3D scaffolds

Several fabrication techniques have been developed for the construction of 3D matrices for TE and regenerative medicine. Two main strategies can be distinguished: “Top-down” and “Bottom-up” TE. While in the top-down approach, cells are seeded on top of the scaffold and are over time creating a tissue, the bottom-up approach uses several methods to generate tissue-building blocks which can be further assembled to form a large tissue consisting of several layers (see Figure 2) (Lu et al., 2013). The fabrication of thin tissues, including skin or bladder, has been successfully established using the top-down strategy, whereas the construction of rather complex tissues, such as liver or kidney, poses a challenge for this traditional approach. The bottom-up strategy holds great potential to be applied in the formation of those tissues (Groeber et al., 2011; Korossis et al., 2009).

![Figure 2: Schematic of “top-down” and “bottom-up” approaches for tissue engineering (figure taken from Lu et al., 2013)](image)
Top-down tissue engineering

Many top-down approaches aim to design biomimetic fibrous scaffolds, to mimic extracellular proteins, such as collagen. It has been shown that the fibrillar structure enhances cellular attachment and proliferation. To fabricate this nanofibrillar structure several methods, such as phase-separation, self-assembly and electrospinning are utilised. Phase-separation is applied to fabricate porous membranes or foams. By regulating the phase separation temperature and polymer concentration, porosity and fibre size can be regulated. Additionally, phase-separated scaffolds can be molded into a variety of shapes. Thermally induced phase separation is based upon the reduction of polymer solubility by lowering the temperature (liquid-liquid phase separation) or by inducing solvent crystallisation (solid-liquid phase separation) (Hutmacher et al., 2008; Lu et al., 2013). Another technique, which has been used to prepare porous scaffolds, is freeze-drying. In this method a frozen sample is placed in a chamber in which the pressure is lowered. In this primary drying process ice in the material is removed. By a second drying step most unfrozen water is taken. By regulating the pressure during the drying process, porosity and pore size can be altered (Lu et al., 2013).

Another common method used in top-down approaches is “self-assembly”. Biological molecules can assemble by noncovalent bonds or weak covalent interactions, such as electrostatic, van der Waals, hydrophobic interactions, ionic, hydrogen, and coordination bonds. A range of nanofibres can be created by adjusting sample parameters, such as pH and ionic concentration of the aqueous solution. However, the poor mechanical properties and high synthesis costs limits its application in TE (Colombo et al., 2007; Lu et al., 2013).

Besides those conventional techniques, other methods emerged in the recent years. Scaffolds can be produced using textile technologies such as knitting, braiding or by extruding a polymer solution through a spinneret. Using electrospinning, fibres with a nanoscale or microscale diameter can be produced. Standard polymers processed within this process include poly(lactic acid) (PLA), PCL, PLGA, collagen, silk fibroin, chitin and chitosan (Hutmacher et al., 2008; Liu et al., 2012; Lu et al., 2013). Other strategies use systems based on laser and ultraviolet light sources. For example, selective laser sintering applies a high-power laser to fuse polymer powder. Layers of powder can be deposited to form a 3D structure. Similarly, 3D printing
creates solid objects by inkjet printing a binder into sequentially deposited powder layers (Hutmacher et al., 2008).

**Bottom-up approaches**

Bottom-up approaches allow the formation of complex 3D constructs. Several methods are applied to fabricate cell-based microtissues that serve as building blocks for larger tissues. For example, by cell-encapsulation in hydrogels, cell aggregation, the formation of cell sheets or cell printing. The microtissues can be further assembled using magnetic assembly (Derby, 2012; Lu et al., 2013). To form a 3D cell-scaffold construct using a bioplotter, thermogelling materials are used, such as gelatin or agarose, and combined with cells. After the gelation of one layer the next layer can be printed on top (Fedorovich et al., 2008).
1.7. Injectable scaffolds

Injectable scaffolds offer big opportunities for clinical use as they minimize risk of infections due to minimal incisions, scar formation and costs of treatment. In contrast to preformed scaffolds, injectable materials can fill defects with irregular shapes and allow a homogenous distribution of additional factors, such as cells or growth factors (Chen et al., 2012; Hou et al., 2004; Kohane and Langer, 2008; Wintermantel, 1996). The solidification of the scaffold should take place under, or close to, physiological conditions to avoid damage to the surrounding tissue, the scaffold or the cells embedded in the scaffold and its functionality (Hou et al., 2004). The time for the solidification process also has to be considered as it should allow a distribution of cells within the scaffold and it should permit sufficient time for the surgical procedure. Typical solidification mechanisms include physical as well as chemical cross-linking. Physical cross-linking methods, such as thermal or ionic gelation, are considered to be milder, but are associated with lower mechanical stability (Chen et al., 2012). While chemical cross-linking, such as photopolymerisation, improves the mechanical strength of the scaffold it often incorporates reactive compounds that can lead to cytotoxicity (Chen et al., 2012; Elisseeff et al., 1999; Zheng Shu et al., 2004). The usage of enzymatic protein cross-linking, such as cross-linking by microbial transglutaminase, gained broad attention, as many chemical cross-linking reagents are toxic or may form harmful by-products. Also, enzymes react under physiological conditions and are mostly regarded as biocompatible. The most prominent member among mammalian tissue transglutaminases is the fibrin stabilizing factor XIII, which cross-links antiparallel fibrin chains (reviewed in Heck et al., 2013).

Thermoresponsive scaffolds

An important class of physically cross-linked injectable scaffolds are temperature-sensitive materials. Below a certain temperature, the lower critical solution temperature (LCST), the scaffold is liquid and can be therefore mixed with therapeutic cells or drugs. Increasing the temperature above the LCST of the material results in the formation of a hydrogel (Moon et al., 2012). Examples for thermal gelling polymers include copolymers of N-isopropylacrylamide, polyethylene glycol (PEG)-based amphilic block copolymers, gelatin, agarose, and cellulose (Hou et al., 2004). The sol-to-gel transition temperature should range between 10°C and
40°C to be of biomedical relevance. A thermoresponsive gelation process, as in the case of PEG-based polymers or gelatin, offers the possibility to culture or predifferentiate cells prior to injection.

One of the most prominent synthetic thermosensitive polymers is Poly(N-isopropyl acryl amide) (PNIPAm), but its application is associated with cellular toxicity (Vihola et al., 2005). Therefore biodegradable PEG-based polymers have been developed. The gelation temperature can be modified by varying polymer concentration and block lengths and is thought to be caused by association of micelles (Hou et al., 2004).
1.8. Biomaterials for tissue engineering

Natural polymers, such as fibrin or collagen, served as scaffolds for several decades and are used for several indications in TE. The great advantage, their potential to be degraded by naturally occurring enzymes, stands in opposition to its disadvantages, such as a pathogenic risk and their potential limited availability. Synthetic polymers allow an inexpensive fabrication in different designs and mechanical properties as well as varying degradation rates (Kohane and Langer, 2008; Vacanti and Vacanti, 2000).

**Natural polymers**

The most prominent polymers from natural origin are collagen, alginate, agarose, hyaluronic acid derivatives, chitosan and fibrin (Hutmacher et al., 2001). Collagen, the most described among those biomaterials due to its abundance in the body, differs from other proteins as it has a triple-helical structure formed by polypeptide chains (Kadler et al., 1996). A great variety of members of the collagen family have been reported, that are all based on containing domains with repetitions of the proline-rich tripeptide Gly-X-Y (Gelse, 2003). The most abundant group within this family are the fibril-forming collagen types representing around 90% of the collagen family (Gelse, 2003). While collagen type I and Type V are involved in the bone structure, collagen type II and XI are components of the articular cartilage (Kadler et al., 1996; Mayne, 1990). The fifth collagen in this group is collagen type III, which is distributed in several tissues, such as the skin or vessel walls (Gelse, 2003; Kadler et al., 1996). For TE purposes collagen is usually obtained from animal tissues, which creates concerns regarding the safety of the collagen (Yang et al., 2004). Therefore recombinant sources of human collagen were developed for clinical use (Yang et al., 2004).

Another upcoming approach for the generation of scaffolds is the decellularisation of tissues or even whole organs, such as dermis, the small intestine or heart valves. The decellularisation process allows the preservation of the complex composition and three-dimensional ultrastructure of the extracellular matrix (Crapo et al., 2011).

In a case-study in 2008, a 30-year old woman with end-stage bronchomalacia received a decellularised human donor trachea, that was reseeded with autologous epithelial cells and mesenchymal stem-cell derived chondrocytes. This graft was then
implanted replacing the left airway bronchus, resulting in a functional airway and therefore improved the quality of life of the patient (Macchiarini et al., 2008). After 8 months, a collapse of the most proximal 1 cm of the graft occurred and as a precaution an endoluminal stent was placed (Baiguera et al., 2010).

Although decellularised tissues are promising candidates for TE applications, more studies have to be done to optimise the decellularisation methods to avoid potential immunogenic responses due to remnants of immune-eliciting native material such as DNA from resident cells (Crapo et al., 2011).

**Synthetic polymers**

The most commonly used synthetic polymers in the form of poly(α-hydroxy acid), such as PLA, PGA and their copolymers PLGA and PCL, belong to the class of aliphatic polyesters and have been investigated in its use as scaffold for TE cases that require a longer stability. It has been demonstrated in previous studies that PLA, PGA as well as PCL serve as scaffolds for TE of skin, cartilage, bone, ligament, tendon, vessels, nerves and in the case of PLA and PGA also bladder and liver (Alberti, 2009; An et al., 2012; Bandyopadhyay et al., 2012; Bercovy et al., 1985; Hibino et al., 2002; Leong et al., 2008; Liu et al., 2010; Oh et al., 2012; Rosado et al., 2012; Singh et al., 2012; Williams, 2008; Xu et al., 2009). Their degradation rates can be adjusted to vary from a few months (PGA, PLA) to several years (PCL, PLA) and can be therefore used for a variety of TE strategies (Williams, 2008; Wolfe et al., 2011; Woodruff and Hutmacher, 2010).
In 1934 the synthesis of PCL was described for the first time (van Natta et al., 1934). PCL is degraded by hydrolysis via surface or bulk degradation pathways and might cause minimal inflammation. Its low degradation rate between 2-4 years makes it an attractive candidate for replacing long-term implants (Woodruff and Hutmacher, 2010).
1.9. Cells for Tissue Engineering

As described above, the strategy of TE involves harvesting, culturing and seeding of cells prior to implantation in vivo. Stem cells are defined by their potential for self-renewal and differentiation. They are categorised in two groups: embryonic stem cells and adult stem cells. While embryonic stem cells promote embryonic and fetal development, adult stem cells are able to regenerate and repair damaged tissue and support growth and tissue maintenance (Bianco et al., 2008). Induced pluripotent stem (iPS) cells are obtained by reprogramming adult stem cells and have the ability to differentiate towards any cell type (Gilbert and Blau, 2011).

Embryonic stem cells

Embryonic stem cells (ESCs) are obtained from the inner cell mass of a blastocyst and can differentiate towards cells from all three germ layers (ectoderm, mesoderm and endoderm). Injection of ESCs into nude mice leads to teratoma formation that includes tissues from all three germ layers (Thomson et al., 1998). Early in vitro trials relied on used fibroblasts as feeder cells to keep the cells in an undifferentiated state. Later on several methods were developed to culture the cells without a feeder layer of fibroblasts as they complicate manipulations of ESCs or effect the self-renewal and differentiation of ESCs (Fu et al., 2011; Xu et al., 2001; Yoon et al., 2010). Stem cell therapy could offer treatment of diseases in which a loss of cells occurs, such as Parkinson’s disease, Alzheimer’s disease, spinal cord injury, stroke or heart failure (Chung, 2011). Ethical consideration and the concern of teratoma formation when implanting ESCs have so far limited clinical trials to a small number. Recently the first clinical study using human ESCs was performed, in which a retinal pigment epithelium derived from human ESCs was implanted in patients suffering from Stargardt’s macular dystrophy and dry age-related macular degeneration. The implanted ESCs showed no signs of hyperproliferation, tumourigenicity, ectopic tissue formation, or rejection in the study period. However, no significant visual improvements were stated (Schwartz et al., 2012).

Induced pluripotent stem cells (iPS)

Fully differentiated cells can be reprogrammed to retrieve their pluripotency. This reprogramming can be achieved by transferring nuclear contents of the differentiated...
cell into oocytes, by fusion with ES cells or by the addition of specific factors. The group of Yamanaka showed that the addition of four factors Oct3/4, Sox2, c-Myc, and Klf4 recovers the pluripotency of fibroblasts (Takahashi and Yamanaka, 2006). However, this approach used retroviral transfection to introduce the reprogramming factors, which increases the risk for tumour formation. Other, safer delivery methods, such as the introduction of plasmids or modified RNA, were less efficient (Okita and Yamanaka, 2011). Current research studies the treatment of neurological and neurodegenerative disorders, hematopoietic, cardiovascular and metabolic disorders (Qiang et al., 2013; Unternaehrer and Daley, 2011; Wang et al., 2013).

**Adult stem cells**

Adult stem cells (aSCs) represent a promising source for TE strategies as they are abundantly availability in the human body and easy to access. In contrast to the usage of ES cells or iPS cells aSCs are not raising ethical objections. Several types of aSCs have been identified; the most prominent are hematopoietic stem cells and mesenchymal stromal cells.

**Hematopoietic stem cells**

Similar to all stem cells, hematopoietic stem cells (HSCs) are capable of self-renewal and differentiation to cells of the blood cell lineage, such as erythrocytes, megakaryocytes, myeloid cells and lymphocytes (Orkin and Zon, 2008). HSCs are the best investigated stem cell source as the transplantation of bone marrow including HSCs has been performed in patients suffering from primary immunodeficiencies and hematologic malignancies for more than 40 years (Buckley et al., 1999; de la Morena and Gatti, 2010). In theory, one single HSC is able to reconstitute the entire hematopoietic system of a patient (Orkin and Zon, 2008).

**Multipotent mesenchymal stromal cells**

To unify different isolation and expansion methods the “International Society for Cellular Therapy” defined minimal criteria for MSCs. First of all isolated MSCs have to adhere on cell culture plastic and have to proliferate in standard culture conditions (Dominici et al., 2006). A list of surface molecules was defined, that has to be present on MSCs, including CD105, CD73 and CD90, while the markers CD45, CD34, CD14
or CD11b, CD79α or CD19 and HLA-DR should be absent (Dominici et al., 2006). Last, MSCs are characterised having the potential to differentiate at least towards osteocytes, chondrocytes as well as adipocytes (Dominici et al., 2006).

For TE purposes adult MSCs can be obtained from various sources such as bone marrow or adipose tissue.

**Bone marrow derived MSCs**

BMSCs were first described as bone marrow stromal cells that are able to differentiate to a number of mesenchymal tissues such as bone, cartilage or adipose tissue (Owen and Friedenstein, 1988; Pittenger, 1999). Current research uses mobilized HSCs or transplanted BMSCs to restore infarcted myocardium (Kajstura et al., 2005; Orlic et al., 2001; Yoon et al., 2005). Intravenous injection of BMSC in experimental models of stroke showed beneficial effects such as a reduction in infarct size and improved sensorimotor function (Kocsis and Honmou, 2012; Song et al., 2012; Yang et al., 2012). Recent studies in human stroke patients confirmed the experimental findings as the patients show reduced neurological impairment after BMSC transplantation (K et al., 2012; Li et al., 2013).

A detailed description of the present status of MSCs for musculoskeletal regeneration including the areas of bone defects and nonunions, avascular necrosis of the hip, cysts and benign tumours of the bone, spine, cartilage lesions, tendons and ligaments, was reviewed in Steinert et al. (2012).

**Adipose tissue-derived stromal cells**

ASCs are derived by enzymatic digestion of adipose tissue or liposuction material (Zuk et al., 2001). Equivalent to BMSCs, ASCs have the potential to differentiate into cells and tissues of mesodermal origin. In contrast to bone marrow aspiration, liposuction is less invasive and therefore causes less discomfort for the patient (Mizuno, 2009). Additionally, a higher stem cell yield can be obtained from liposuction material compared to bone marrow aspirate (Fraser et al., 2006).

The isolated cells from the liposuction material are termed stromal vascular fraction. After culturing, a less heterogenous cell population, ASCs, can be obtained (Bourin et al., 2013; Gimble et al., 2011). While the age of the donor showed no differences in the amount of ASCs, increased body mass index was associated with
a decreased number of ASCs and a reduced differentiation potential (Gimble et al., 2011; van Harmelen et al., 2003; Wu et al., 2013). Interestingly, the adipogenic differentiation potential was unrelated to donor age, while a distinct relationship between osteogenic differentiation potential and age was described (Gimble et al., 2011; Zhu et al., 2009).

To characterize the cells, the presence or absence of specific markers (table 2) is determined. The antigen profile of hASCs resembles the immunotype of BMSCs (with more than 90%) and skeletal muscle-derived cells (Gimble et al., 2007). The isolation of subpopulations of cells within the stromal vascular fraction can be performed using immunomagnetic beads or flow cytometry by positive or negative selection of specific markers. For example, by the selection of the marker CD31, endothelial precursor cells can be excluded from the population (Bourin et al., 2013; Gimble et al., 2007). It has been suggested to identify ASCs in the SVF by the positivity for the marker CD34 and the negativity for CD45, CD235a and CD31. In culture, ASCs retain markers in common with other MSCs including CD90, CD73, CD105, and CD44 and remain negative for CD45 and CD31. They can be distinguished from BMSCs by the positive selection for CD36 and by excluding cells with the marker for CD106 (Bourin et al., 2013).

<table>
<thead>
<tr>
<th>Antigen Category</th>
<th>Surface-Positive Antigens</th>
<th>Surface-Negative Antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adhesion molecules</td>
<td>CD9 (tehraspin), CD29 (β1 integrin), CD43 (αv, integrin), CD54 (VCAM-1), CD105 (endogline), CD166 (ALCAM)</td>
<td>CD11b (αv integrin), CD18 (β2 integrin), CD50 (ICAM-3), CD56 (NCAM), CD62 (E-selectin), CD104 (αv Integrein)</td>
</tr>
<tr>
<td>Receptor molecules</td>
<td>CD44 (hyaluronate), CD71 (transferrin)</td>
<td>CD18 (Fc receptor)</td>
</tr>
<tr>
<td>Enzymes</td>
<td>CD10 (common acute lymphocytic leukemia antigen), CD13 (aminopeptidase), CD73 (5′-nucleotidase), CD63 (dehydrogenase)</td>
<td></td>
</tr>
<tr>
<td>Extracellular matrix molecules</td>
<td>CD90 (Thyl), CD146 (Muc18), collagen types I and III, osteopontin, osteonectin</td>
<td></td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>n-smooth muscle actin, vitronectin</td>
<td>CD14, CD31, CD45</td>
</tr>
<tr>
<td>Hemostaticopeic</td>
<td>CD52 (decay-accelerating factor), CD59 (protectin)</td>
<td>HLA-ABC</td>
</tr>
<tr>
<td>Histocompatibility Antigen</td>
<td>CD90, CD34, AB032</td>
<td>HLA-DR</td>
</tr>
<tr>
<td>Stem cell</td>
<td>CD34, CD73, CD90, CD166</td>
<td></td>
</tr>
<tr>
<td>Stromal</td>
<td>CD29, CD44, CD73, CD90, CD166</td>
<td></td>
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</table>

Table 2: Immunophenotype of passaged human ASCs (table taken from Gimble et al., 2007)

In vitro, ASCs have the potential to differentiate towards several cell types, including adipocytes (Peterbauer-Scherb et al., 2012; Zuk et al., 2001), cardiomyocytes (Planat-Bénard et al., 2004; Song et al., 2007), chondrocytes (Betre
et al., 2006; Erickson et al., 2002; Hildner et al., 2009; Ogawa et al., 2004; Zuk et al., 2001), endothelial cells (Miranville et al., 2004), myocytes (Mizuno et al., 2002; Zuk et al., 2001), neuronal-like cells (Safford et al., 2002, 2004), Schwann-like cells (Razavi et al., 2012), and osteoblasts (Ahn et al., 2009; Arrigoni et al., 2009; Leong et al., 2008; Ogawa et al., 2004).

**Cell-lines**

Continuously growing cell lines have been developed to serve as model for *in vitro* cell differentiation. Murine calvarial MC3T3-E1 cells allow the differentiation towards osteocytes within 16 days (Quarles et al., 1992). Another example for a cell line is the species C3H 10T1/2 cell line, which is able to differentiate towards myocytes, chondrocytes, osteocytes or adipocytes (Braun et al., 1989; Reznikoff et al., 1973; Roy et al., 2010; Taylor and Jones, 1979). The murine C2C12 cell line is also commonly used as it represents immature myoblasts that have the potential to differentiate towards myogenic, osteogenic as well as chondrogenic pathways (Shin et al., 2000). The addition of BMP2 mediates osteogenic differentiation by activating Smad5 (Nishimura, 1998). Specific cell lines, such as the C2C12 cell line, have the potential to differentiate towards certain cell types similar to primary cells, but do not completely replicate them. Therefore key experiments should be repeated with primary cells (Kaur and Dufour, 2012).

Established cell lines are cost efficient and allow the reproducibility of studies without considering donor effects of primary cells.
1.10. **In vitro versus in vivo tissue engineering**

Although *in vivo* studies are essential to evaluate future therapeutic strategies, *in vitro* studies offer several advantages. *In vivo* studies often require sacrificing animals and therefore raise ethical concerns. Therefore *in vitro* studies are often used as replacement of *in vivo* studies according to the “3Rs” (reduction, refinement, and replacement; see chapter 1.12 “Ethical considerations”). *In vitro* studies allow drug screening, especially at the low end of dose-response curves. Additionally, only a few studies consider the different sexes and life stages of the animals (Hartung and Daston, 2009; Polli, 2008). *In vitro* studies allow a high number of replicates and low costs. The reduction to one or two cell types facilitates the interpretation of results.

**Imitation of the in vivo situation**

The goal of *in vitro* experiments is to mimic the *in vivo* situation and facilitate the examination of processes such as the differentiation of cells or allow toxicity assays. To imitate the *in vivo* trigger of differentiation, several factors are added to the cell culture. The amount of factors needed depends on the cell system used. While some cells are stimulated by one factor only, such as BMP for the osteogenic differentiation of C2C12 cells, others, especially primary cells, require a cluster of factors. Not only the combination, but also the dose plays an important role. For example, by changing the dose of dexamethasone, a synthetic glucocorticoid, the differentiation of ASCs towards adipocytes, osteocytes, chondrocytes and myocytes is effected (Gimble et al., 2007).

The artificial environment of *in vitro* cultures has its disadvantages. The low cell density compared to tissues impairs intracellular signalling. The culture conditions are not homeostatic and the oxygen supply is inefficient. Also the growth media are designed to stimulate a rapid cell proliferation (Hartung and Daston, 2009). *In vivo*, scaffold are subjected to remodelling, a process in which the material is degraded and replaced by new host tissue. Cells can be stimulated via adhesion and bound growth factors of the ECM to remodel the scaffold (Gaalen et al., 2008). Scaffolds composed of ECM are very successful in many TE applications. Therefore, synthetic materials aim to mimic ECM characteristics. In contrast to injectable scaffolds, thermoreponsive hydrogels allow the cultivation and predifferentiation of cells *in*
vitro. Due to the thermoresponsive properties, the scaffold-cell construct, including the newly formed ECM, can be transferred to the defect site.
1.11. Performance of ASCs in clinical trials

ASCs are abundantly available in easily accessible subcutaneous fat depots and have a multilineage differentiation potential. Together with their intrinsic properties and the possibility to use them as autologous substitutes for tissue defects, ASCs are promising candidates for indications in preclinical as well as clinical studies (Stillaert et al., 2008).

Soft tissue defects

Clearly, treatment of soft tissue defects is the most prominent application area for using ASCs, as the cells return to their tissue of origin (Gimble et al., 2011). Several studies report the usage of ASCs in clinical studies within this area. In 2008, ASCs were combined with hyaluronic acid-based scaffolds and implanted subcutaneously in humans. It was possible to generate a volume-stable tissue, but no adipogenic differentiation of ASCs was observed (Stillaert et al., 2008). The group of Yoshimura used SVF-enriched fat-grafts as cosmetic breast augmentation material. In 10% of the patients, cyst formation or microcalcification was observed. However, the authors claim that satisfactory clinical results were obtained without any major complications (Yoshimura et al., 2007, 2010). The same group treated facial lipoatrophy by injecting aspirated fat or SVF-enriched aspirated fat and report clinical improvements, especially in those receiving SVF-enriched lipoaspirate (Yoshimura et al., 2008). One of the major concerns using SVF or ASC cells for breast augmentation or reconstruction is the possible immunosuppressive and tumour promoting function of SVF cells and ASCs that could facilitate the recurrent growth of residual cancer cells in the breast tissue of postmastectomy patients. Therefore, surgeons are cautious to use SVF cells or ASCs in cancer patients (Donnenberg et al., 2010; Gimble et al., 2011; Perez-Cano et al., 2012; Scioli et al., 2013). Oncologic radiation therapy can cause irradiation-induced lesions that lead to a decreased range of movement and pain. Lipoaspirate transplantation resulted in a progressive regeneration with enhanced range of motion and reduced pain (Akita et al., 2010; Rigotti et al., 2007). One recent study compared the survival of fat grafts that were enriched with ex-vivo expanded ASCs to the survival of fat grafts without ASC enrichment. The fat grafts were injected subcutaneously as a bolus to the posterior part of the right and left upper arm and measured by MRI immediately after injection and after 121 days.
before surgical removal. Grafts that were enriched with ASCs showed a significantly higher residual volume compared to the control grafts (Kølle et al., 2013). In some reports, free fat grafting led to several complications, including cyst formation, calcification and severe breast deformity. These complications were associated with poor lipoinjection techniques performed by the physician. Especially calcifications have to be considered critically, as they were difficult to distinguish from breast tumours and could therefore complicate the detection (Hyakusoku et al., 2009; Mizuno and Hyakusoku, 2010). As the techniques were improved over time, the American Society of Plastic Surgeons announced in 2009 that fat grafting could be considered for cosmetic and reconstructing breast surgery. However, the results largely depend on the technique and surgeon expertise (Mizuno and Hyakusoku, 2010).

### Bone defects

The first report using ASCs to treat widespread traumatic calvaria defects was published in 2004. Autologous ASCs were applied in a single operative procedure in combination with autologous fibrin glue and two macroporous sheets for mechanical fixation. After three months, new bone formation was detected (Lendeckel et al., 2004). Another study combined ASCs with βTCP granules in a preformed titanium cage for maxillary reconstruction. After 7 months, a biopsy confirmed bone regeneration (Mesimäki et al., 2009). Thesleff et al. (2011) performed a cranioplasty in 4 patients by combining ASCs with βTCP granules. This study yielded satisfactory outcomes in ossification and good results in clinical examinations (Thesleff et al., 2011). In 2010, bilateral orbitozygomatic defects of a 14-year old adolescent boy were treated with engineered bone made from a combination of human bone allograft, adipose-derived mesenchymal stem cells, bone morphogenic protein-2, and periosteal grafts. After 6 months, a biopsy showed healthy, lamellar bone formation (Taylor, 2010). In 2011, Pak reported 2 cases of patients with hip osteonecrosis of the femoral head and 2 patients suffering from knee pain due to osteoarthritis. All patients received a combination of ASCs, hyaluronic acid, platelet-rich plasma and calcium chloride. The osteoarthritis patients were in addition treated with a nanogram dose of dexamethasone. After three months, all patients revealed improved function and less pain, which was underlined with MRI evidence (Pak, 2011).
Gastrointestinal lesions

Crohn’s disease, an inflammatory bowel disease, often leads to anorectal fistula formation. A Spanish group evaluated the use of ASCs in a proof-of-concept phase I clinical trial in fistulising Crohn’s disease and a phase II clinical trial in fistulas of cryptoglandular origin associated with Crohn’s disease. 17 out of 24 patients, showed fistula healing, whereas treatment fibrin glue alone lead to healing only 5 out of 25 patients (Garcia-Olmo et al., 2005, 2009). In a recent phase III trial, they further investigated the effectiveness and safety of ASCs in the treatment. Three treatment-options were tested: ASCs alone (64 patients), ASCs in combination with fibrin glue (60 patients) and fibrin glue alone (59 patients). All therapies applying ASCs achieved moderate healing rates. Nevertheless, no beneficial effects were reported compared to treatment with fibrin glue alone (Herreros et al., 2012). The safety of ASCs for the usage in clinical trials was reaffirmed in a long-term follow-up. Data was available from 12 patients that were treated with ASCs plus fibrin glue resulting in a complete closure. Out of those, 5 patients with closure after the procedure showed a recurrence after more than 3 years of follow-up (Guadalajara et al., 2011). A phase II clinical trial evaluated the efficacy and safety of ASCs in patients with Crohn’s fistulae. ASCs were combined with fibrin glue and injected intralesional. 8 weeks after the injection, 27 out of 33 patients showed complete fistula healing. However, these results were not compared to other therapies (Yong Lee et al., 2013). A phase I study evaluated the safety for the treatment of Crohn’s fistula using three dosages. No patient in the group (n=3) that received $10^7$ ASC/ml showed a complete closure of the fistula, whereas two out of four patients receiving $2 \times 10^7$ ASC/ml and one out of three patients treated with $4 \times 10^7$ ASC/ml showed complete healing after 8 weeks. No grade 3 or 4 severity adverse events related to the study drug were observed (Cho et al., 2013).

Immune disorders

The immunosuppressive characteristics of ASCs were used in a study of leukemic patients with steroid-refractory acute graft-versus-host disease (GVHD) after receiving hematopoietic stem cell transplantation. The study included six patients that were treated with allogeneic ASCs (either from haplo-identical family donors or
unrelated mismatched donors). In five patients acute GVDH disappeared completely. After a median follow-up of 40 months, four patients out of the five responding to the ASC therapy survived and are in good clinical conditions and in remission of their haematological malignancy. The patient unresponsive to ASC treatment died of multiorgan failure, while the other patient died due to a relapse of leukaemia. However, the infusion of ASCs did not result in any negative side effects (Fang et al., 2007). Three patients with multiple sclerosis, an inflammatory disease, were injected twice with SVF cells. The treatment was well tolerated and resulted in significantly improved overall conditions, including balance, coordination, energy level and memory function (Riordan et al., 2009).

Another study reports about the systemic administration of ASCs to ten patients with autoimmune associated tissue damage. The treatment with ASCs resulted in clinical benefit in all treated patients. In six cases, immunological and blood status samples were also measured showing a decrease in inflammatory responses and eosinophil counts (Ra et al., 2011).

**Cardiovascular diseases**

In an ongoing clinical trial, ASCs are used for cardiovascular treatment in patients with acute myocardial infarction or chronic myocardial ischemia (Sanz-Ruiz et al., 2009). However, inconsistent results in clinical trials of cardiac cell therapy require more research in a number of issues such as appropriate type and procedure of cell delivery (Tobita et al., 2011).

**Other indications**

Currently, several clinical trials using ASC for various indications are performed. The search term “adipose AND stem AND cells” at the clinical trials website http://clinicaltrials.gov hosted by the US National Institute of Health (NIH) gives 77 results, of which 5 are not involving ASCs. The studies performed include research on peripheral vascular diseases, limb ischemia, liver cirrhosis, diabetes mellitus, erectile dysfunction, autism, diffuse brain lesions, osteoarthritis, chronic obstructive pulmonary disease, Buerger’s disease, Parkinson’s disease, renal failure, stroke, Romberg’s disease, osteoporotic fractures and articuler lesions of the femur condyle.
are ongoing (http://clinicaltrials.gov (date of search: 14.02.2013), (Gir et al., 2012; Mizuno, 2009).
1.12. Ethical considerations

There exist several controversial areas in TE raising ethical concerns. As mentioned above, the isolation of embryonic stem cells at the blastocyst stage results in destroying the embryo. Highly debated has been the question of when life begins and, due to religious or moral believes, some people claim that human life begins with conception (Lo and Parham, 2009). This concern can be circumvented by the usage of iPS cells or aSCs. Adult human material is obtained by donation and therefore requires the patients' consent.

Researchers are asked to design animal studies according to the “3Rs”. However, many studies that have been approved by an institutional nonhuman animal trial committee use high numbers of animals in invasive research (Balcombe et al., 2013). If alternative testing methods, such as in vitro studies, can generate equally valid results, animal studies should not be performed (Andreasen and Andersson, 2011). It is contentious, to which extent animal studies laboratory animal outcomes can be extrapolated to humans. Out of 20 published reviews examining clinical utility of toxicology assays, only two cases showed potential to predict clinical interventions (Knight, 2008).
1.13. **Aims of the study**

A thermoresponsive scaffold based on PCL-particles and the polymer polyPEGMA-EE$_{246}$ was developed that liquefies below its LCST of 20°C and is solid above its LCST.

Aims of the study were

- to establish a method that allows the histological processing of the thermoresponsive scaffold

- to evaluate the scaffold *in vitro* for its support of cellular differentiation using cell lines as well as primary cells

- to investigate the effect of the scaffold on cellular migration and vascular formation in an *in vivo* angiogenesis model
2. Prologue

Temperature-sensitive materials offer several possibilities, such as the incorporation of therapeutic cells or drugs by mixing them into the scaffold in its liquid phase. Above the LCST of the material the scaffold forms a hydrogel that can be used as matrix for TE purposes. Ideally, the sol-to-gel transition temperature should range between 10°C and 40°C to be of biomedical relevance.

The preparation of scaffolds for routine histological processing requires the exposure of the scaffold to temperatures above 50°C (for paraffin embedding) or below 4°C (for cryosectioning). Decreasing the temperature for cryosectioning would result in the liquefaction of the scaffold and would therefore alter the cell-matrix interactions.

Elevating the temperature above 50°C could mechanically damage some biomaterials, including PCL.

Therefore a method was established, which can be used to prepare thermoresponsive scaffolds for histological examinations.

All parts of chapter 2.1 have been written by the author. Alexandra Meinl contributed with her experience in histological processing by assisting in the development of the histological procedures. The authors from Nottingham University contributed by producing the thermoresponsive scaffold. Heinz Redl as well as Susanne Wolbank consulted during the whole project. The author prepared the material and the cells, including the embedding of the samples in gelatin, for histological processing. The author took the pictures of the histological sections and evaluated them.

All authors proof-read the article prior to publication.
2.1 Gelatin embedding for the preparation of thermoreversible or delicate scaffolds for histological analysis

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CHAPTER TWO: RESULTS

COMMUNICATION

Gelatin embedding for the preparation of thermoreversible or delicate scaffolds for histological analysis

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Abstract
Thermoreversible hydrogels for tissue engineering (TE) purposes have gained increased attention in recent years as they can be combined with cells and drugs and directly injected into the body. Following the fate of transplanted cells in situ is essential in characterizing their distribution and survival, as well as the expression of specific markers or cell–matrix interactions. Existing histological embedding methods, such as paraffin wax embedding, can mechanically damage some biomaterials during processing. In this study, we describe a broadly applicable preparation protocol that allows the handling of delicate, thermoreversible scaffolds for histological sectioning. The gelatin solution permits the embedding of samples at 37 °C, which suits the solid phase of most TE scaffolds. A thermoreversible scaffold of polycaprolactone microparticles, combined with poly(ethylene glycol) methacrylate ethyl ether and containing human adipose-derived stem cells, was prepared for histology by an initial gelatin embedding step in addition to the standard cryosectioning and paraffin processing protocols. Sections were evaluated by hematoxylin eosin staining and immunostaining for human vimentin. The gelatin embedding retained the scaffold particles and permitted the complete transfer of the construct. After rapid cooling, the solid gelatin blocks could be cryosectioned and paraffin infiltrated. In contrast to direct cryosectioning or paraffin infiltration, the extended protocol preserved the scaffold structure as well as the relevant cell epitopes, which subsequently allowed for immunostaining of human cells within the material. The gelatin embedding method proposed is a generalizable alternative to standard preparations for histological examination of a variety of delicate samples.

(Some figures may appear in colour only in the online journal)

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CHAPTER TWO: RESULTS

1. Introduction

Both biodegradable hydrogels and chemically or physically crosslinked polymers are widely used in tissue engineering (TE) applications because of their three-dimensional (3D) network, their tissue-like water content, their structural stability, biocompatibility and homogeneous cell encapsulation [1–3].

Among hydrogels, scaffolds with a temperature dependent sol–gel transition have attracted increasing attention in recent years. At a low temperature, these materials turn into homogeneous solutions and can therefore be injected, mixed with drugs or combined with cells. Upon increasing the temperature, these materials solidify and serve as constructs for TE [2, 4–6]. In this study, we selected a thermoreversible scaffold consisting of polycaprolactone microspheres combined with poly(polyethylene glycol methacrylate ethyl ether) (polyPEGMA-EE36o) which had a transition temperature at 20 °C [7]. This matrix has recently been proven to support the cellular attachment and proliferation of NIH 3T3 cells [7].

To follow cell fate in 3D thermoreversible scaffolds, it is possible to use quantitative methods on mRNA or protein levels (for example qRT-PCR or enzymatic assays). It is also feasible to examine the structure of the scaffold, its interactions with cells, the cell distribution and cell identity using histology. However, due to the physical properties of the material the use of staining methods to characterize cells in situ within the 3D constructs is difficult.

There are several reports demonstrating that the histological analysis of thermogelling scaffolds can be performed using both cryozone sectioning of frozen samples or paraffin embedded sectioning. In the study of Expander et al. human adipose tissue derived stromal cells (ASCs) were used in conjunction with the injectable hydrogels Extracel-SS, HyStem-HP and HyStem-CSS (Glycosan BioSystem Inc, USA). For histological examination, the samples were fixed in a cryoprotective medium in preparation for cryosectioning [8]. In another study, chondrocytes encapsulated within a thermoreversible gel that solidifies above 7 °C were evaluated using histology on standard paraffin sections [9]. Some other studies evaluated materials (e.g. poly(N-isopropylacrylamide)-g-methylcellulose) that require lower temperatures for sample embedding but still allow sectioning with a microtome [10]. In this case, an embedding resin system based on glycol methacrylate-Technovit 8100 (Kulzer, Germany) was used, making follow-up immunohistology difficult.

Several studies lack histological analysis due to the specific properties of the investigated materials [11, 12]. For example, the evaluation of an alginate-based composite system containing beta-tricalcium phosphate granules was limited to 3D cell proliferation assays and qRT-PCR [12]. Also, in the study of Choi et al where MSCs were combined with injectable PLGA spheres, the cell–scaffold interaction was examined using SEM and cell differentiation analyzed by Oil Red O quantification [11].

In our study, the properties of the scaffold chosen complicates histological analysis using cryo- or paraffin wax embedding. Lowering the temperature results in a liquefying of the scaffold, which would lead to the disruption of the established cell–matrix interactions. Fragile samples can usually be embedded prior to histological processing using HistoGel™ (Thermo Scientific, Belgium), which is added to the sample in its liquid state (above 50 °C) and solidifies at room temperature. However, the melting temperature of polycaprolactone particles is around 60 °C, which is problematic for both paraffin embedding or embedding in HistoGel™ [13, 14].

An alternative method that stabilizes the scaffold and requires lower temperatures for scaffold infiltration is gelatin embedding [15]. The sample is infiltrated with gelatin at 37 °C, which matches the gel (solid) phase of the thermoreversible PCL-polyPEGMA-EE36o scaffold. During rapid cooling, the gelatin retains the scaffold structure and allows further treatment for histological preparations. The chosen gelatin concentration of 25% results in solid constructs that compare to those obtained by using HistoGel™, facilitating the sectioning process.

The aim of this study was to establish and optimize histological methods to allow the combined analysis of cell fate and cell–scaffold interaction. Therefore we compared direct embedding of the scaffold in cryogel or paraffin wax with embedding in gelatin prior to cryogel or paraffin wax processing.

2. Materials and methods

Polycaprolactone particles, as well as the polyPEGMA-EE36o, were prepared as previously described [7]. For each sample, 3 × 10⁶ ASCs were seeded within 300 µl of a cooled 30 wt% PCL microparticle suspension, containing a final concentration of 3 wt% polyPEGMA-EE36o [16]. After an incubation of 20 min at 37 °C, a prewarmed culture medium consisting of DMEM/HAM’s F12, 1% L-Glut, 1% Penicillin/Streptomycin (all from Sigma-Aldrich, Austria) and 1 ng ml⁻¹ basic FGF (Peprotech, Austria) was added. On the following day, the medium was removed and the scaffolds were prepared for histological analysis.

To obtain histological sections, we embedded the samples either in cryomedium for cryosectioning or in paraffin for microtome sectioning, with or without prior gelatin incorporation. As the scaffold itself is rather soft and porous, the complete, intact scaffold could not be transferred to cryogel or into the histological chamber.

The scaffolds were covered with cryomedium and immediately frozen in liquid nitrogen. Histological sections were produced by embedding the frozen scaffold samples in a OCT™ compound (Tissue-Tek, The Netherlands) and subsequently sliced with a cryotome, at a thickness of 10 µm. For embedding into paraffin wax, the scaffolds were fixed with 4% formaldehyde solution overnight. Samples were then processed to paraffin sections (Tissue Tek, The Netherlands) according to routine practice and sectioned at a thickness of 4 µm using a microtome (Microm, Germany) [17].

For prior gelatin embedding (illustrated in figure 1) of the scaffolds, 4% formaldehyde was added on top of the scaffold
and left for 24 h at room temperature. Formaldehyde solution was replaced by prewarmed water and incubated at 37 °C for 24 h. The scaffold was incubated in 12.5% gelatin solution for 24 h at 37 °C, followed by an incubation in 25% gelatin solution for 24 h at 37 °C and then 15 min at 4 °C to harden the gelatin-scaffold block. The construct was transferred to a larger well and warm 25% gelatin solution was added. After 24 h incubation at 37 °C, the gelatin-scaffold block was kept at 4 °C for 15 min. The block was then transferred to 4% formaldehyde solution at room temperature for 24 h and was finally stored in distilled H2O at room temperature. Hematoxylin and eosin (HE) staining was performed using an automated slide stainer (Tissue-Tek, The Netherlands) according to standard protocols [18]. Cryosections were fixed for 10 min in cold, 4% buffered formaldehyde and subsequently washed for 4 min in distilled H2O prior to HE staining. To perform immunostaining for human vimentin, the gelatin-scaffold blocks embedded in paraffin were placed twice for 10 min in xylol, followed by an ethanol gradient from 100% to 50%. After washing with distilled H2O, the sections were blocked with 3% H2O2 in tris-buffered saline (TBS) for 10 min. The sections were steamed for 20 min with Target Retrieval Solution (Dako, Austria) and washed with TBS. Monoclonal mouse anti-vimentin (Clone V9 3B4, Dako) was added in REAL™ Antibody Diluent (Dako; 1:400) and incubated for 1 h at room temperature. After washing with TBS, the secondary antibody (Envision + System-HRP, Anti-Mouse, Dako) was added and again incubated for 30 min at room temperature followed by washing with TBS. The sections were developed with 3,3’-diaminobenzidin-tetra-hydrochlorid for 6 min and stopped with water.

3. Results

The construct composed of PCL-polyPEGMA-EE250 and ASCs was transferred to cryogel (figure 2(a)), prepared for paraffin wax embedding (figure 2(b)) or embedded in gelatin (figure 2(c)) prior to cryosectioning or paraffin wax processing.

When scaffolds were embedded directly in cryomedium, it was possible to perform cryosections but the visible structure was not representative of cell morphology or scaffold design (figure 3(a)). Direct embedding in paraffin was nonfeasible because, after the inevitable dehydration steps, the scaffold sample was too brittle to proceed with the subsequent steps, even though the samples were treated carefully. After gelatin embedding it was possible to make sections of the constructs on both a cryotome (figure 3(b)) or, embedded in paraffin wax, on a microtome (figure 3(c)). The gelatin infiltrated the complete scaffold and therefore stabilized the material for the complete transfer of intact scaffolds, histological preparations, sectioning and staining. Using HE staining, cells could be easily identified and showed intact interactions with the matrix in both cryosections as well as paraffin sections. At the chosen timepoint (24 h), the cell morphology was rather round in shape and a limited cell-cell interaction could be seen (figures 3(b) and (c)). HE staining also slightly stained the infiltrated gelatin, but this did not affect the interpretation of the sections as the gelatin could be easily distinguished from cells.

For TE purposes, immunohistological stainings are required to adequately characterize the cells and material structure. To evaluate whether embedding in gelatin prior to paraffin processing allows subsequent immunohistological staining, the sections were stained for human vimentin, a standard marker that is frequently used to identify human

![Figure 2. Methods to embed thermoreversible scaffolds. Transfer of the scaffold in a cryogel for cryosectioning (A), placement of the scaffolds in a well for paraffin embedding (B) and embedding of the material within gelatin for paraffin embedding or cryosectioning (C).](image-url)
cells after transplantation into rodents. As visible in figure 4, human ASCs could be immunohistologically detected within the gelatin-embedded paraffin sections of scaffold constructs. The extended protocol, embedding the scaffold in gelatin prior to paraffin preparation, preserved the relevant epitopes which hence allows staining with immunoreagents.

To further test whether the gelatin embedded scaffolds are stable to be stored, cryosectioning as well as paraffin embedding of the gelatin blocks was repeated after 3 weeks. No difference could be observed in the scaffold structure or cell morphology as well as in the degree of the staining itself compared to that observed before, suggesting that the gelatin embedding allows the storage of samples for several weeks.

4. Discussion and conclusion

Handling thermoreversible scaffolds requires the adaption of analysis methods including the protocols for histological sectioning. Due to the properties of the selected scaffold, PCL-polyPEGMA-EE226, processing temperatures above its sol-gel transition temperature of 20 °C but below the melting temperature (of the constituent PCL particles) of 55 °C are required to retain the scaffold structure and cell/scaffold interaction. Currently most methods work either below (e.g. cryoembedding) or above this temperature range (e.g. paraffin embedding or HistoGe³™ embedding). Gelatin solutions are liquid at 37 °C and therefore match the solid phase of the thermoreversible scaffold. By subsequent cooling, the
gelatin preserves the structure of the material and stabilizes the scaffold for subsequent transfer for cryosectioning and paraffin infiltration. In addition to the thermoreversible material, we have also successfully applied the method to two other materials, which are difficult to transfer, fibrin scaffold of very low component concentration (3 mg ml\(^{-1}\) fibrinogen, 2 U ml\(^{-1}\) thrombin), as well as FloSeal\textsuperscript{©} particles (Baxter BioSurgery, Austria) (data not shown). Several studies completely lack histological analysis due to material characteristics [11, 12]. The presented method allows for the histological analysis of the included cells as well as cell–scaffold interactions. The evaluation of TE constructs should however be accompanied by molecular biological methods for more detailed analysis of extracellular matrix composites, which would be difficult to characterize within the gelatin construct.

To conclude, it was possible to establish a suitable method to prepare thermo-responsive scaffolds for histological examinations. Gelatin is an inexpensive, convenient compound that is perfectly suitable to conserve scaffolds with complex physical properties. Gelatin embedding represents a generally applicable method which offers an alternative to standard preparations for histological examinations of a variety of delicate samples.

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References

2.1.1 Interlude

The PCL-polyPEGMA-EE$_{246}$ scaffold was evaluated for its suitability to support bone and soft TE using both a cell line and primary cells. The scaffold was combined with the C2C12 cell line, which has the potential to differentiate towards the myogenic as well as osteogenic lineage. As a source of primary cells human adipose-derived stem cells (ASCs) were chosen, as they have the potential to differentiate towards cell types of mesodermal origin, including adipocytes and osteoblasts.

The suitability of the scaffold to support cellular attachment, survival, distribution, persistence as well as differentiation was characterised. The myogenic as well as osteogenic differentiation of C2C12 cells in the 3D scaffold was compared to their differentiation on standard cell culture plastic. Similarly, the adipogenic as well as osteogenic differentiation of ASCs was evaluated.

All parts of chapter 2.2 have been written by the author. The authors from Nottingham University contributed by producing the thermoresponsive scaffold. Georg Feichtinger assisted in the design of all qRT-PCR primers used. Heinz Redl as well as Susanne Wolbank consulted during the whole project.

All authors proof-read the manuscript.
2.2 A thermoresponsive polycaprolactone scaffold for in vitro 3D stem cell differentiation

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A thermoresponsive polycaprolactone scaffold for *in vitro* 3D stem cell differentiation

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Keywords
PCL, ASCs, C2C12, stem cells, differentiation

Short title
3D cell differentiation in thermoreversible PCL-scaffold
Abstract

Tissue engineering (TE) strategies aim to imitate the natural process of regeneration by using bioresorbable scaffolds that support cellular attachment, migration, proliferation and differentiation. Based on the idea of combining a fully degradable polymer (Poly(ε-caprolactone) with a thermoresponsive polymer (polyethylene glycol methacrylate), a scaffold was developed, which liquefies below 20°C and solidifies at 37°C. In this study, this scaffold was evaluated for the ability to support C2C12 cells and human adipose derived stem cells (ASCs) to generate an expandable 3D construct for soft or bone TE.

As a first step, biomaterial seeding was optimised and cellular attachment, survival, distribution and persistence within the 3D material were characterised. C2C12 cells were differentiated towards the osteogenic as well as myogenic lineage, while ASCs were cultured in control, adipogenic or osteogenic differentiation media. Differentiation was examined using qRT-PCR for the expression of osteogenic, myogenic and adipogenic markers and by enzymatic- or immunoassays. Both cell types attached and were found evenly distributed within the material. C2C12 cells and ASCs demonstrated the potential to differentiate in all tested lineages under 2D conditions. Under 3D osteogenic conditions for C2C12 cells, only osteocalcin expression (fold induction: 16.3 ± 0.2) and alkaline phosphatase (ALP) activity (p<0.001) were increased compared to the control C2C12 cells. 3D osteogenic differentiation of ASC was limited and donor dependent. Only one donor showed an increase in the osteogenic markers osteocalcin (p=0.027) and osteopontin (p=0.038). In contrast, differentiation towards the myogenic or adipogenic lineage showed expression of specific markers in 3D at least at the level of the 2D culture. In 3D culture, strong induction of myogenin (p<0.001) as well as myoD (p<0.001) were found in C2C12 cells. The adipogenic differentiation of one donor showed greater expression of peroxisome proliferative activated receptor gamma (PPARg) (p=0.004), fatty acid binding protein 4 (FABP4) (p=0.008) and adiponectin (p=0.045) in 3D compared to 2D culture. Leptin levels in the supernatant of the ASC cultures were elevated in the 3D cultures in both donors at day 14 and day 21. In conclusion, the thermoresponsive scaffold was found suitable for 3D in vitro differentiation towards soft tissue.
Introduction

There is a high demand for injectable biomaterials for tissue engineering (TE) as they offer advantages, such as their non-invasive and simple application into a defect site or their ability to incorporate cells, growth factors or DNA without prior incubation\textsuperscript{1-3}. Of those, thermoreversible materials additionally allow for the easy non-destructive transfer of a material-cell construct by simply changing the temperature. By this means, a precooled material can fill the defect site \textit{in situ} when still in its liquid phase at a low temperature, and solidifies following its injection as it warms up to body temperature. Thermosensitive polymers based on polyethylene glycol (PEG) have been employed as biomaterials\textsuperscript{4-6}. In a recent study, a PEG based polymer poly(PEG methacrylate ethyl ether) (polyPEGMA-EE\textsubscript{246}) was combined with PCL particles to form a thermoreversible scaffold that is suitable to be combined with cellular components\textsuperscript{7}. At temperatures below their characteristic lower critical solution temperature (LCST), PEG based polymers are water-soluble and can be used to coat suspended biodegradable particles\textsuperscript{7}. Raising the temperature above its LCST leads to a network of aggregated particles, converting the suspension into a gel\textsuperscript{7}. In this study, we aimed to apply polyPEGMA-EE\textsubscript{246} as biomaterial component for an injectable TE therapeutic. For this we have used the material as a composite together with stem cells and evaluated its ability to support the \textit{in vitro} 3D differentiation towards cells of bone or soft tissue.

Polycaprolactone (PCL) is a widely used biomaterial which can be used in a diverse range of scaffold designs and has excellent properties as a tissue engineering matrix because of its biodegradability-rate and good biocompatibility\textsuperscript{8-14}. Suspensions of PCL particles of a size below 10 µm combined with 3% wt polyPEGMA-EE\textsubscript{246} exhibited thermoreversible gelation\textsuperscript{7}. The thermophysical properties allowed for the distribution of cells below 20°C within the liquid scaffold material, and for the \textit{in vitro} culture of solid 3D cell-material constructs at 37°C.

For the cellular component we selected the murine C2C12 cell line, which possesses both myogenic and osteogenic differentiation potential, and is therefore an ideal characterisation cell line to test the materials for support of cell differentiation. It has already been published, that electrospun PCL fibers or laser-sintered PCL scaffold allowed myogenic differentiation and an injectable starch-PCL scaffold supported the osteogenic differentiation of C2C12 cells \textit{in vitro}\textsuperscript{13,15,16}.

To further investigate the relevance of PCL-polyPEGMA-EE\textsubscript{246} for TE purposes, human adipose-derived stem cells (ASCs) were chosen to evaluate its suitability to support osteogenic as well as adipogenic differentiation. For application as cell-therapeutic agent in regenerative medicine, ASCs have several advantages, including quantity and cell yield, easy accessibility and the potential for use in an autologous setting. In previous studies, PCL-tricalcium phosphate (TCP) materials combined with fibrin glue or lyophilised collagen,
and PCL/ poly(lactic-co-glycolic acid) (PLGA)/ collagen scaffolds were found suitable to support osteogenic differentiation of human ASCs\textsuperscript{17,18}. Also the adipogenic differentiation of human ASC on PCL meshes, as well as the endothelial differentiation on PCL coated dishes has been reported\textsuperscript{14,19}.

The aim of this study was to evaluate the thermoresponsive PCL- polyPEGMA-EE\textsubscript{246} scaffold for its ability to support of cell viability, 3D osteogenic as well as myogenic differentiation of the cellline C2C12 as well as adipogenic and osteogenic differentiation of ASCs.
**Material and Methods**

If not indicated otherwise, all reagents were obtained from Sigma-Aldrich (Austria) and of analytical grade quality.

**Preparation of the scaffold**

**Preparation of PCL microparticles**

The single emulsion solvent evaporation method was used. Polycaprolactone (Mn . 10 000, 300 mg) dissolved in dichloromethane (DCM, 10 ml) was added to a 100 ml of 0.3 wt% polyvinyl alcohol (PVA, Mw . 13–23 kDa) solution in water. The mixture was homogenised at 24 000 rpm for 2 minutes and then left to stir at 400 rpm for 6 hours, for DCM evaporation and microparticles hardening. The microparticles were collected by centrifugation at 3000 rpm for 4 minutes, and then freeze dried. Yields were 63 ± 3.78% (mean ± SD, from 5 different batches).

**Synthesis of polyPEGMA**

Free radical polymerization was used to synthesize polyPEGMA. The monomer PEGMA (Mn. 246, 10 g, 40 mmoles) was weighed into a round bottom flask to which 1-dodecanethiol (0.05 g, 0.25 mmoles) and butanone (15 ml) were added. AIBN (0.05 g, 0.3 mmoles) was added last, and the mixture was degassed with argon for 15 minutes. The flask was then immersed in an oil bath, and the polymerization was conducted at 70°C for 1 hour. Poly-PEGMA-EE was precipitated into a large excess (10 fold) of hexane and purified by dialysis against deionized water for 7 days in a cold room (5°C, dialysis membrane of a molecular weight cut off: 6000). The Polymer was freeze dried for 2 days and stored at 8°C.

The PCL particles were sterilised for 1 h under UV light. PCL particles were mixed with 3% wt PEGMA to form a 30% wt suspension.

Aliquots of 150 µl were transferred into a 48 well suspension plate (Greiner Bio-One, Austria) and cooled to 4°C prior to cell seeding.

**C2C12 cells**

C2C12 cells (DSMZ, Germany) were cultured in DMEM-HG medium supplemented with 5% fetal calf serum (FCS) (PAA, Austria), 1% L-Glutamin and 1% Penicillin/Streptomycin to a subconfluent state. For 3D seeding, 2.5 x 10^5 cells were mixed with the cold scaffold. After an incubation at 37°C for 20 min, prewarmed culture medium was added. As control, cells at a density of 1.1 x 10^4/cm² were seeded in 2D on standard cell culture plastic. For myogenic differentiation the FCS concentration was lowered to 1% for 7 days. For osteogenic differentiation 300 ng/ml recombinant BMP2 (PeproTech, Austria) was added to the C2C12...
medium containing 1% FCS and the cells were incubated for 7 days. Medium was changed every 48 h –72 h.

**ASC harvest and cultivation**

The collection from adipose tissue material was approved by the local ethical board. Adipose-derived stem cells were harvested and isolated as described by Peterbauer-Scherb et al.\(^2^0\). Cells for experiments were used between passages 2-3.

ASCs were expanded in endothelial growth medium 2 (Lonza, Belgium) to a subconfluent state. For 3D cell seeding 10^6 cells were mixed into the scaffold below 20°C. After an incubation time at 37°C for 20 min prewarmed control medium (DMEM/HAM's F12 supplemented with 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin) was added. As control, cells were seeded in 2D at a density of 5.3 x 10^3/cm^2 on standard cell culture plastic in control medium. ASCs from two individuals were selected to study donor specific responses to 3D conditions.

**ASC differentiation**

One day after seeding the cells under 3D or 2D conditions, medium was changed to control, osteogenic or adipogenic differentiation medium. For the osteogenic differentiation, ASCs were incubated in DMEM-LG-medium containing 10% FCS, 1% L-Glutamin, 1% penicillin/streptomycin, 0.01 µM dexamethasone, 150 µM ascorbat-2 phosphate and 10 mM β-glycerophosphate (STEMCELL Technologies, France) for 21 days. For adipogenic differentiation, ASCs were cultured in adipogenic differentiation medium consisting of DMEM-HG, 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin, 1 µM dexamethasone, 0.5 mM IBMX, 0.5 µM hydrocortisone (STEMCELL Technologies, France) and 60 µM indomethacin for 21 days. Cell medium was changed twice per week.

**Histology**

The 3D constructs were fixed in 4% formaldehyde overnight and then washed with distilled water for at least 1 h. The scaffold was then embedded in low concentration of gelatin to allow for diffusion throughout the scaffold for 24 h. This was followed by a higher concentration to make stronger embedding as described by Hruschka et al.\(^2^1\). The blocks were cut to appropriate sizes, covered with 4% formaldehyde overnight, and stored in distilled water. The samples were dehydrated through a graded series of ethanol, transferred to xylol followed by an infiltration in paraffin wax. Sections were cut at a thickness of 4 µm on a microtome (Microm, Germany).
Cell viability assay

Cytotoxicity was measured using a lactate dehydrogenase (LDH) assay (Invitrogen, Austria). The 3D PCL-polyPEGMA scaffolds were resuspended in medium by repeated pipetting and then transferred to a 1.5 ml microcentrifuge tube. The sample was centrifuged at 300 g for 5 min. From both 2D as well as 3D samples the supernatant was aspirated. 1x Lysis Solution was added and incubated at 37°C for 1 h. The lysed samples were centrifuged for 4 min at 2500 g. The supernatant of the 3D samples was transferred and the centrifugation was repeated. All further steps were performed following the manufacturers protocol.

Alkaline phosphatase (ALP) assay

Samples were lysed (0.25% Triton in ALP Buffer (0.5 M 2-amino-2methyl-1-propanol, 2 mM magnesiumchloride, pH 10.3)) and intracellular ALP activity was measured. Cell lysates were incubated with 20mM para-nitrophenyl phosphate in ALP buffer. The reaction was stopped with 0.2 M NaOH solution and the absorption was measured at 405/620 nm.

Quantitative Real Time PCR (qRT-PCR)

RNA was isolated using TriFast Reagent (Peqlab, Germany) according to the manufacturer’s protocol. DNA digestion using RQ1 Rnase-free DNAsse (Promega, Austria) as reported in the manufacturer’s protocol. Total RNA complementary cDNA synthesis was performed using the DyNAmo cDNA Synthesis Kit (Biozym, Austria) as described in the manufacturers protocol using an Oligo dT18 primer and random hexamers. For qRT-PCR 40 ng cDNA template were mixed with the primers (Microsynth, Switzerland), KAPA SYBR fast green kit (Peqlab, Germany) and ddH2O. Primer sequences specific for the quantified transcripts and spanning exon-intron borders or flanking intron sequences were generated using Beacon Designer Software (Premier Biosoft, US) and are listed in supplementary Table 1. For each primer the appropriate molarity and annealing temperature was determined in advance using standard curve and melting curve analysis (data not shown) and sample Cts were determined in triplicates Gene expression was quantified using the comparative Ct method \( 2^{-\Delta\Delta Ct} \). Normalized gene expression was calculated relative to the hypoxanthine guanine phosphoribosyl transferase (for murine samples) or glyceraldehyde-3-phosphate dehydrogenase (for human samples) housekeeping genes individually for each sample using \( 2^{-\Delta Ct} \) and then depicted as folds relative to the respective control samples.

The C2C12 differentiation control for the osteogenic marker expression were cells differentiated towards the myogenic lineage for the same period, while cells differentiated
towards the osteogenic lineage served as control for myogenic marker. The osteogenic and adipogenic marker expression of ASCs were compared to cells cultured in control medium.

**Leptin ELISA**

To evaluate human leptin in the tissue culture medium, the supernatant of the 2D and 3D samples was collected after 72h of incubation. The leptin ELISA (Abcam, United Kingdom) was performed according to the manufacturer’s protocol.

**SEM**

The scaffold was morphologically characterised using scanning electron microscopy (SEM). SEM analysis was performed on gold-coated samples (Agar Sputercoater 108, United Kingdom) and using a Philips XL20 microscope (Philips, The Netherlands). Particle size measurements were obtained from several micrographs acquired in the SEM.

**Statistical testing**

To perform statistical analysis IBM SPSS Statistics (IBM, USA) was used. Gaussian distribution was assumed and one-way ANOVA was performed to determine statistical significance (α=0.05). Tukey test was used for post-hoc analysis. Data were represented as mean ± standard deviation (n = 3/group; Graphpad Prism, USA).
Results

Cell number and distribution

Cell viability throughout the scaffolds was investigated by quantitative LDH assays comparing cells proliferated in 3D PCL-polyPEGMA-EE$_{246}$ scaffolds to standard 2D cell culture on plastic conditions. After an initial adaption phase of the cells seeded in 3D, C2C12 cells resumed proliferation in parallel with the 2D culture (Figure 1A). ASC cell number increased when seeded in 2D, while the number of ASCs seeded in 3D remained steady after seven days of culture (Figure 1B). Depending on the scaffolds’ properties, cell growth can be limited particularly in the centre of the scaffold due to decreased nutrient supply.

Cell distribution was analysed by microscopic examination of the cells and by histological sectioning. ASCs were distributed evenly throughout the PCL matrix (Figure 1C), which was confirmed by SEM (Figure 1D), demonstrating attachment and tight interaction of ASCs with the PCL particles. ASCs showed an average diameter of 10 µm and presented a flattened morphology.

Differentiation of C2C12 cells

The murine C2C12 cell line has the ability to differentiate towards the osteogenic as well as the myogenic lineage, which makes it ideal to study the scaffold-support of cell differentiation.

Osteogenic differentiation

C2C12 cells, differentiated towards bone in 2D cell culture, displayed the typical osteocyte phenotype (Figure 2A). The degree of differentiation was determined by measuring alkaline phosphatase (ALP) activity and the expression of osteogenic markers (Figure 2E). Under osteogenic conditions, both the 2D and 3D cultures showed increased ALP activity compared to the control (p<0.0001). However, 2D samples had a significantly higher ALP activity (p<0.0001) compared to the 3D samples. To obtain a more detailed profile we quantified mRNA expression of three typical osteogenic markers (Figure 2C). In contrast to protein activity, mRNA expression of ALP was only increased in 2D (p=0.003) compared to the 3D culture, which remained at control levels. Similarly, collagen-I mRNA levels were slightly increased in 2D compared to control levels, while the 3D levels were significantly decreased compared to both, the 2D culture (p=0.002) and the control (p=0.035). Osteocalcin mRNA was equally upregulated in differentiation culture (fold increase for both 2D and 3D: 16.3 ± 0.2). These heterogenous results suggest a limited potential of the scaffold to support osteogenic differentiation of C2C12 cells.
Myogenic differentiation

Cells differentiated in 2D on cell culture plastic showed a distinct myotube formation, a typical sign of myogenic differentiation (Figure 2B). To evaluate the degree of differentiation, the expression of established myogenic markers was examined (Figure 2D). In both 2D and 3D differentiation cultures all three myogenic markers were upregulated. The marker myogenin was found highly upregulated in both 2D as well as 3D culture (fold change compared to control: 50.3 ± 2.0). MyoD expression was significantly increased in 3D compared to the control (p=0.001), as well as to the 2D culture (p=0.0000). In the marker expression of Tnt1, no significant increase could be detected under both differentiation conditions.

Differentiation of ASCs

To mimic an in vivo TE situation the behaviour of primary cells, human ASCs, was evaluated in vitro for osteogenic and adipogenic differentiation. Typical signs of differentiation include the mineralisation of ASCs differentiated towards the osteogenic lineage, demonstrated by von Kossa staining, or lipid accumulation, stained with Oil Red O, of cells differentiated towards the adipogenic lineage. As confirmed by these stainings, both chosen ASC donors presented a high differentiation potential, while cells treated with control medium showed neither mineralisation nor lipid accumulation, demonstrating that the adequate donor was chosen. In 2D, very large mineralisation was found after three weeks of culture in osteogenic differentiation medium (Figure 3 B and E), while a high degree of lipid accumulation was demonstrated after stimulation with adipogenic differentiation medium (Figure 3 C and F).

Osteogenic differentiation

Although mineralisation indicated excellent differentiation potential for both donors, at the expression level of osteogenic markers, donor specific differences were found.

Upon osteogenic stimulation of donor A (Figure 4A), the mRNA expression was induced in 2D compared to the 3D conditions for the osteogenic markers Osteocalcin (p=0.018) and ALP (p=0.006). Also, the expression of Osteopontin (p=0.016) and Osteocalcin (p=0.013) was upregulated in 2D compared to the control, while it remained at equal levels as the control in 3D culture. ALP expression was not differentially regulated compared to the control samples.

In donor B the expression of Osteocalcin was upregulated in the 2D samples (p=0.001) and 3D samples (p=0.027) compared to the control (Figure 4B). Interestingly, Osteopontin expression was significantly upregulated in 3D compared to the 2D samples (p=0.038) and
CHAPTER TWO: RESULTS

the control (p=0.014). ALP expression was upregulated in 2D compared to the 3D samples (p<0.001) and the control (p<0.001). However, an increase in ALP expression in 3D culture compared to the control could be seen. To summarise the results obtained from donor A, a statistically significant increase compared to the control was observed in the expression of osteocalcin and osteopontin in the 2D culture samples, while no difference was detected between the 3D culture samples and the control. In donor B a significant increase of the osteogenic markers expression, Osteocalcin and Osteopontin, in the 3D culture samples compared to the control was observed. However, the overall markers expression was elevated in the 2D compared to the 3D culture conditions. The results of the qRT-PCR revealed a successful osteogenic differentiation of samples differentiated under 2D conditions, but only minor signs of 3D osteogenic differentiation in 3D culture. The quantitative ALP assay (Figure 4C) showed an increase in ALP activity upon osteogenic stimulation only in cells cultured under 2D conditions (p<0.001), confirming the qRT-PCR findings. ALP activity in cells cultured in 3D was significantly decreased compared to both 2D (p<0.01) and the control (p=0.01) cultures.

Adipogenic differentiation

3D adipogenic differentiation of ASCs was evaluated via mRNA expression analysis. FABP4, PPARγ and Adiponectin, three markers specific for adipogenic differentiation, were highly induced in both 2D and 3D culture of both donors, indicating the suitability of the scaffold for adipogenic differentiation. Donor A (Figure 5A) showed equally increased marker expression of PPARγ (fold increase: 10.6 ± 0.5), FABP4 (fold increase: 96024.2 ± 24753.6) and Adiponectin (fold increase: 4994.4 ± 2044.5). For donor B higher expression of the markers PPARγ (p=0.004), FABP4 (p<0.001) and Adiponectin (p<0.001) was detected in 3D culture compared to 2D culture (Figure 5B). Similar to donor A, the 2D as well as 3D differentiated donor B samples showed a pronounced fold change compared to the control. To further analyse the 3D adipogenic differentiation, the human Leptin expression in the supernatant was evaluated. Already after 2 weeks significantly higher levels of human Leptin were found in the 3D culture samples compared to the 2D culture samples in donor A (p=0.003, Figure 5C) as well as donor B (p<0.001, Figure 5D). The increase was even more evident after 3 weeks in both donor A (p<0.001) and donor B (p<0.001).
Discussion

The studied thermoreversible PCL-polyPEGMA-EE<sub>246</sub> scaffolds have shown potential for 3D cultivation and differentiation of cells with stem cells characteristics.

Cell adhesion and growth within a 3D scaffold are dependent on the pore size, pore interconnectivity and the ratio of surface area to volume of the scaffold <sup>23</sup>. After 3D seeding, C2C12 cells showed a lag phase in proliferation for 3 days followed by resuming cell growth, while ASC numbers remained steady over the tested 7 days. A previous study employed a variety of scaffolds, including PCL scaffolds and PLGA meshes for the 3D expansion of cord blood derived CD34<sup>+</sup> progenitor cells. They observed a decrease in cell number at day 2 followed by an increase up to day 14 for cells seeded on PCL scaffolds. In contrast, PLGA meshes were not able to support the proliferation of cord blood-derived hematopoietic stem cells, which they attributed to lack of cell adhesion, which could not be improved by changing scaffold stiffness, porosity or fibre size <sup>24</sup>.

Other studies showed that cell proliferation could be increased by changing the surface energy, e.g. by surface alkali treatment of the material, to improve cellular attachment <sup>25–27</sup>. Therefore, the surface characteristics of the investigated PCL-polyPEGMA-EE<sub>246</sub> scaffold were altered, by exposing the constituent PCL particles to sodium hydroxide treatment to achieve superior cell attachment, as previously described <sup>7</sup>. Histology and SEM analysis showed that human ASCs were attached and homogenously distributed throughout the scaffold, which indicates suitable surface properties, and at least a sufficient porosity to guarantee nutrient supply in the centre of the scaffold. Zeltinger et al. investigated the effect of changing the pore size of poly(L-lactic acid) scaffolds on the proliferation of three primary cell types (canine dermal fibroblasts, vascular smooth muscle cells and microvascular epithelial cells). They showed that larger pore sizes above 38µm resulted in increased cellular proliferation <sup>25</sup>. Hence, the low cell proliferation rate observed in our study might partly be explained by the pore size of the PCL-polyPEGMA-EE<sub>246</sub> scaffold, which were found below 5 µm.

Alternatively, it has been suggested that there is a reciprocal relationship between cell proliferation and differentiation. For various cell types it has been described that differentiation results in the suppression of the proliferation and the upregulation of the differentiation in a tissue- and species-specific manner <sup>28,29</sup>. Therefore, we suggest that the limited cell proliferation in the chosen PCL-polyPEGMA-EE<sub>246</sub> scaffold could partly be a result of early differentiation induced by the 3D culture.

C2C12 cells as well as ASCs have proven differentiation potential in the chosen directions under 2D conditions, which therefore served as control for the level of differentiation <sup>30</sup>. Even
though PCL based scaffolds are commonly used for bone TE, the mechanical properties of
the PCL particles-polyPEGMA-EE\textsubscript{246} scaffold resemble the characteristics of soft
tissues\textsuperscript{7,10,31}. Therefore the potential of the scaffold to support soft tissue as well as bone
tissue engineering was evaluated. The expression of early (MyoD, Myogenin) myogenic
C2C12 markers was higher under 3D and 2D differentiation conditions compared to the
control. C2C12 myogenic differentiation resulted in significantly elevated MyoD expression
under 3D conditions, while Myogenin and the late marker Tnt1 were found on equal levels in
2D and 3D culture. For the adipogenic differentiation of ASCs, both donors showed equal
(donor A) or increased (donor B) mRNA expression of the three adipogenic markers in 3D
compared to 2D culture. The Leptin ELISA confirmed that the 3D adipogenic differentiation
was more advanced than the 2D differentiation. These results suggest that the PCL-
polyPEGMA-EE\textsubscript{246} scaffold is suitable for TE of soft tissues such as fat and muscle.

In contrast to adipogenic and myogenic differentiation, the scaffold showed limited ability to
support osteogenic differentiation. While under 2D conditions all three markers were elevated
compared to the control, in the 3D environment, the early markers ALP and Collagen I were
equal to the control or even down regulated, while the late marker Osteocalcin was increased
compared to the control. ALP activity was present in C2C12 cells stimulated with osteogenic
medium in 2D as well as 3D conditions. However, the 3D scaffold did not efficiently induce
osteogenic differentiation and is therefore unsuitable for osteogenic differentiation of C2C12
cells.

Interestingly, a different osteogenic marker profile (Osteocalcin, ALP activity) was found in
C2C12 cells compared to ASCs in 3D cultures. These results underline the importance of
using primary cells beside cell lines, as cell-lines may have lost the genuine properties of
primary cells and therefore allow only limited evaluation of biomaterials\textsuperscript{32}. Another important
issue to take into account is donor to donor variability of primary cells. Two human ASC
donors were assessed, which both showed excellent signs of differentiation in standard 2D
differentiation assays (von Kossa, Oilred O staining). However, the two donors reacted quite
differently with regards to specific marker expression, especially in 3D culture. Data were not
pooled as it is important to know how different donors respond to the material\textsuperscript{33}.

Several injectable materials have been developed and applied for TE \textit{in vitro} as well as \textit{in vivo}\textsuperscript{34–36}. Various studies have shown that PCL based scaffolds support bone, muscle as
well as adipose TE\textsuperscript{10,17,19,37,38}, and in combination with ASCs those scaffolds were evaluated
for chondrogenesis, neural or endothelial differentiation\textsuperscript{14,39–44}. Injectable hydrogels based on
PCL for use in TE have also been reported\textsuperscript{45–49}, with a limited number of studies reporting
hydrogels based on PCL for TE purposes with thermoresponsive properties\textsuperscript{37,49–51}. Fu et al.
prepared an injectable and thermosensitive scaffold composed of triblock PEG-PCL-PEG
(PECE) copolymer, collagen and nano-hydroxyapatite and implanted it in cranial defects of New Zealand White rabbits. The hydrogel showed a good capacity for guided bone regeneration \(^{50}\). Another study showed that the PECE copolymer is able to support

*in vivo* incision wound healing after primary tumour removal in mouse \(^{49}\). Besides support of bone and wound healing, it has been shown that the PECE hydrogel prevents postoperative adhesions in rats \(^{51}\). In the study of Kim et al. muscle regeneration was achieved when combining ASCs with a methoxyPEG-PCL scaffold *in vivo* \(^{37}\).

Compared to the previously reported scaffolds based on PCL, the design of our scaffold based on PCL particles and polyPEGMA-EE\(_{246}\) is unique and offers distinct advantages for TE applications. Its thermoreversible gelling behaviour allows for it to be conditioned in vitro. And for the subsequent minimally invasive injection of the cell- scaffold constructs in vivo. The reversible thermoresponse behaviour also allows for the enzyme-free expansion of cultured cells as previously described \(^{7}\). The constituent PCL particles can serve as controlled release depots for various factors that could promote cell behaviour such as growth factors or other small chemicals.

In our study we showed that the developed PCL-polyPEGMA-EE\(_{246}\) scaffold rather supports soft TE than bone TE. A recent study demonstrated that the osteogenic differentiation of ASCs depends on the mechanical scaffold properties \(^{31}\). By increasing the compressive tangent moduli from 15 kPa up to 194 kPa, increased calcium production as well as osteocalcin gene expression could be found under osteogenic conditions \(^{31}\). We suggest that the PCL-polyPEGMA-EE\(_{246}\) scaffold with an elastic modulus of approximately 10 kPa \(^{7}\) matches the cellular requirements for differentiation towards soft tissue lineages including adipose tissue \(^{52,53}\).

A recent study transplanted solid-free form fabricated PCL meshes seeded with human ASCs into the dorsal subcutaneous pockets of mice resulting in volume-stable adipose tissue formation *in vivo* \(^{54}\). However, the transplantation of solid scaffolds requires invasive procedures and therefore injectable materials are favourable \(^{1,3}\). Additionally, the fit and integration of injected material with the surrounding tissue at the defect site is expected to be superior as compared to a prefabricated scaffold. To our knowledge, it has not yet been demonstrated that ASCs have the potential to differentiate towards the adipogenic lineage in a 3D injectable PCL-based scaffold.

In conclusion, C2C12 cells as well as ASCs were found attached and evenly distributed in the presented thermoreversible PCL-polyPEGMA-EE\(_{246}\) scaffold. The scaffold supports the differentiation of the C2C12 cell line towards myocytes and the adipogenic differentiation of ASCs, and has therefore the potential for use as an injectable biomaterial for soft TE.
For future studies, the characteristics of the PCL-polyPEGMA-EE_{246} scaffold could further be optimised to enhance cellular attachment, proliferation and differentiation. By altering biochemical cues within the scaffold, for example by the addition of fibronectin or laminin, which would improve cell adhesion and signalling \(^{31}\). Pore size, which has been identified as critical parameter for cell proliferation, could be increased by modification of the material composition. To ameliorate the suitability for further applications within the area of TE, including bone TE, the stiffness of the scaffold could be elevated, possibly by blending with other injectable particles or by use of an alternative polymer composite.

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### Supplementary Table 3 Primer sequences

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Figure 4 Cell viability of C2C12 cells (A) and ASCs (B) measured using LDH assay. ASC cell distribution was examined by Hematoxylin & Eosin staining of paraffin embedded sections (C) and by SEM (D). Size bar equals 20 µm (C) or 10 µm (D).
Figure 5 Cellular phenotype of C2C12 cells after 7 days 2D osteogenic (A) and myogenic (B) differentiation. qRT-PCR results for osteogenic markers (C) as well as myogenic markers (D) revealed the suitability of the scaffold for myogenic differentiation. ALP activity (E) confirmed the limited support for 3D osteogenic differentiation. Values are shown as fold change compared to the differentiation control (* p-value < 0.05 to control, # p-value < 0.05 to 3D; Size bar equals 500 µm; n=3)
Figure 6 ASCs cultured in 2D conditions: Representative pictures of cells in control medium (A and D) show no mineralisation or lipid droplet accumulation. Cells stimulated with osteogenic (B and E) differentiation medium show advanced mineralisation after von Kossa staining. Oil Red O staining of cells stimulated with adipogenic differentiation medium (C and F) shows high lipid droplet formation. Size bar equals 100 µm.
Figure 7 Differentiation of ASC cultured in the 3D scaffold compared to those cultured in 2D tissue culture plastic for two different donors: qRT-PCR results for osteogenic markers (A and B) demonstrated increased markers under 2D culture conditions compared to the 3D culture within the scaffold. Values are shown as fold change compared to the differentiation control. Analysis of differentiation at protein level: ALP activity of ASCs stimulated with control or osteogenic differentiation conditions (C): An increase in ALP activity was found only in 2D culture conditions with osteogenic stimulation. (* p-value < 0.05 to control, # p-value < 0.05 to 3D; n=3)
Figure 8 ASC adipogenic differentiation in the 3D scaffold compared to 2D culture on tissue culture plastic for two donor A (A) and donor B (B) cells. Analysis of differentiation at protein level: The amount of Leptin in enriched supernatant from control or adipogenic differentiation conditions: Donor A (C) showed a higher response than donor B (D). In both donors Leptin levels were increased in the 3D compared to the 2D differentiation at both timepoints. Values are shown as fold change compared to the differentiation control. (* p-value < 0.05 to control; # p-value < 0.05 to 3D; n=3)
2.2.1 Interlude

*In vitro* studies allow the evaluation of biomaterials regarding its support for cellular proliferation and differentiation. However, additional *in vivo* studies should be performed to give clinically relevant results. In a further study, the PCL-polyPEGMA-EE$_{246}$ scaffold was injected into a pre-defined space of a silicone tube, which was placed around the inferior epigastric vascular bundle of Sprague Dawley rats, to follow cellular migration and angiogenesis.

The authors from Nottingham University contributed by producing the thermoresponsive scaffold. Chapter 2.3 have been written by Paul Slezak, except the description of the material and its background in the Introduction, the preparation of the material in the Materials & Methods section, and by contributing to the discussion. The author prepared the thermoresponsive scaffold for its *in vivo* use and for the analysis using SEM. The author conducted the SEM analysis.
2.3 A simple in vivo approach to assess angiogenesis and cell migration in a PCL based, thermosensitive scaffold

Authors: Paul Slezak, Cyrill Slezak, Joachim Hartinger, Veronika Hruschka, Rainer Mittermayr, Heinz Redl
A simple *in vivo* approach to assess angiogenesis and cell migration in a PCL based, thermosensitive scaffold.

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Abstract:
We show how a minimal invasive *in vivo* model in Sprague Dawley rats can be used in conjunction with open access image analysis software to achieve a comprehensive assessment of vascularization and cell migration in an injectable, thermosensitive scaffold. In this study, the inferior epigastric vascular bundle of rats was sheathed with a silicone tube which was filled with a polycaprolactone (PCL) particle based biomaterial which was assessed over a period of 4 weeks. The morphology, spatial distribution and dynamics of the ingrowths of cells and vascular structures into the matrix, originating from the central vessel were assessed from histologically stained samples. We detected a peak vascularization with subsequent reabsorption after 2 weeks as well as a bulkmigration of cells within the first week.

In summary, a profound analysis of a biomaterial in an *in vivo* situation can be realized with reduced expenses and a minute workload, profoundly covering fundamental aspects like cell migration and angiogenesis.
Introduction

Biomaterial scaffolds and their interaction with pre-seeded or migrating cells are a cornerstone of modern tissue engineering techniques. In this context, the role of biomaterials cannot be solely restricted to their primary scaffolding function. A large array of properties governs the behavior of biomaterials in tissue engineering, including degradability, porosity, surface morphology, and various chemical attributes, which together define their suitability in tissue engineering (Pérez et al., 2013). While many of these properties can be studied individually though in vitro methods, the combined assessment of cellular and vascular ingrowth is ideally achieved by in vivo models. Despite recent advancements in bioreactors, in vivo models are still unparalleled in their ability to offer clinically relevant results concerning the suitability of a biomaterial and in enabling a quick transition from research to application (Norrby, 2006). At the same time, moral and ethical considerations concerning animal testing must take a strong position and animal models are rightfully challenged in many aspects. Not only are they required to produce robust and in-depth scientific results, but they are also expected to economize on material used, work load, and analytic expenses – all while keeping animal stress, drop-out rates, and surgical procedures to a minimum. We have taken these requirements in consideration and have modified a recently established animal model on angiogenesis to include cell migration in biomaterials and have shown that the model is an efficient tool to characterize a newly developed injectable biomaterial.

Injectable materials have gained increased attention as they allow the application of the material in a minimally invasive manner. A promising subclass of these injectable materials are thermosensitive materials which are characterized by their liquid-solid phase transition. Temperatures below their lower critical solution temperature (LCST) result in the liquefaction of the material, while temperatures above the LCST lead to solidification (Hruschka et al., 2013).

Recently, a new injectable scaffold has been developed based on polycaprolactone (PCL) particles in combination with a thermosensitive polymer (polyethylene glycol methacrylate ethyl ether (polyPEGMA-EE246). It has been shown that this biomaterial supports the adhesion as well as growth of NIH 3T3 cells (Cheikh Al Ghanami et al., 2010) and therefore may be a promising candidate for tissue engineering purposes. For this reason, the proliferation and differentiation of cells with stem cell characteristics (adipose-tissue derived stromal cells (ASCs) and C2C12 cells) has been evaluated in vitro. While the material showed limited potential to support cellular proliferation of both ASCs and C2C12 cells, the scaffold allowed the adipogenic differentiation of ASCs as well as the myogenic
differentiation of C2C12 cells (Hruschka et al., 2013). It remained unclear whether this 3D construct would allow in vivo cellular infiltration, persistence and differentiation.

The aim of the study was to apply a minimal invasive animal model in conjunction with an automated analytical routine in a comprehensive evaluation of a thermosensitive PCL scaffold. We showed that this economical and easily implementable in vivo approach was able to assess the dynamics of cellular migration and vascularization as well as the spatial distribution of cells and vessels inside the scaffold.
CHAPTER TWO: RESULTS

Materials and Methods

Scaffold
PCL particles as well as polyPEGMA-EE\textsubscript{246} were produced as described previously (Cheikh Al Ghanami et al., 2010). To constitute the thermoresponsive material, PCL particles were suspended in 3\% wt polyPEGMA-EE\textsubscript{246} resulting in a 30\% wt suspension. The material presented itself homogenously in structure, opaque and white in color. It was fluid and easily applied after being stored on ice. A particle size ranging from 0.2 µm to 5 µm was reported by the manufactures as well as a porosity of 60-75\% for the solidified compound.

SEM analysis
Scanning electron microscopy (SEM) was performed to morphologically characterize the scaffold prior/post implantation. SEM analysis was performed on gold-coated samples (Agar Sputercoater 108, United Kingdom) using a Philips XL20 microscope (Philips, The Netherlands).

Study Layout
In this study a modified and enhanced minimal invasive model of angiogenesis in rats was applied (Cronin et al., 2004; Slezak et al., 2013). This work employed the same basic surgical procedure; however the analytic steps were extended to facilitate cell detection. In brief, 9 Sprague Dawley rats (weight ~ 350 g) underwent surgery, receiving two silicone tube implants each (Figure 1A). The silicone tubes were placed around the epigastric pedicle on each side in order to create a pre-defined space around the vein and artery (Figure 1B-C). After implantation these tubes were sealed with sutures and filled with cooled, liquid PCL-PEGMA-EE\textsubscript{246} which solidified at body temperature within seconds. The surgical wounds were then closed and analgesia was provided for 3 days (Slezak et al., 2013).

The implanted silicone tubes measured 1.5 cm in length with an inner diameter of 4 mm and a wall thickness of 1 mm and were well tolerated by the animals. No drop outs were registered and the implants caused no signs of discomfort in the animals. The application of the biomaterial proved very convenient utilizing a pre-cooled syringe and needle, which kept the material fluid during the application process. After injection into the tube the material engulfed the central vascular bundle in its full circumference, with the biomaterial being easily identifiable.

The study setup consisted of 3 groups (n = 6) that all received the same amount of the same biomaterial in the same fashion. Each group was sacrificed and their implants removed after
one week, two weeks and four weeks. In addition a 4th group (n = 6), providing zero-week sample which represented the vascular situation right after surgery was introduced. Since in this specific case no vascular or cellular ingrowth was to be observed, surgery was performed on dead rats of the same weight and strain and standard fibrin glue (Artiss, Baxter) was used as a bioscaffold. The zero week histologic samples were obtained and conserved immediately after the biomatrix application.

In all 4 groups no tissue ingrowth from the outside was observable on any of the obtained samples. Each implanted tube yielded a solid, cylindrical sample containing the centric vascular bundle and newly formed tissue within the biomaterial surrounding it. No degradation of the material was observed at any time point. The cylindrical samples were fixated in 4% formaldehyde solution and conserved in 70% ethanol after 24 hours. Histologic slices were produced with the section plane chosen perpendicular to the central axis. The resulting circular sections were immuno- histologically stained for smooth muscle actin (SMA) as well as hematoxylin & eosin (HE). Subsequently, full scale, high resolution microscopic scans were produced at a magnification of 200x. Based on these scans a computational image analysis was performed with the freely available software “Cell Profiler” to detect vessels and cells (Kamentsky et al., 2011). The detection rate of vessels was improved by manually marking their histologic cross sections in a commercial image editing software. Additionally a custom made image processing pipeline was applied in order to augment cellular contrast for the detection of cell nuclei. Collected data included the position and ellipsoid-approximated shape of each cell and vessel in the whole section of the sample.

In detail, the individual images were resampled to a size of 3750x3750 pixels and a colourunmix procedure was applied, utilizing the “Cellprofillers” preset for hematoxylin stainings. This accentuated stained nuclei within the histologic image and allowed subsequent object detection. To identify pixels qualifying as potential cell nuclei a Otsu thresholding algorithm was applied which was further optimized by introducing a manual thresholding factor ensured a correct detection. When identifying individual objects as cell nuclei a minimum size of 2 µm as well as a maximum size of 12 µm was defined. Objects found by the thresholding procedure out of this size range were discarded. To verify our cell detection procedure, the amount of cells found in 10 randomly selected sample regions within the cellularized scaffold of randomly selected slices was manually counted and results were compared to the automated analysis. In average, automated cell counts were off by 13%, however the total of all manually counted cells in 10 samples merely differed by 3% from the total of cells detected by automated image analysis. Concerning vascular detection,
since vessel cross sections were manually marked before image analysis, an accurate
computational detection was ensured.

Analysis
In brief, the spatial information of the vessels cross sections was used to calculate distance
plots in regard to the central vein as well as vessel diameter distributions. Further the total
number of vessels in each sample was detected and average values were calculated. In
order to quantify the vascularized area a virtual perfusion radius of 150 µm was introduced.
To calculate the vascularized area, each vessel cross section was expanded by this radius
and the resulting perfused areas subsequently superimposed; overlapping contributions were
single counted. This closely resembles the area of perfused tissue within the samples, and
as such was finally used to calculate vascular density. A more detailed description of the
obtained vascular parameters can be found in (Slezak et al., 2013).

In this work we enhanced the analytical capacities of our established image analysis routine
to obtain spatial data on each cell nucleus within the sections. This allowed for the
introduction of new parameters to the model. As such the average total cell count in each
group was calculated as well as the average cellularized area. Mirroring the analysis of
vascular structures we further obtained distance plots of the cell population in regard to the
central vein, revealing potential distribution patterns within the scaffold.
Results

In general, no signs of inflammation were observed. SEM imaging comparing the scaffold prior to post implantation showed that the scaffold was infiltrated with cells after 4 weeks (Figure 2A-B). An encapsulation of the scaffold occurred after week 2 in regions in which cells had completely traversed through the PCL material (Figure 3). In the specific boarder regions between the populated and unpopulated scaffold in which cellular infiltration took place an increase of SMA positive structures was detected. These structures did not represent competent vasculature but probably cellopodia of migrating cells (Figure 3). Further distal and beyond the cellularized areas, only a few cells were detected, either in small groups or individually, among cell debris and apoptotic cells (Figure 3).

Cellular Migration

Over the timeframe of 4 weeks, a homogenous and dense cellularization and vascularization of the scaffold was observed. Figures 4 and 5 show dynamic cellular growth. A very low cell count of $0.16 \pm 0.031 \times 10^4$ per sample in our baseline sample of week zero indicated a successful surgery with only residual cells remaining in the sample. In addition, these cells were located in close proximity to the central vein indicating little to no intrusion into the scaffold as shown in Figure 6. After a rapid increase in the cell count after one week ($3.73 \pm 0.37 \times 10^4$ per sample) we noted no statistically significant changes at the 95% level $[F(2,15=0.69,p=0.52)]$ in consecutive weeks two and four ($4.25 \pm 0.36 \times 10^4$ and $4.30 \pm 0.41 \times 10^4$). We observed an identical behavior when considering the areal cellular spread as measured by the total cellularized tissue area. The zero-week value of $0.28 \pm 0.05 \text{mm}^2$ was in line with our assertion of a careful and successful implantation. The rapid cellular growth over the first week was also evident by the large tissue area ($5.35 \pm 0.57 \text{mm}^2$) but also remains relatively stable thereafter. No statistically significant change at the 95% level $[F(2,15=1.58,p=0.24)]$ in consecutive weeks two and four ($6.41 \pm 0.47 \text{mm}^2$ and $6.54 \pm 0.51 \text{mm}^2$) was observed.

Figure 6 shows the histogram of the cell count as a function of distance to the central vein. Besides the aforementioned cellular residue from the implantation in the close vicinity of the main vessel (less than 0.5 mm) at week zero, we were able to obtain an estimate of the dynamical cellular distribution. All cellular structure up to a distance of 11 mm remained unchanged after week one. The highly linear slope of the histogram was indicative of a highly uniform cellularization throughout our sample. All features beyond this length scale were artifacts from the off-axial localization of the vein within the cylinder and the thus created variation of the histological samples. This analysis was consistent with our results when
directly evaluating the average cell density for each sample. Figure 7 shows the average cell count densities for each week. The initial sample showed a slightly, yet statistically significantly (p<0.05), lower density which we attributed to the spreading of residual cells into the scaffold during surgery. However, for the remaining weeks the overall density showed no significant differences ([F(2, 15=1.41, p=0.27)]).

**Vasculature**

In the previous section we have seen that the cellularization of the sample is essentially complete after one week. However, the dynamical timescale for angiogenesis appeared to be significantly different. Samples from week one showed distinct vascularization inside the surrounding scaffold, reaching well beyond the central region. The average number of total vessels since implantation had increased from 49 ± 10 to 107 ± 28 (Figure 8). Applying a virtual perfusion area, obtained by expanding the cross section of each detected vessel by a radius of 150 µm, we found a virtually perfused to also increased from 0.86 ± 0.11 mm² to 4.32 ± 0.85 mm². This level of perfusion remained statistically unchanged at the 95% level for the samples obtained in consecutive weeks 2 and 4 (Figure 9).

After 2 weeks total vessel count had risen to 199 ± 61 virtually perfusing an area of 5.24 ± 0.052 mm². After 4 weeks, vascular resorption was expected to have set in reducing the total vessel count which was found to be 157 ± 37 perfusing 4.80 ± 0.70 mm². It is important to note that there was no statistically significant difference at the 95% level in the vessel counts past week zero ([F(2, 15=1.068, p=0.368)]). Most of the changes in vessels numbers past week one was seen in the domain of capillaries with a diameter ranging from 15 µm to 30 µm (Figure 10).
Discussion

In this study we have improved an *in vivo* model originally developed for the study of angiogenesis to include individual cell detection, thus expanding its suitability for biomaterial assessment. We measured vascularization and cellularization dynamics within a newly developed thermosensitive PCL based injectable scaffold. The scaffold itself was recently tested in various *in vitro* experiments on ASCs and C2C12 cells and had shown to allow the adipogenic differentiation of ASCs as well as the myogenic differentiation of C2C12 cells. However, a limited potential to support *in vitro* cell growth over a period of seven days was reported (Hruschka et al., 2013). It was discussed that the lack of proliferation *in vitro* might be either due to the small pore size of the scaffold (below 5 µm) or as a result of early differentiation within the scaffold. *In vitro*, the cells were mixed into the scaffold and subsequently differentiated (Hruschka et al., 2013). It remained unknown whether the cells are able to infiltrate the scaffold. Further, though *in vitro* data on the specific material already suggested that no cell toxicity was present and that the material was suitable for soft tissue engineering approaches, it was finally unclear if these results would translate into an *in vivo* model (Hruschka et al., 2013). Therefore we were interested to evaluate the PCL-polyPEGMA-EE scaffold in an *in vivo* model that allows following cellular migration as well as differentiation.

In our *in vivo* approach we expanded the time of observation to 4 weeks, as initially it was unclear, if the material would encourage quick cell migration. Also, with a particle size of up to 10 µm and a calculated porosity of 60-75% proper cell propagation and adequate vascularization were of critical concern. After the first week a considerable population of cells was already found in the scaffold, indicating good biocompatibility. During these first 7 days a strong migration of cells into the surrounding scaffold had taken place and the average total cell count had already reached 87% of the 4 week cell count. The material showed properties appealing to cells and promoting cell proliferation. This finding was further supported via SEM imaging, revealing a dense compound of cellular material engulfing the original scaffold. Over the whole 4 week observation time, total cell count showed a continuous trend of a non linear, inverse exponential increase. This was also mirrored in cellularized area which translated to tissue volume in 3 days.

During the first weeks when cells were actively migrating into the scaffold, we observed the emergence of SMA positive structures in the border zone between tissue and acellular scaffold regions. These structures occurred solely in the interface between the scaffold and
the cellularized area and were usually oriented away from the cellularized center towards the empty scaffold. We consider these formations of SMA positive structures to be cellopodia, facilitating the migration process of the observed cells. Our \textit{in vivo} findings confirmed that the scaffold supports the differentiation of cells, as has been previously studied \textit{in vitro} (Hruschka et al., 2013). It could be presented in this study that cells are able to migrate into the scaffold \textit{in vivo}. The proliferation \textit{in vitro} could be influenced by insufficient media supply. The small pore size of the scaffold seemed to be irrelevant for cellular infiltration \textit{in vivo}. However, further studies should be performed to evaluate the influence of the pore size in the presented model.

In summary we can conclude that the tested thermosensitive PCL-PEGMA-EE$_{246}$ material was supportive of tissue ingrowth and suitable for tissue engineering approaches. No signs of inflammation were observed and it was capable of supporting a homogenous and dense population of cells, based on a newly formed vascular network inside the scaffold. Additionally the modified model proved efficient and practicable in acquiring the sought parameters in an easy and quick manner.
CHAPTER TWO: RESULTS

Literature


Figure 1. Anatomical position and surgical procedure. A) the implanted silicone tube with a length of 15 mm. B) Inguinal incision after preparation of the epigastric vascular bundle. C) Silicone tube in situ after implantation.
Figure 2. A) Scanning electron microscope image of the scaffold prior to implantation. B) Scanning electron microscope image at the same scale, 4 weeks post implantation.
**Figure 3.** Histologic section, perpendicular to the central vessel. SMA & Haematoxylin stained. A) Acellular PCL Scaffold. B) Centric artery and vein. Note the vascular network and dense formations of SMA positive structures in the border zone of cellular migration with individual cells advancing into the scaffold. C) Boarderzone with cells reaching the perimeter of the scaffold. Note that an encapsulation of the scaffold only takes place where full cellularization has occurred. Once fully cellularized, a reduction in SMA positive structures can be observed.
Figure 4. The average number of individual cells per sample as detected by our image analysis tool. Note the massive increase in cells during the first week after implantation, followed by a flattening increase thereafter.

Figure 5. The average area of each sample which was detected as being cellularized. This parameter is closely linked to cell number and showed a strong increase in week 1.
Figure 6. Cell distribution histogram as a function of distances to the central vein. Note the linear increase up to 1100 µm, indicating a homogenous cellularization of the samples.

Figure 7. The average cell count density within the cellularized area of each sample.
Figure 8. The average number of vessels per sample in each group. Note the increase up to week 2 with a following decrease. Vessels in at week 0 indicate the number of vessels present around the vascular bundle after surgery.

Figure 9. The average area per sample, potentially perfused by the vascular tree. This is a virtual parameter and based on a defined perfusion distance of 150 µm. Note the strong increase in week one which peaks after 2 weeks.
Figure 10. Normalized, smoothed histogram of vessel distribution. The different distances to the central vein of the vessels within a sample were plotted as a fraction of the total amount of cells within each sample. Note the shift of the statistical weight to smaller vessel diameters between week 0, week 1 and week 2.
3.1 General Discussion

Injectable scaffolds are promising candidates to be used in the clinics since - due to the small incisions required - the infection risk is minimized, scar formation is reduced and treatment costs are low. The possibility to combine injectable biomaterials with therapeutic cells, growth factors or additional factors allows a variety of application fields (Chen et al., 2012; Hou et al., 2004; Kohane and Langer, 2008; Wintermantel et al., 1996). In this study an injectable matrix based on PCL and the thermoresponsive polymer polyPEGMA-EE\textsubscript{246} was chosen and its potential for TE applications evaluated. The \textit{in vitro} study showed that the PCL-polyPEGMA-EE\textsubscript{246} scaffold supports the 3D myogenic differentiation of C2C12 cells as well as the adipogenic differentiation of human ASCs. In contrast, a limited osteogenic differentiation was found in C2C12 cells, while ASCs did not differentiate towards the osteogenic lineage when seeded into the scaffold. In an \textit{in vivo} angiogenesis model the scaffold was infiltrated with cells, newly formed vessels were detected and no signs of inflammation were observed.
Injectable scaffolds

Working with injectable scaffolds has several advantages, but also disadvantages. The most obvious disadvantage is their limited mechanical stability. Biomaterials that solidify via physical cross-linking methods, show low stability, low mechanical strength and fast degradation rates (Chen et al., 2012). In contrast to physical cross-linking, chemical cross-linking methods have been shown to result in higher stability (Amini and Nair, 2012). However, most chemical cross-linking methods require the incorporation of reactive compounds or photoirradiation, which might cause toxicity problems (Amini and Nair, 2012). In contrast to this, the specific properties of the chosen PCL-polyPEGMA-EE$_{246}$ matrix allow gelling to occur under physiological conditions in vivo which should therefore not irritate or damage the surrounding tissue.

Biomimetic scaffolds should serve as temporary and artificial ECM to improve cell adhesion and to guide tissue formation to allow regeneration of tissues. Hydrogels are promising candidates as they can withhold large amounts of water or nutrients while showing certain mechanical strength to simulate living tissue (Fu et al., 2012). The mechanical properties of a scaffold should ideally be as similar as possible to the host tissue at the site of implantation. Therefore it is unlikely that a single material is suitable to serve as scaffold for engineering many tissues (Kretlow et al., 2007; Temenoff and Mikos, 2000). One mean to measure the stiffness of a tissue is the determination of the shear modulus. It has been described that human femoral bone has a shear modulus of approximately 7 GPa, human muscle has a modulus ranging between 2.99 and 4.5 kPa, the modulus of human fat is around 0.29 kPa, and the modulus of a rat aorta is about 137 kPa (Deng et al., 1994; Evans, 1978; Lacourpaille et al., 2012; Sopher et al., 2011; Spears et al., 2007). At 37°C, the PCL-polyPEGMA-EE$_{246}$ scaffold has a shear modulus of approximately 10 kPa, which would resemble the shear modulus of soft tissues. It may hence be well suited to temporarily replace soft tissues that need to be regenerated in vivo without leading to the formation of a fibrotic capsule, while providing the required mechanical strength. The low shear modulus of the PCL-polyPEGMA-EE$_{246}$ scaffold might be responsible the limited osteogenic differentiation in vitro.
Degradation of the scaffold

An ideal scaffold should degrade in parallel with new tissue formation. A too slow degradation could limit the function of the newly formed tissue as it influences the formation and distribution of ECM (Hou et al., 2004). The degradation rate of synthetic polymers varies between 1 week and several years. PCL has been described as slowly degrading polymer with a degradation time of two to four years, depending on the initial molecular weight of the scaffold (Woodruff and Hutmacher, 2010). Depending on the site and degree of injury, bone fracture healing might last for several months or years (Lin et al., 2013). Therefore, the degradation of scaffolds based on PCL depending on the formulation may match the rate of bone formation (Fu et al., 2012; Yeo et al., 2010b). However, it has been reported that PCL fragments, with a particle size ranging between 53-500 nm, were rapidly degraded inside the phagosomes of macrophage and giant cells within 13 days after a subcutaneous implantation in rats (Woodward et al., 1985). Ji et al. (2012) evaluated the in vitro degradation of an electrospun PCL/PLGA scaffold with a fibre diameter of 731 to 780 nm. After 8 weeks, the molecular weight of PLGA could not be detected in the scaffold, whereas the molecular weight of PCL decreased from 150.6 to 26.9 kDa (Ji et al., 2012).

Soft tissue injuries were described to take place in three phases. The first phase of acute inflammation last for up to 7 days and is followed by a proliferative phase of fibroblasts, synovial cells, and capillaries, lasting for up to 21 days. The last phase of maturation and remodeling may take as long as 6 to 12 months for the final maturation in the case of tendon and ligament tissue. New collagen fibres can resist near-normal stress after 6-8 weeks (Kannus, 2000). Therefore, the degradation rate of PCL particles might be more suitable for the regeneration of soft tissue like muscle or adipose tissue. Therefore it will be essential to characterise the in vivo degradation time of the PCL-polyPEGMA-EE246 to determine the suitability of the scaffold for the regeneration of a specific tissue type.

Another requirement for scaffolds to be used for TE purposes is that its degradation products are nontoxic. In contrast to substances such as PLGA, which releases acidic degradation products, the degradation of PCL results in non-acidic free caprolactone and its dimers and trimers (Hakkarainen, 2002; Ji et al., 2012). A study evaluating the toxicity of methoxy PEG/PCL nanospheres demonstrated no
signs of cytotoxicity in vitro using human fibroblasts. Also after injecting the nanospheres intraperitoneally in mice, no significant pathological changes were observed (Kim et al., 2003). Another study reported a mild toxicity of PCL on MG63 cells, when cultured in direct contact. However, no significant difference was found when the cells were cultured in extract of the scaffold compared to control medium (Salgado et al., 2012a). In a similar study the authors injected a biodegradable PCL-sebacic acid gel in a tibia defect. After 30 days, an inflammatory response was observed, which was less prominent after 60 and 90 days. After 30 days mineralized tissue was formed, which developed into a more organised structure over time. The authors discuss that the inflammation may be part of a normal healing response (Salgado et al., 2012b). Taken together this data and our own observations of the tube model, in which the scaffold was evaluated for four weeks, no signs of toxicity could be observed at the site of integration, suggesting that the PCL-polyPEGMA-EE$\text{EE}_{246}$ scaffold is suitable for TE purposes.
Porosity and pore size

Several studies have been done to evaluate the influence of porosity and pore size on tissue formation. It has been reviewed by Zhong et al. (2012) that increasing the pore size enhances cellular infiltration in electrospun scaffolds (Zhong et al., 2012). Underwood et al. Measured studied the epithelial ingrowth into rods of sphere-templated porous poly(2-hydroxyethyl methacrylate) by percutaneous implantation in mice. The presented a shorter average keratinocyte migration distance through 20 µm pores compared to 40 and 60 µm pores (Underwood et al., 2011). Heterogenous reports are found concerning the influence of porosity on in vivo bone formation. While some studies using orthotopic bone models and various implantation materials, e.g. PLGA or poly(propylene fumarate), report increased bone formation with high porosity, others do not find a statistically significant difference between several porosity levels tested (Fisher et al., 2002; Karageorgiou and Kaplan, 2005; Roy et al., 2003). Two studies that implanted hydroxyapatite-based scaffolds subcutaneously in rats evaluated the effect of pore size for bone TE and presumed that pore sizes around 300 µm are optimal for bone formation (Kuboki et al., 2001; Tsuruga et al., 1997). Also in vivo cartilage repair was found to be improved using a PCL scaffold with pores of 400 µm and 200 µm compared to 100 µm after implanting the scaffold into an osteochondral defect in rabbits (Im et al., 2012). A study compared the in vitro osteoblast phenotype expression using titanium scaffolds with three different pore sizes (62 µm, 130 µm and 312 µm). Interestingly, the smallest pore size of 62 µm yielded the highest osteoblastic gene expression together with a decrease in proliferation (Teixeira et al., 2012). Higher porosity in vitro facilitated the nutrient supply to the cells and therefore improved proliferation. Therefore, it has been suggested that in vitro cell differentiation is not dependent on pore size, but that lower porosity enhanced osteogenic differentiation (Karageorgiou and Kaplan, 2005; Takahashi and Tabata, 2004). Scaffolds for soft tissue defects are frequently composed of several layers with different porosities to mimic the anatomical structures of tissues with a dense basement membrane in combination with a loose and highly porous connective tissue. Cells can easily infiltrate the porous areas of the scaffold, while the second layer serves as physical barrier (Knight et al., 2013; Leong et al., 2010; Pok et al., 2013). A deeper cell migration of bladder smooth muscle cells was found improved in electrospun PLGA scaffolds with a porosity of 80.9±1.5%.
compared to a porosity of 73.1±2.9%. A bladder-like wall structure with urothelial lining and cell infiltration was observed in both groups *in vivo*. Scaffolds with a higher porosity had a significantly increased microvessel density (Maya et al., 2013). A recent study confirmed that increasing the porosity, but also pore sizes resulted in an increased vascularisation (Mehdizadeh et al., 2013). In contrast, it has been reported that a pore size of approximately 30 µm allows for the healing of silicone elastomer structures or cross-linked poly(2-hydroxyethyl methacrylate) into soft tissues with significantly enhanced vascularity and reduced fibrosis. Pores with a size below 20 µm or above 60 µm lead to avascular, fibrotic healing in subcutaneous sites, in heart muscle and percutaneously in the skin (Galperin et al., 2010; Ratner, 2007). Calculations of the specific PCL-polyPEGMA-EE scaffold indicated that the void volume in the scaffold is equivalent to a porosity of 60-75% and therefore would be in the range of scaffold-porosity suited for both, bone and soft TE (Cheikh Al Ghanami et al., 2010; Hidalgo-Bastida et al., 2007; Karageorgiou and Kaplan, 2005; Maidhof et al., 2012; Son et al., 2011). The used PCL-polyPEGMA-EE scaffold showed no signs of inflammation and advanced vascularisation *in vivo*, suggesting that the combination of porosity and pore size is suitable for soft tissue healing.
**Predifferentiation of cells**

Stem cells represent a promising candidate for TE strategies. In general, the less manipulation is performed on the cells ex vivo, the less expensive is the procedure and the easier it is to obtain an approval for the clinics. Therefore it is relevant to know whether there is a need for *in vitro* predifferentiation before application to the site of tissue regeneration. Only a few studies have been done to evaluate the relevance of the cellular maturity at the time of implantation *in vivo*. The implantation of fibrin matrices containing *in vitro* predifferentiated preadipocytes resulted in increased adipose tissue formation, compared to the usage of undifferentiated preadipocytes (Cho et al., 2006). In another study, an *in situ* gelling agarose was combined with predifferentiated or undifferentiated BMSCs to form a cartilaginous matrix in the growth plate. In contrast to predifferentiated BMSCs, undifferentiated cells were able to restore the growth function and could therefore correct the limb length discrepancy (Coleman et al., 2013). Miot et al. (2012) compared the influence of *in vitro* maturation of engineered cartilage to generate osteochondral repair. Autologous chondrocytes were predifferentiated in hyaluronic acid scaffolds for 2 days, 2 weeks or 6 weeks and subsequently implanted above hydroxyapatite/hyaluronic acid sponges into the defect site. After 8 months, poor cartilage architecture was found in the empty defects or in the defects with cell-free implants. While the implants precultured for 6 weeks resulted in the highest type II/I collagen ratio, inferior architecture within the defect and lower integration was detected. Therefore, the defects treated with 2 weeks predifferentiated grafts displayed a good compromise between the cartilage maturity and structural properties (Miot et al., 2012).

The importance of cellular maturity was studied by implanting a human striatal neural stem cell line (STROC05) into a mouse model of a neurodegenerative genetic disorder (Huntington’s disease). Cells were either predifferentiated towards the DARP-32 neurons or implanted undifferentiated. The outcome was not improved compared to a vehicle control injection, which might be due to poor cell survival and neuronal differentiation (El-Akabawy et al., 2012). Another study evaluated predifferentiated ASCs as well as naïve ASCs for the treatment of spinal cord injury. They found a significant improvement in function recovery and locomotor activity in both groups. The predifferentiated cells showed an increase in survival and
interacted closely with the host tissue. However, no neuronal markers were detected. Further, they did not observe a difference in functional improvement between the groups and therefore claim that the repair was induced mainly through paracrine mechanisms (Arboleda et al., 2011).

The studies comparing the usage of predifferentiated and naïve cells suggest that it might not be mandatory to predifferentiate cells prior to implantation. As for the specific PCL-polyPEGMA-EE\textsubscript{246} used, a sufficient vascularisation was observed, suggesting that the material can support cellular differentiation of endothelial cells. Depending on the tissue of interest, factors that stimulate cellular differentiation (e.g. BMP2 for bone TE) can further be added to the scaffold. Therefore it might be possible to avoid a time-consuming and expensive predifferentiation of cells \textit{in vitro}.
3.2 Conclusion and future prospects

In this study, the suitability of an injectable scaffold that is based on PCL particles and the thermoresponsive polymer PEGMA to serve as TE construct was evaluated.

In the first study a method for the histological analysis of delicate scaffolds, such as thermoresponsive or injectable scaffold, was established. Using gelatin-embedding prior to sectioning, the scaffold can be stabilised which further allows the complete transfer of the intact scaffold, histological preparations, sectioning and staining.

In the second study, the scaffold was combined with cells with stem cell characteristics, such as the C2C12 cell line or ASCs. It was presented, that the scaffold shows limited support of osteogenesis for neither C2C12 cells nor ASCs. In contrast, the scaffold was suitable for the myogenic differentiation of C2C12 cells as well as the adipogenic differentiation of ASCs.

As the formation of tissues necessitates the formation of blood vessels, the ability of the scaffold to support angiogenesis was evaluated in an in vivo model. The results show a high number of newly formed vessels in the scaffold, confirming its applicability for TE purposes.

Taken together, the scaffold might be a candidate to be used in the field of soft TE in its present form as it supports cellular growth as well as differentiation in vitro as well as in vivo. No signs of inflammation at the site of integration were observed. In future studies, the scaffold could be altered to match the requirements for specific areas of interest, such as by increasing the stiffness for its use in bone TE.
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APPENDIX

APPENDIX

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