ADVANCED SCAFFOLD FOR ADIPOSE TISSUE RECONSTRUCTION

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Abstract

Adipose tissue is a large loose connective tissue with functions comprising thermogenesis, homeostasis, metabolic activity and cushioning of bordering tissue layers. The tissue is predominantly composed of adipocytes and importantly contributes to the individual body shape. Loss of the fatty tissue due to trauma or disease often results in disfigurement, which affects patients physically as well as emotionally. Different surgical and scientific approaches have been studied to address this problem; in this regard tissue engineered scaffolds have received increased attention. Although there has been significant development in the efforts to address tissue loss, the reconstruction and maintaining of tissue with large dimensions remains a challenge. Hence, the overall aim of this project was to engineer a bulk scaffold suitable for adipose tissue reconstruction.

The scaffold, cell source and extracellular signalling aspects of a tissue engineering strategy were approached to design a scaffold suitable for restoring adipose tissue defects of large dimensions. A particulate leaching method was developed and combined with the application of different freezing temperatures to produce a range of microporous macroporous gelatin scaffolds. Constructs were physically characterised for suitability in cell based studies. Scaffold design was investigated through biological characterisation using adipose derived stem cells (ADSCs) and an artificial ADSC stem cell niche was created through the utilisation of extracellular matrix components. The artificial environment was combined with the scaffolds and evaluated to support adipogenesis.

A range of novel microporous macroporous scaffolds were produced, differing in micropore size range. Selection of scaffolds with defined features resulted in two constructs with a physical and biological profile suitable for adipose tissue construction. The scaffolds displayed high porosity and the materials supported ADSC viability and proliferation. Furthermore, cells were easily absorbed throughout the whole construct. The final resulting composite scaffold consisting of scaffold and artificial ADSC stem cell niche displayed in vitro support of adipogenesis.

Concluding, a platform of novel composite adipogenic regeneration scaffolds were constructed that support adipogenesis as well as the preservation of ADSC stemness. Further, the gelatin sponges display a physical and biological profile suitable for adipose tissue reconstruction.
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AUTHOR’S DECLARATION

Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed

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<td>ADSC</td>
<td>Adipose derived stem cell</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Alginate</td>
<td>Alginate acid sodium salt</td>
</tr>
<tr>
<td>aP2</td>
<td>Adipocyte protein 2</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
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<tr>
<td>BMI</td>
<td>Body-mass index</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
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<tr>
<td>BMSC</td>
<td>Bone marrow derived mesenchymal stem cell</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin Fraction V</td>
</tr>
<tr>
<td>C/EBPs</td>
<td>CCAAT enhanced binding proteins</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine monophosphate</td>
</tr>
<tr>
<td>CCK-8</td>
<td>Cell counting kit 8</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CD13</td>
<td>Aminopeptidase N, stem cell marker</td>
</tr>
<tr>
<td>CD29</td>
<td>Integrin β1, stem cell marker</td>
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<tr>
<td>CD31</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>CD34</td>
<td>Epithelia cell marker, possible adhesion molecule with a role in haematopoiesis</td>
</tr>
<tr>
<td>CD45</td>
<td>Protein tyrosine phosphatase, receptor type C, marker for haematopoietic cells</td>
</tr>
<tr>
<td>CD68</td>
<td>Glycoprotein which binds low density lipoprotein, macrophage marker</td>
</tr>
<tr>
<td>CD90</td>
<td>Thymocyte antigen (glycosylphosphatidylinositol -anchored membrane glycoprotein), Stem cell marker</td>
</tr>
<tr>
<td>CD168</td>
<td>Hyaluronan-mediated motility receptor, expressed in remodelling macrophages</td>
</tr>
<tr>
<td>CD180</td>
<td>Expressed in pro-inflammatory macrophages</td>
</tr>
<tr>
<td>CHO</td>
<td>Carboxyl group</td>
</tr>
<tr>
<td>Col</td>
<td>Collagen</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic AMP response element- binding protein</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DAT</td>
<td>Decellularised adipose tissue</td>
</tr>
<tr>
<td>DB</td>
<td>Digestion Buffer</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle's medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDHB</td>
<td>Ethyl-3,4-dihydroxybenzoate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>FABP</td>
<td>Fatty acid binding protein</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>Fib</td>
<td>Fibronectin</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
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<tr>
<td>H + E</td>
<td>Haematoxylin and Eosin</td>
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<tr>
<td>H2O</td>
<td>Water</td>
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<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
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<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>Hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>hMSCs</td>
<td>human Mesenchymal stem cells</td>
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<tr>
<td>HuNu</td>
<td>Human nucleolus</td>
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<tr>
<td>IL 17</td>
<td>Interleukin 17</td>
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<tr>
<td>ILGF</td>
<td>Insulin like growth factor</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirit</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>JNK</td>
<td>C-jun N terminal kinase</td>
</tr>
<tr>
<td>Lam</td>
<td>Laminin</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
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</table>
M 1  Pro-inflammatory macrophages
M 2  Remodelling macrophages
MAPK  Mitogen-activated protein kinase
MHC  Major histocompatibility complex
MIQE  Minimum Information for Publication of Quantitative Real-Time
        PCR Experiments
MMP  Matrix metalloproteinases
NaOH  Sodium hydroxide
NH₂  Amino group
P/Q  Perm/Quench solution
PA  Polyglycolic acid
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PDGF  Platelet derived growth factor
PEG  Poly(ethylene glycol)
PEGDA  Poly(ethylene)glycol-diacrylate
Pen/Strep  Penicillin/Streptomycin
PFA  Paraformaldehyde
PGAS  PBS, gelatin, azide, saponin solution
PI3K  Phosphatidylinositol-3 kinase
PKB  Protein kinase B
PLA  Poly(lactic acid)
PLGA  Poly(lactic-co-glycolic) acid
PLLA  Poly(L-lactic-co-glycolic)
PPARG  Peroxisome-proliferator-activated receptor-γ
PTC  Patched receptor
qPCR  Quantitative PCR
qRT-PCR  Quantitative real time PCR
RQ  Relative expression
RUNX 2  Runt-related transcription factor 2
Scaffold I  Scaffold prepared at RT
Scaffold II  Scaffold prepared by pre-freezing at -20°C, subsequent freeze drying
Scaffold III  Scaffold prepared by pre-freezing at -80°C, subsequent freeze drying
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<td><strong>SMO</strong></td>
<td>Smoothened</td>
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<tr>
<td><strong>SOX 9</strong></td>
<td>Transcription factor SOX 9</td>
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<tr>
<td><strong>SVCs</strong></td>
<td>Stromal vascular cells</td>
</tr>
<tr>
<td><strong>SVF</strong></td>
<td>Stromal vascular fraction</td>
</tr>
<tr>
<td><strong>TCP</strong></td>
<td>Tissue culture plastic</td>
</tr>
<tr>
<td><strong>TGF</strong></td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td><strong>TIMPS</strong></td>
<td>Tissue inhibitors of MMPs</td>
</tr>
<tr>
<td><strong>UCP-1</strong></td>
<td>Uncoupling protein 1</td>
</tr>
<tr>
<td><strong>VEGF</strong></td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td><strong>vWF</strong></td>
<td>von Willibrand factor</td>
</tr>
<tr>
<td><strong>WAT</strong></td>
<td>White adipose tissue</td>
</tr>
<tr>
<td><strong>2D</strong></td>
<td>Two dimensional</td>
</tr>
<tr>
<td><strong>3D</strong></td>
<td>Three dimensional</td>
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Chapter 1: Introduction

1.1. The Need for bulk scaffolds in adipose tissue reconstruction

Adipose tissue is part of the skin, where it forms the subcutaneous fat layer (Figure 1.1). The subcutaneous layer preserves the mobility of surrounding tissue layers, acts as cushioning for vital structures and shapes the body (Smahel, 1986, Bucky and Percec, 2008, Choi et al., 2010a, Frerich et al., 2012) (Figure 1.1). Subcutaneous adipose tissue can be lost due to a number of factors including age, disease, trauma, tumour removal, congenital malformation and severe burns. This can lead to deep contour defects that affect the dermis and epidermis of the skin which is aesthetically undesirable (Figure 1.2) (Patrick, 2001).

Figure 1.1: Subcutaneous adipose tissue is part of the skin. The human skin can be divided into three main layers: the epidermis, dermis and hypodermis (subcutaneous fat layer). Among the functions of the epidermis is the provision of a barrier for environmental factors such as infection, UV radiation and water. Functions of the dermis include structural and nutritional support of the skin as well as thermoregulation of the body where sweating or vasodilation prevents the body from overheating or cooling. The deepest layer of the integumentum, the hypodermis or subcutaneous fat layer functions include cushioning of vital organs, retaining of the mobility of the surrounding tissue layers and giving the body its individual shape. Loss of the deepest layer of the skin causes contour defects (Palastanga et al., 1994, Bologna et al., 2003, Porth and Matfin, 2009). Figure is adapted from (Cox, 2015).
These defects can influence the patient not just physically in terms of severe scarring resulting in motion restriction and loss of body contour, but also psychologically (Smahel, 1986, Patrick, 2001, Bucky and Percec, 2008, Tabit et al., 2012, Partridge, 2015). Currently, the most common techniques used to re-bulk tissue defects are based on using the patient’s own tissue and synthetic materials. These procedures include fat grafting (lipofilling/ lipoinjection/ micrografts) and the insertion of synthetic materials such as hyaluronic acid based fillers (Brayfield et al., 2010).

These methods suffer from a number of limitations including toxicity and rejection of the synthetic materials. The transplantation volume of autologous tissue is also restricted by necrosis, mostly of the centre part of large transplants, which limits the survival of the graft (Dong et al., 2015). In addition, the resorption of autologous fat transplants is known to be between 30-70% of the original transplanted volume and therefore the successful maintenance of volume requires multiple operations (Gomillion and Burg, 2006, Tabit et al., 2012). This loss of transplant volume can have a negative effect on aesthetic appearance. Furthermore, the long term results are unpredictable and often aesthetically and psychologically unacceptable to the patient as the natural contour is not restored (Smahel, 1986, Sommer and Sattler, 2000, Patrick, 2001, Rosen and MacDougald, 2006, Bucky and Percec, 2008, Tabit et al., 2012).

However, the number of surgical procedures which is currently undertaken to restore those defects shows an increasing trend. From 2010 to 2014 a rise of 18% in plastic and reconstructive surgeries was recorded for the UK. Of the reported 45,406 plastic and aesthetic surgeries in 2014, over 25% are breast augmentations and fat transplants (Surgeons, 2015). Hence, the unsatisfactory outcomes of the current techniques, paired with the increasing number of surgeries, lead to an increasing need for a new approach to restore tissue contour and to re-bulk tissue defects.
Figure 1.2: Common causes for soft tissue defects. Soft tissue defects can occur due to traumatic accidents such as severe burns. (A) Visible contour defect of a severely scarred arm of a 40 year old woman who was burned during childhood (2 years old). Furthermore, the removal of tumours such as a breast tumour can leave disfigurements. (B) The upper body of a 41 year old woman after breast tumour removal. In addition, a common cause for soft tissue defects is congenital malformation. (C) A congenitally deformed left hand of a 1.5 year old child. (Beth, 2007, Hasan, 2012, Deune, 2013).

1.1.1. Current treatment methods

The most common technique used to rebulk soft tissue injuries/defects is fat grafting or lipofilling (also called micrograft, lipoinjection, liposculpture) (Figure 1.3A) (Tremolada et al., 2010, Bucky and Percec, 2008, Yoshimura et al., 2011, Tabit et al., 2012). In which the patient’s own fat is used to rebulk the defect. Mostly, abdominal fat is aspirated from the patient and, after centrifugation to separate blood and debris from the fat, is re-transplantated into the patient in the area of need (Tabit et al., 2012). This procedure uses autologous fat that has advantages of being non-toxic, biocompatible and is easily available. The short term results are generally satisfying (Bucky and Percec, 2008). However, the long term outcomes are unpredictable (Bucky and Percec, 2008, Tremolada et al., 2010, Tabit et al., 2012, Philips et al., 2012).

The post-operative cellular events and timeframe of fat grafting are illustrated in Figure 1.4, which highlights the unsatisfactory results of fat grafting. Failure of the
engraftment can occur and long term survival of larger grafts is often not homogenous but creates irregularities and contour defects (Tremolada et al., 2010). The graft can also result in necrosis of the inner parts of the transplanted tissue due to insufficient vascularisation during the first 6 months (Tabit et al., 2012). The most concerning disadvantage of fat grafting is the high resorption rate which can be up to 70% of the transplanted volume (Bucky and Percec, 2008, Tremolada et al., 2010, Tabit et al., 2012). The volume loss of fat graft over time is partially due to the adipocytes, which are the main cells in fat. These cells are already damaged during grafting due to their low resistance to pressure. Adipocytes also have a low tolerance to ischaemic conditions which are predominant in thicker grafts after re-transplantation (Yoshimura et al., 2011). The study of Nishimura et al. showed that initial fat graft volume increases during the first weeks post-transplantation due to fibrosis but rapidly declines over subsequent months. The decline in graft volume is also partially related to insufficient vascularisation in the first days of post-transplant, which causes cell death and lipid leakage. The dead cells and lipids are thought to be removed by macrophages which contributes to the observed volume loss (Nishimura et al., 2000).
To overcome the vascularisation problem the Coleman lipostructure technique is employed. This technique applies centrifuged fat through several injections at different depths to the defect area. Here, smaller volumes of fat are applied which are more likely to be immediately supplied with oxygen (Tabit et al., 2012, Philips et al., 2012, Tremolada et al., 2010, Coleman, 2001). This method, however, can only be used for mild to moderate defects like wrinkles (Tabit et al., 2012).

To overcome the high resorption rate and volume loss, fat grafts and liposuctions have been enriched with stem cells from adipose tissue – known as adipose derived stem cells (ADSCs) (Yoshimura et al., 2011, Tabit et al., 2012) (for more examples see the review by (Philips et al., 2012)). The stem cells were demonstrated to be present in fat grafts and predicted to support the positive outcome of fat re-transplantation (Tremolada et al., 2010, Stillaert et al., 2010, Philips et al., 2012).

Figure 1.3: Current treatment methods for adipose tissue reconstruction with their limitations on the basis of breast augmentation. (A) Lipofilling is the predominant technique to rebulk soft tissue injuries in which the patient’s own adipose tissue is applied to the defect. The long term outcomes are unpredictable due to high resorption rates up to 70%. (B) Filling with synthetic materials such as hyaluronic acid is limited through transplant resorption, migration and allergenic effects. Figure is adapted from (Mediclude Co., 2007).
Cellular events after fat grafting.

After filling the defect with the graft material, adipocytes die in the first 24 hours due to insufficient blood supply, followed by vascular endothelial cells and blood derived cells. A slight volume increase that declines rapidly can be observed in the first month resulting from fibrosis. Through infiltration of macrophages the dead cells are phagocitosed (Inflammation). The adipose derived stem cells (ADSCs) can endure hypoxic conditions longer and better and therefore are able to contribute to the tissue repair process. This occurs mainly in the first 3 days after transplantation and can go on for 3 months (tissue repair and regeneration). If ADSCs also die it leads to necrosis and scar formation. Partially necrotic tissue does not lose its volume due to non-absorbance of dead adipocytes which can stay on as lipid droplets (weeks till month) (oil absorption). These lipid droplets can form cystic walls and also calcify (cyst wall formation) (Tissue stabilisation). Furthermore, the tissue size change in volume over time is given (modified from (Yoshimura et al., 2011)).

Stillaut et al. described through in vitro experiments of graft biopsy cultured in 3D matrices the outgrowth of cells of the biopsy that are able to proliferate and differentiate towards the adipogenic lineage (Stillaut et al., 2010). Furthermore, the graft enrichment with ADSCs represented a decreased resorption rate. Nevertheless, outcomes are also unpredictable and there is a risk of tumour formation (Alperovich
et al., 2014). Also, it is a new technique and the long-term complications remain unknown (Tabit et al., 2012). In addition, fat grafts are highly dependent on the technical skills of the surgeon, thickness of the graft, ratio of ADSCs to adipocytes in the graft and donor age and site (Geissler et al., 2014, Bucky and Percec, 2008, Lee et al., 2013). Furthermore, use of this technique is dependent on the availability of the patient’s own fat (Bucky and Percec, 2008).

In severe cases like large burns or lipoatrophy there may not be enough fat to graft. Here, synthetic fillers including silicon, hyaluronic acid and calcium hydroxylapatite based materials can be used (Bucky and Percec, 2008, Philips et al., 2012). But they lack in long term durability and are associated with high costs, an unnatural texture, a risk of implant migration and implant leakage and can be allergenic (Philips et al., 2012, Bucky and Percec, 2008, Brayfield et al., 2010) (Figure 1.3B). Therefore, the current state of available treatment methods confirms the clear need for a low cost and easy to handle new approach in soft tissue reconstruction.

1.1.2. Tissue engineering for the reconstruction of adipose tissue

Current treatment methods do not provide an optimal solution for the replacement of lost adipose tissue with a clear lack of appropriate filler for the repair and reconstruction of adipose tissue defects. The most promising strategy to approach this problem is tissue engineering.

Tissue engineering, alongside cell therapy, is a main application area of regenerative medicine. Regenerative medicine is defined as “the engineering and growth of functional biological substitutes in vitro and/or the stimulus to regeneration and remodelling of tissues in vivo for the purpose of repairing, replacing, maintaining, or enhancing tissue and organ functions.” (Muraca et al., 2007). Tissue engineering was first popularised by Vacanti and Langer in 1993 (Langer and Vacanti, 1993). They defined it as “a multi-disciplinary area which combines engineering and the life sciences with the aim of developing a biological substitute which restores lost tissue” (Langer and Vacanti, 1993). There are already a small number of tissue engineered applications of biological substitutes in medical use including artificial bladder, airways and skin (Table 1.1) (Burke et al., 1981, Stoddart et al., 2009,
Macchiarini et al., 2008, Polak, 2010). Indeed, skin substitutes are one of the most widely clinically used tissue engineered products (Muraca et al., 2007).

Table 1.1: Examples of tissue engineered products in clinical use.

<table>
<thead>
<tr>
<th>Organ/Tissue</th>
<th>Material/ Cells</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder</td>
<td>Collagen scaffold/ autologous urothelial, muscle cells</td>
<td>(Atala et al., 2006)</td>
</tr>
<tr>
<td>Airways</td>
<td>Human donor trachea/ autologous epithelial cells, mesenchymal stem cell-derived chondrocytes</td>
<td>(Macchiarini et al., 2008)</td>
</tr>
<tr>
<td>Skin</td>
<td>Silastic (silicon-plastic material), collagen-chondroitin 6-sulfate/ autologous fibroblasts, bovine dermal matrix, e.g. Matriderm® human dermal matrix, e.g. Alloderm®</td>
<td>(Burke et al., 1981, Halim et al., 2010)</td>
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Tissue engineering therefore, provides a potential strategy to address the problem of restoring lost tissue contour. The idea of tissue engineering is to harvest cells from patients and amplify them \textit{in vitro}. These cells can be seeded onto a support structure (scaffold), which assists cell proliferation and growth and delivers a frame work for the regenerating tissue structure. This cellularised construct can then be implanted into the patient. After transplantation the scaffold should either degrade or stay as a framework and guide tissue regeneration (Casadei et al., 2012). Therefore, the 3 main aspects of tissue engineering are: 1. an engineered matrix (scaffold) which acts as a shape and support structure, 2. cells which are capable of proliferation and differentiation to replace the missing tissue 3. the microenvironment that delivers appropriate signals to the cellular component for growth and tissue formation with the aim of restoring or replacing damaged/diseased tissue or organs (Choi et al., 2010a).

For the engineering of adipose tissue a material is needed that mimics the natural feel, composition and structure of adipose tissue, cells that are capable of differentiating into adipose cells and a microenvironment similar to the natural adipose tissue environment that provides appropriate cues for the cells to proliferate, differentiate and support tissue formation. Therefore the investigation of all three aspects is required.
The first step in designing an engineered construct to replace missing native tissue is to study and understand this tissue. The following overview and project outline provide a brief summary of adipose tissue biology and current approaches in adipose tissue engineering including cell sources and suitable materials. A detailed outline of the project is provided which aims to develop an appropriate cell-seeded scaffold for adipose tissue reconstruction.

1.2. Adipose tissue

Adipose tissue is a loose connective tissue (also known as fatty tissue (Smahel, 1986)) and is the largest tissue in the body (Patrick, 2001). In adults adipose tissue typically comprises 15-25% of the total body weight (Smahel, 1986) although in cases of obesity this can reach up to 70% (Cinti, 2005). One of the principal functions of adipose tissue is the storage of energy (lipogenesis) whereby excess energy is stored in the form of triglyceride in fat cells (adipocytes). This energy storage process is regulated by lipogenic enzymes. In the case of energy shortage this reservoir is released through enzymatic reactions. Here, the adipocyte’s lipase (lipoprotein lipase (LPL)) breaks the triglyceride into fatty acids and glycerol (lipolysis). These are transported through the blood system to the liver and muscle where the energy is released through fatty acid oxidation (Sethi and Vidal-Puig, 2007). Adipose tissue, however, is not just a reservoir for energy storage. It is also known as an endocrine organ that secretes adipose tissue derived factors termed adipokines (Choi et al., 2010a). These factors have effects in endocrine, paracrine and autocrine signalling pathways on lipid and carbohydrate metabolism, appetite, thermogenesis, the immune system, reproductive and further metabolic functions (Smahel, 1986, Sethi and Vidal-Puig, 2007, Bucky and Percec, 2008, Choi et al., 2010a, Cinti, 2012, Frerich et al., 2012).

Adipose tissue also has a high plasticity and can expand or regress dependent on nourishment (Cao, 2010, Sun et al., 2011). Imbalance in adipose tissue can result in adipose dysfunction such as obesity (too much fat) or lypodystrophies (too little fat as such seen in anorexic people or in old age), which is closely related to the occurrence of diabetes mellitus type 2, cardiovascular diseases and reproductive dysfunction (Niemelä et al., 2007, Hajer et al., 2008, Bucky and Percec, 2008, Cinti,
2012). Fat further acts as cushioning for vital structures and organs due to its location, preserves the mobility of the tissue layers due to its texture properties and modulates the body shape because it gives volume to the outer body contour (Smahel, 1986, Bucky and Percec, 2008, Choi et al., 2010a, Frerich et al., 2012).

1.2.1. Types of adipose tissue

There are two types of adipose tissue, brown adipose tissue (BAT) and white adipose tissue (WAT). BAT’s function is in non-shivering thermogenesis and can be found predominantly in infants in the thorax and neck area (Sethi and Vidal-Puig, 2007, Choi et al., 2010a). It is essential for heat generation after birth. Brown adipocytes are multilocular and rich in mitochondria. They express the uncoupling protein 1 (UCP-1). UCP-1 dissipates the proton gradient in the inner mitochondria membrane at the expense of ATP resulting in heat generation (Rosen and MacDougald, 2006). BAT reduces during maturation and in adults it is found scattered in WAT (Cannon and Nedergaard, 2004, Rosen and MacDougald, 2006, Sethi and Vidal-Puig, 2007, van Marken Lichtenbelt et al., 2009, Choi et al., 2010a, Illouz and Sterodimas, 2011). This project focuses on WAT, which comprises the majority of adipose tissue within the body (Bucky and Percec, 2008, Choi et al., 2010a, Illouz and Sterodimas, 2011).

1.2.1.1. White adipose tissue (WAT)

The predominant components of WAT are tightly packed spherical adipocytes (size range from 25 μm up to 190 μm (Skurk et al., 2007)) with one large lipid droplet (unilocular) (Cinti, 1999, Cinti, 2005, Lee et al., 2010, Wronska and Kmiec, 2012) and a peripheral nucleus, leaving just a thin rim of visible cytoplasm (Cinti, 1999). Adipocytes have a life span of up to 10 years (Yoshimura et al., 2011) and comprise 35-70% of the adipose tissue mass (Wetterau et al., 2012) (Figure 1.5). The maturation of fat cells from precursor cells occurs throughout life with approximately 10% of adipocytes renewed each year (Niemelä et al., 2007, Wronska and Kmiec, 2012). Adipocytes are surrounded by stromal vascular cells (SVCs) such as preadipocytes (cells with a fibroblast character that are committed to the adipocyte lineage (Farmer, 2006)) and represent 15-50% of WAT cells (Wronska and Kmiec, 2012). Furthermore, endothelial cells (these form an extensive vasculature),
fibroblasts (providing structural support), immune cells (resident macrophages that participate in normal remodelling and T-cells) (Lee et al., 2010), pericytes (vascular endothelial cells) (Zimmerlin et al., 2010), mast cells (influence angiogenesis and remodelling) and ADSCs (cells which are multipotent) are also found in WAT. The cells are interconnected through the extracellular matrix (ECM) (Bucky and Percec, 2008). WAT further contains lymph nodes, nerves and an extensive vascularised system with blood vessels (Smahel, 1986, Cinti, 2005, Bucky and Percec, 2008, Choi et al., 2010a, Wronska and Kmiec, 2012) whereby in thick WAT layers every single adipocyte has direct contact with a dense network of capillaries (Figure 1.5). WAT can be distinguished in 2 major depots according to location; the subcutaneous and the visceral (intra abdominal) fat that surrounds organs and other WAT sites (Choi et al., 2010a, Wronska and Kmiec, 2012) (Figure 1.5). The subcutaneous adipose tissue is located in the hypodermis layer (subcutaneous fat layer) of the skin (Figure 1.1) (Patrick, 2001, Cinti, 2005, Sobajo et al., 2008).

1.2.1.2. Vascular system

Thick (about 10 mm) and thin fat layers of WAT have a different pattern of blood vessel distribution. Thick WAT tissue has a dense system with each adipocyte in contact with a vessel. Thin layers are mainly supplied by diffusion and do not have such a dense connection to blood vessels (Smahel, 1986, Cinti, 2005). The maximum diffusion limit is 200 µm, displaying the utmost distance between capillaries (Novosel et al., 2011). Through the dense vascular system the adipose tissue is under a high partial oxygen tension (Yoshimura et al., 2011). The main function of a vascular system is to supply nutrients and oxygen to support growth, maintenance and waste removal. The vasculature within adipose tissue supplies plasma enriched with growth factors and cytokines, which trigger growth and survival signals in adipocytes to maintain function and promote expansion. The vascular system also supports the infiltration of monocytes and neutrophils into adipose tissue which, among others, play a role in wound healing (Cao et al., 2006, Sun et al., 2011). Furthermore, the vascular system is a release route for hormones, cytokines and various
Figure 1.5: Overview of the white adipose tissue (WAT). (A) WAT can be distinguished in 2 major depots: the visceral adipose tissue including omental (superficial around intestines), mesenteric (deeply buried around intestines) and perirenal (around the kidney) depots and the subcutaneous fat located in the hypodermis of the skin. (B) Adipocytes are the predominantly found cell type in WAT. They harbour a unilocular large lipid droplet with a peripheral nucleus. WAT further locates stromal vascular cells such as fibroblasts, preadipocytes, macrophages, ADSCs and immune cells. The adipose tissue is also rich in blood vessels and nerves. The figure is modified from (Wronska and Kmiec, 2012, Ouchi et al., 2011).
other biological agents (Wronska and Kmiec, 2012). The formation of blood vessels is driven by the proliferation of endothelial cells which is regulated by the release of growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and platelet derived growth factor (PDGF) by the stromal vascular cells (Ye, 2011).

1.2.1.3. Adipogenesis – development of mature adipocytes

Adipogenesis is the process of stem cells differentiating into mature adipocytes (Figure 1.6). The transformation involves the development from fibroblast like mesodermal stem cells via preadipocytes to lipid laden spherical adipocytes (Bucky and Percec, 2008). The process is thought to be closely coupled to angiogenesis due to the secretion of angiogenic factors such as VEGF, hepatocyte growth factor (HGF) and transforming growth factor (TGF) by ADSCs and mature adipocytes (Scioli et al., 2014). Furthermore, the adipogenic differentiation process involves a highly orchestrated gene expression leading to the transcription and translation of adipocyte specific genes (Sethi and Vidal-Puig, 2007, Niemelä et al., 2007, Bucky and Percec, 2008). Multiple endogenous and exogenous signals influence the differentiation of stem cells to adipocytes, such as hormones, cell-cell interaction, cytoskeleton changes, extracellular matrix compositions, bone morphogenetic protein (BMPs), TGF β superfamily, insulin like growth factor 17 (IL 17), FGF 1, 2 and the elevation of cyclic adenosine monophosphate (cAMP) (Rosen and MacDougald, 2006, Lowe et al., 2011, Bauer-Kreisel et al., 2010, Fernández-Real, 2012, Ali et al., 2013). Adipogenesis further consists of 2 major phases (determination and terminal differentiation phase), a subsequent growth arrest and the involvement of more than 2,000 genes (Gregoire et al., 1998, Rosen and MacDougald, 2006, Mariman and Wang, 2010).

In the determination phase, stem cells commit towards the adipogenic lineage triggered through endogenous and exogenous signals. Here, they are no longer able to differentiate towards different lineages (Rosen and MacDougald, 2006). The events that promote the determination of stem cells towards preadipocytes are, to date, not well understood compared to the events of the second phase. These centre around the peroxisome proliferator activated receptor gamma (PPARG) and CCAAT
enhanced binding proteins (C/EBPs) with subsequent activation of adipocyte specific gene expression. However, it is accepted that bone morphogenic protein 2 (BMP 2) and insulin signalling promote, while the Wnt and hedgehog (Hh) signalling pathways inhibit the determination of ADSCs towards the adipogenic lineage (Rosen and MacDougald, 2006, Lowe et al., 2011, Tang and Lane, 2012) (Figure 1.6B).

BMP2 activates the translocation of Schnurri-2 (SHN2) to the nucleus where it binds with SMAD1,4 and C/EBP together on the PPARG promoter (Rosen and MacDougald, 2006). The insulin pathway is known to be involved as well. The hormone insulin binds to the insulin growth factor -1 receptor which activates insulin receptor substrate (IRS). This activates phosphatidylinositol-3 kinase (PI3K) as well as AKT (also called protein kinase B, (PKB)). The phosphorylation of IRS signalling promotes and triggers the phosphorylation of cAMP response element-binding protein (CREB). CREB is involved in the expression of PPARG.

The activation of the canonical Wnt signalling pathway has the binding of Wnt to the membrane bound frizzled receptor and lipoprotein –receptor related protein-5 or 6 as a consequence. This binding prevents the phosphorylation of β-catenin. Consequently, β-catenin accumulates in the cytoplasm and translocates into the nucleus where it attaches to lymphoid-enhancer binding factor/ T-cell specific transcription factor family. This activates Wnt target genes resulting in inhibition of PPARG and C/EBP expression, hence adipogenesis. Deactivation or blockage of this pathway triggers adipogenesis (Rosen and MacDougald, 2006, Choi et al., 2010a).

The Hh signalling pathway is not well understood in terms of adipogenesis (Lowe et al., 2011). This pathway is known to act through the binding of Hh to patched receptor (PTC). Through the binding the seven transmembrane protein smoothened (SMO) is released, which suppresses the expression of adipogenesis genes such as PPARG (Rosen and MacDougald, 2006). A few other pathways show association to the differentiation of adipocytes and can be reviewed in Lowe et al. (Lowe et al., 2011).
However, the change from ADSC towards a committed cell is morphologically not visible. The committed cell has the same fibroblastic phenotype as the stem cell but has lost its ability to differentiate into different lineages and cell types (Rosen and MacDougald, 2006) (Figure 1.6A). After commitment of the cells towards the adipogenic lineage they enter the terminal differentiation phase. Here, the pre-adipocytes become mature adipocytes (Rosen and MacDougald, 2006). This process starts with a growth arrest triggered by contact inhibition and the expression of early adipogenic differentiation genes like PPARG and C/EBPs (Rosen and MacDougald, 2006, Villanueva et al., 2011, Rosen et al., 2014). C/EBPβ and C/EBPσ are expressed earlier than PPARG and promote its expression through promoter binding (Niemelä et al., 2007). The key early adipose transcription receptor/ factor PPARG and C/EBPβ, α promote the expression of adipogenic specific proteins whereby C/EBPα is expressed before the expression of adipogenic genes such as enzymes and
adipokines (Lowe et al., 2011, Gregoire et al., 1998) like LPL (involved in lipid accumulation), glucose transporter 4 (GLUT4) (which regulates glucose diffusion in adipocytes) (Huang and Czech, 2007) and fatty acid binding protein (FABP4) (Lowe et al., 2011, Rosen et al., 2014). In addition, morphological changes take place triggered through the decreased expression of collagen I and III and increased expression of glycosaminoglycans (Bucky and Percec, 2008) (described in section 1.2.1.4). The pre-adipocytes become rounder and accumulate triglycerides which increases in the final differentiated mature adipocytes (Gregoire et al., 1998). The lipid droplets in the developing adipocytes become larger and coalesce into one major droplet (Rosen et al., 2014) which gives the adipose tissue its yellowish colour.

1.2.1.4. Extracellular matrix (ECM)

Adipose tissue, like other tissues, contains a complex extracellular matrix which connects the cellular components. Cells adhere to ECM via cell surface receptors such as integrins (α1β1, α2β1, α3β1, α6β1, α7β1) (Lam and Longaker, 2012), discoidin domain receptors and syndecans (Heino, 2007, Harburger and Calderwood, 2009, Durbeej, 2010). In this way cells can sense mechanical cues (mechanotransduction) via cytoskeleton microtubules and actin filaments and respond (change cell shape, size, differentiation and proliferation) to the changing ECM (Lygoe et al., 2007, Meran et al., 2007, Divoux and Clément, 2011). This process is also reciprocal with cells influencing the composition of ECM and therefore changing the extracellular environment (Watt and Huck, 2013). Hence, the ECM is a highly dynamic structure, which is constantly remodelled (Frantz et al., 2010).

The general function of the ECM is acting as a physical support structure of the cellular components, regulating of the intracellular communication and the initiation of biochemical and biomechanical cues, which are necessary for tissue morphogenesis, differentiation and homeostasis (Frantz et al., 2010, Divoux and Clément, 2011). Malfunction of the ECM can lead to severe syndromes including osteogenesis imperfect and Marfan syndrome (Järveläinen et al., 2009, Frantz et al., 2010).
Each tissue has its own ECM with its unique composition that arises through the communication between cells and ECM. Through its individual make up it provides the biochemical and mechanical properties of the niche of that particular tissue (tensile and compression strength, buffering) (Frantz et al., 2010). For instance cartilage ECM has a hyaluronan and proteoglycan rich composition to facilitate absorption of pressure. Bone ECM is rich in fibrillar collagen with calcium phosphate deposition to ensure rigidity (Mariman and Wang, 2010, Votteler et al., 2010, Watt and Huck, 2013). Furthermore, the composition changes according to the physiological state of the tissue and cells. The ECM of a particular tissue can further vary depending on the location and function. An example of this is in adipose tissue, where subcutaneous tissue is softer and this supports tissue expansion due to the predominant function in triglyceride storage, and visceral fat is rich in fibril collagen to provide shock protection of inner organs (Alkhouli et al., 2013).

In the adipose tissue the cells are supported and surrounded by an ECM which contains fibrillar, network and bead forming collagen types I, III, IV, V, VI and various classes of adhesion proteins including fibronectin, laminin, elastins and proteoglycans (Kadler et al., 2007, Mariman and Wang, 2010, Divoux and Clément, 2011, Watt and Huck, 2013). Subcutaneous adipose tissue is low in fibronectin (Divoux and Clément, 2011). This ECM composition rich in fibrillar collagen makes it a soft substance which can endure high pressure and therefore cushions the cells. The adipose ECM is particularly important for the survival of adipocytes which contain a single large lipid droplet. The lipid droplet is surrounded by a sole lipid monolayer. This fragile structure might burst if not surrounded by a strong external structure such as the ECM provides (Mariman and Wang, 2010, Wronska and Kmiec, 2012). The ECM which surrounds adipocytes (the basal lamina) differs from the ECM of the fibrillar stromal vascular fraction (SVF) in terms of thickness and molecular composition. The basal lamina is especially thick and rich in collagen type IV and laminin (Bucky and Percec, 2008, Mariman and Wang, 2010, Choi et al., 2010a, Divoux and Clément, 2011, Aoyagi et al., 2012). Hence, the basal lamina and ECM proteins reduce external forces by absorption and by spreading the force over the whole tissue, hence providing structural and functional integrity to the adipose tissue (Mariman and Wang, 2010, Wronska and Kmiec, 2012).
Adult adipose tissue expands and shrinks throughout adulthood (Cao, 2010). Normal growth and expansion of adipocyte size may have no negative consequences if accompanied by a flexible ECM and a proportional increase in blood flow and oxygenation (Lee et al., 2010). The ECM is a highly active component of the adipose tissue and is secreted by its stromal vascular cells, adipocytes and ADSCs (Mariman and Wang, 2010). These cells produce the different components of the ECM and therefore determine its composition which plays an important role in the development and function of the adipose tissue (Lee et al., 2010). Adipose tissue cells also secrete enzymes which are involved in ECM remodelling (Mariman and Wang, 2010, Wronska and Kmiec, 2012). The remodelling occurs via degradation of the existing ECM and secretion of new ECM components (Divoux and Clément, 2011). There are two main degradation enzymes: fibrinolytic plasminogen and plasmin, and matrix metalloproteinases (MMP) degrade ECM and tissue inhibitors of MMPs (TIMPS) inhibit degradation of ECM (Mariman and Wang, 2010, Divoux and Clément, 2011).

The ECM is also involved in other processes such as adipogenesis and storage of triglycerides in adipocytes. Collagen is a prerequisite component for adipogenesis (Mariman and Wang, 2010). During adipogenesis the fibrillar ECM rich in collagen I and III transitions to a laminar ECM rich in laminin and collagen IV (Aratani and Kitagawa, 1988). In vitro, mesenchymal stem cells cultured on a soft surface mimicking a soft ECM environment become rounder and are more likely to differentiate towards the adipogenic lineage (Lowe et al., 2011). Nakajima et al. showed that the initiation of preadipocyte differentiation and the storage of triglyceride is partially dependent on the availability of collagen V and VI (Nakajima et al., 2002).

Additionally, the ECM provides a transport system for nutrients and waste products and is involved in tissue formation and remodelling through C-jun N terminal kinase (JNK) and Mitogen-activated protein kinase (MAPK) signalling pathways (Mariman and Wang, 2010, Divoux and Clément, 2011). In the case of obesity, the ECM interacts with hypertrophic adipocytes (increase of lipid droplet leads to increase of adipocyte size) (Mariman and Wang, 2010) and activates the JNK or MAPK

- 18 -
pathway which leads to apoptosis. Furthermore, the loss of collagen VI can result in uncontrolled adipocyte expansion (Divoux and Clément, 2011).

In addition, the ECM is involved in wound healing, regeneration and provides intercellular communication via integrins (Frantz et al., 2010, Divoux and Clément, 2011, Schultz et al., 2011). These in turn trigger a chemical cascade that includes the activation of tyrosine kinase, serine/threonine kinase and protein kinase C (Rawhow, 1994) to the adjacent cells and the cytoskeleton (Divoux and Clément, 2011). Through this cell-ECM interaction cells are primed to differentiate, proliferate or remain quiescent and assume the architecture and function of the healing tissue (Schultz et al., 2011).

1.2.1.4.1. ECM components within adipose tissue

As described in previous section 1.2.1.4, the ECM that surrounds the cells of the adipose tissue harbours growth factors, integrins, MMPs and consists of various proteins which can be divided in two main classes of macromolecules; proteoglycans and fibrous proteins (Frantz et al., 2010, Mariman and Wang, 2010, Kular et al., 2014). Proteoglycans have functions in buffering, hydration, binding and providing force-resistance. They are covalently linked with negatively charged sulphated glycosaminoglycan (GAGs) chains, which bind individual protein cores that classify them (Prydz and Dalen, 2000, Frantz et al., 2010). Proteoglycans include chondroitin sulphate, heparan sulphate GAGs and hyaluronic acid (Votteler et al., 2010). The main structural element of the ECM is made up by the fibrous proteins including collagen, laminin and fibronectin (Frantz et al., 2010, Votteler et al., 2010). These components exist in a variety of forms gained through post-transcriptional modification (Kadler et al., 2007, Aziz-Seible and Casey, 2011, Aumailley, 2013). They are important components in processes such as adipogenesis, pre-adipocyte differentiation and regeneration. Therefore, they are described in detail below.

Collagens are triple helical macromolecules and the major components of the extracellular matrix. These homo- or heterotrimeric are composed of 3 chains with gly-X-Y triplet structures, where usually glycyl residues are located at every third position and X and Y are repeatedly proline and hydroxyproline. The chains are connected
via hydrogen bonds (Kadler et al., 2007) and can assemble in fibrils and fibres, beaded filaments or networks. Within the collagen structure are integrin binding sites which provide communication points between cells and ECM (Kadler et al., 2007, Heino, 2007). They are involved in scaffolding cells, adhesion, migration, angiogenesis, tissue morphogenesis and repair. In vertebrates 28 types of collagen can be found which are grouped according their function and structure (Heino, 2007). The groups of collagen including fibrillar, bead forming and network forming collagens (Heino, 2007, Kadler et al., 2007). All are represented in the adipose tissue (Mariman and Wang, 2010, Divoux and Clément, 2011). The majority of the adipose ECM consists of fibrillar collagens. They contribute to the relaxed network matrix which can withstand a wide range of tensile stresses (Kadler et al., 2007, Frantz et al., 2010, Mariman and Wang, 2010). Adipocytes, multipotent stem cells and the stromal vascular fraction cells produce the collagens (Mariman and Wang, 2010). The beaded-filament forming collagen VI was shown to play an important role in adipocyte size restriction where it interacts with collagen IV of the basement membrane (Khan et al., 2009, Divoux and Clément, 2011). Collagen I is represented in various artificial ECM constructs mimicking the adipose ECM (von Heimburg et al., 2001). Collagens are a pre-requisite for adipogenesis. Ibrahimi et al. showed that the inhibition of collagen synthesis with ethyl-3,4-dihydroxybenzoate (EDHB) during pre-adipocyte culture prevented the expression of terminal adipogenic differentiation genes such as LPL and adipocyte protein 2 (aP2). Hence, collagen is essential for the terminal differentiation phase of adipogenesis (Ibrahimi et al., 1992). It also has been demonstrated that collagen I and III synthesis decrease during adipogenesis while collagen V and collagen IV synthesis increases (Divoux and Clément, 2011).

Laminin is known as the major component of the basal lamina that surrounds the adipocytes and consists of α, β and γ chains coiling into an α-helical central arm. The heterotrimer has a characteristic structure of globular and rod-like domains. The different laminin types are named after their chains whereby there are 5 α, 3 β and 3 γ chains in vertebrates. So far 18 different vertebrate laminin types have been described. The macromolecule can self-assemble or bind with other laminins. Laminin also contains binding sites for ECM molecules and integrins (Durbeej, 2010). In tissue engineering laminin is an important component in the creation of a natural adipose tissue matrix including Matrigel (Stillaert et al., 2010). Laminin-411
is expressed in excess during adipocyte differentiation (Chiu et al., 2011), and additionally, the heterotrimer is involved in enhancing ADSC attachment and proliferation (Lam and Longaker, 2012) and inducing adipogenesis (Aratani and Kitagawa, 1988, Divoux and Clément, 2011). During adipocyte maturation, a transition from a fibrillar to laminar form takes place resulting in a rough composition of the ECM. Hence, laminin plays an important role in maintenance and remodelling of adipose tissue (Choi et al., 2010a).

Another important component of the adipose ECM is fibronectin. This glycoprotein is an abundant soluble constituent of plasma, other body fluids and of the ECM (Pankov and Yamada, 2002, Aziz-Seible and Casey, 2011). The dimer consists of 2 identical subunits which are linked through disulphide bonds (Frantz et al., 2010). Each monomer consists of 3 types of repeat-types I, II and III. In humans there are 20 different fibronectins known which are created through alternative splicing. Two different soluble forms exist: a soluble plasma fibronectin that is found in the plasma dominantly synthesised by the hepatocytes of the liver and a less soluble cellular fibronectin. Fibronectins contain binding sites for integrins, gelatin and collagen, which makes it an optimal component for cell culture use and interaction with gelatin. Thus, fibronectin is used in combination with gelatin coated surfaces and gelatin based materials (Pankov and Yamada, 2002, Aziz-Seible and Casey, 2011). The dimer is involved in cell adhesion (Lam and Longaker, 2012) and migration during development and wound healing (Krammer et al., 2002). Fibronectin also influences the cytoskeleton and contributes to ADSC proliferation (Lam and Longaker, 2012), growth and differentiation (decreases during preadipocyte differentiation towards adipocytes) (Divoux and Clément, 2011). The role of fibronectin in adipocyte differentiation is controversial. Pre-adipocytes cultured on fibronectin showed tubular morphology and suppression of differentiation through influencing the actin cytoskeleton (Spiegelman and Ginty, 1983). In contrast, Hemmrich et al. observed enhanced differentiation when pre-adipocytes were cultured on fibronectin coated dishes in differentiation medium, compared to cells on uncoated TCP (Hemmrich et al., 2005a). Thus, the influence of fibronectin on the suppression of adipogenic differentiation is overcome through the use of differentiation medium which induces cell rounding. Therefore, fibronectin can
suppress as well as support adipogenesis which is dependent on cell spreading or cell rounding (Smas and Sul, 1995, Gregoire et al., 1998).

It is clear that the features and functions of the introduced macromolecules are involved in cell attachment, adipogenesis and pre-adipocyte differentiation. Therefore, they display important components of the non-cellular mass of the fatty tissue which should be considered when designing a construct for adipose tissue regeneration.

1.2.1.4.2. ECM is part of the stem cell niche

The ECM is part of the three dimensional (3D) environment of the stem cell, the so called stem cell niche (Figure 1.7A). This specialised local environment regulates stem cell fate through cell-niche-cell (interaction via adherence and gap junctions), soluble and immobilized factors within the niche, cell-surface and cell-ECM (via integrins) interaction (Votteler et al., 2010, Gattazzo et al., 2014). In the niche, cells and ECM are in constant interplay (Even-Ram et al., 2006, Votteler et al., 2010, Watt and Huck, 2013). The ECM provides structural integrity of the tissue. It is also involved in development and metabolic function. Additionally, the non-cellular mass plays a role in stem cell survival, migration and regulates cell morphology through ECM-cell contact via integrins (Votteler et al., 2010, Gattazzo et al., 2014).

The interaction of stem cells with the surrounding ECM molecules takes place via integrin receptors (Even-Ram et al., 2006). Integrins, the heterodimeric transmembrane receptors, connect the stem cell cytoskeleton directly with the ECM via formation of focal adhesions. The focal adhesion is in contact through focal adhesion proteins such as vinculin with the actin cytoskeleton of the stem cell (Watt and Huck, 2013). If mechanical force is applied on the ECM the force is transmitted via integrins towards the cell and its cytoskeleton which activates various signalling pathways. The process is called mechanotransduction (Votteler et al., 2010, Gattazzo et al., 2014). This interaction leads to a balance of tensional forces at the cell-matrix interface (Even-Ram et al., 2006, Trappmann et al., 2012).
Hence, via tissue stiffness stem cell fate can be decided (Gattazzo et al., 2014). Cell-ECM contact on soft surfaces via integrins forms a more flexible bond, and therefore less force is applied, which allows the cells to become rounded and differentiate towards the adipogenic lineage. In contrast, if the ECM is more rigid the cell-ECM contact is stiffer and more force is applied on the focal adhesion. Consequently, the cell shape spreads and flattens which can promote differentiation towards the osteogenic lineage (Even-Ram et al., 2006, Trappmann et al., 2012, Watt and Huck, 2013) (Figure 1.7B). Thus, this makes the ECM stiffness a controllable key component in determining stem cell fate (Even-Ram et al., 2006, Votteler et al., 2010, Trappmann et al., 2012).

Therefore through the variation of the ECM compositions, which affects the ECM stiffness, the stem cell fate can be modulated (Spiegelman and Ginty, 1983, Engler et al., 2006, Even-Ram et al., 2006, Li et al., 2008, Lam and Longaker, 2012, Huebsch et al., 2010, Trappmann et al., 2012, Young et al., 2013). Engler et al. reported the differentiation of human mesenchymal stem cells (hMSCs) towards bone, neuronal and muscle cells when cultured on different stiffness surfaces which were produced through coating polyacrylamide gels with collagen (Engler et al., 2006). Trappmann et al. showed that hMSCs cultured on soft polyacrylamide hydrogels coated with collagen differentiate into adipocytes (Trappmann et al., 2012). Furthermore, Young et al. showed ADSC differentiation towards the adipogenic lineage when ADSCs were cultured on adipose extracellular matrix at different stiffnesses. The surface which mimicked the stiffness of adipose tissue (2 kPa) combined with the native ECM composition, led to a visible rounding of ADSCs and differentiation to lipid containing adipocytes (Young et al., 2013) (Figure 1.7C).

Thus, through the modulation of the ECM composition and the use of ECM components, cell attachment and proliferation can be influenced (Spiegelman and Ginty, 1983, Li et al., 2008, Lam and Longaker, 2012). Lam and Longaker analysed the attachment and maintenance of stemness in diverse stem cell lines, such as ADSCs, embryonic stem cells and induced pluripotent stem cells. They revealed that laminin and fibronectin improved ADSC attachment and proliferation compared to tissue culture plastic (TCP). They also showed that ADSCs grown on laminin maintained their stemness (Lam and Longaker, 2012). Li et al. observed similar
results when culturing ADSCs on collagen and fibronectin where attachment and proliferation was increased (Li et al., 2008). Another study suggested that pre-adipocytes grown on fibronectin at different concentrations lead to cell spreading and inhibition of adipogenic differentiation (Spiegelman and Ginty, 1983).

All of these findings make the ECM of the adipose tissue a target for controlling the ADSC fate. Through the use of individual or combinations of ECM components an environment could be created, which maintains ADSC stemness and supports ADSC differentiation towards the adipogenic lineage.

Figure 1.7: The ECM is part of the stem cell niche and its stiffness influences stem cell fate. (A) The overview of a stem cell niche where soluble factors, stem cell-cell interaction and stem cell-ECM interaction influences stem cell fate (after Votteler et al., 2010)). (B) The ECM communicates with the stem cells via mechanotransduction where the stiffness of the matrix surrounding influences the cell cytoskeleton. A soft (low collagen content) or stiff (high collagen content) surface applies less or more force on the integrin formed focal adhesion between the cells and the ECM. This force is transmitted via the cytoskeleton to the cells resulting in morphological changes (adapted from Watt and Huck, 2013)). Hence, soft surfaces support cell rounding or spreading of stem cells. (C) Presents cell rounding and spreading of ADSCs on soft (2 kPa) and stiff (20 kPa) surfaces (modified from Young et al., 2013). Scale bar 50 µm. Green = actin cytoskeleton, blue = nucleus.
1.3. Adipose tissue engineering

Due to the unsatisfactory results of current clinical strategies for adipose tissue reconstruction there remains a clear clinical need for improved treatment options (reviewed in section 1.1). Tissue engineering provides the potential for such a treatment as described in section 1.1.2.

Tissue engineering can be divided into 3 main work platforms: the cells, the microenvironment and the scaffold (Figure 1.8).

Figure 1.8: Tissue engineering strategy can involve cells, microenvironments and scaffolds. The represented tissue engineering strategy is based on gaining donor stem cells, expanding them in vitro, combining them with the engineered scaffold and providing a suitable microenvironment. The final step would be the implantation into the recipient patient (modified from (Amranto, 2010)).

It is important that the cells, the microenvironment and the engineered scaffold can be transplanted and integrate into the patient if used clinically (Gomillion and Burg, 2006, Bauer-Kreisel et al., 2010). For adipose tissue engineering a number of suitable cell types, microenvironments and different scaffolds have been reported, with the aim of mimicking the soft, smooth feel, extracellular and cellular structure of the native tissue (Hong et al., 2005, Hong et al., 2006, Gomillion and Burg, 2006,
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Bauer-Kreisel et al., 2010, Lesman et al., 2010, Vernejulden et al., 2010, Frerich et al., 2012). All 3 aspects present their own challenges and the success of the tissue engineered constructs depends on addressing each of these.

1.3.1. Approaches in adipose tissue engineering

The diverse structure, the dense vascular system of the adipose tissue and the complex surrounding environment of its cells provide a challenge for developing an appropriate scaffold. In the last few years there have been a number of approaches in the field of adipose tissue engineering, especially, in the use of stem cells which are capable of replacing the missing tissue.

1.3.1.1. Cell source for adipose tissue engineering

The cell source for tissue engineering is essential to establish a durable, native and clinically usable reconstructed tissue. The cells have to fulfil different requirements, such as: availability to be extracted and cultured in bulk, proliferation to restore tissue volume, differentiation into cells resembling native tissue as well as compatibility with the host (Gomillion and Burg, 2006, Wong et al., 2010, Bauer-Kreisel et al., 2010).

For restoring adipose tissue defects autologous fat, adipocytes and preadipocytes have been used but each had their limitations. In autologous fat transplantation (containing ADSCs and SVF (such as adipocytes and preadipocytes)), up to 90% of adipose tissue cells, among them adipocytes, are damaged during the liposuction process and cannot fill the damaged tissue volume. The rest of the cells have a high risk of forming cysts and dying by necrosis (Patrick, 2001). The damage of cells due to isolation and the hypoxic conditions in large fat grafts results in a high resorption rate of the transplanted fat of up to 70% of the initial volume (Tabit et al., 2012). Therefore, autologous fat is not a sufficient cell source for adipose tissue reconstruction.

Mature adipocytes are the predominant cells in adipose tissue (Wronska and Kmiec, 2012) and therefore, an obvious cell source for adipose tissue engineering (Huber et al., 2015). However, they are easily damaged by harvesting methods due to their
fragile nature as they contain a large lipid droplet (Nishimura et al., 2000). Adipocytes also cannot endure hypoxic conditions which are present in larger fat transplants (Yoshimura et al., 2011). Additionally, the mature cells are differentiated and incapable of proliferation (Gomillion and Burg, 2006, Bauer-Kreisel et al., 2010). Mature adipocytes are therefore an unfavourable cell source for engineering adipose tissue replacements.

Preadipocytes can be expanded (low proliferation rate) and it is possible to induce adipogenesis through stimulation with growth factors such as FGF and hormones including insulin (Gregoire et al., 1998, Bauer-Kreisel et al., 2010). The use of these cells is limited by their slow proliferation cycle, their commitment to one lineage and they are easily damaged during the isolation process (Gregoire et al., 1998, Bauer-Kreisel et al., 2010, Choi et al., 2010a).

Thus, there is a need for cells, which can be easily isolated to gain a simple and standardised access to the cell source. They should have an increased proliferation rate to obtain a high cell number in an appropriate timeframe. In addition, the ideal cell source should be able to differentiate towards the adipogenic lineage to support and promote tissue regeneration. Furthermore, the cells should be involved in vessel formation to support vascularisation of thick areas (Illouz and Sterodimas, 2011). They should also induce signals for angiogenesis and adipogenesis to replace a whole complex tissue such as adipose tissue. Therefore, a promising source which is increasingly reported in the tissue engineering literature are ADSCs found in the SVF of the adipose tissue and first described by Zuk and co-workers (Zuk et al., 2001).

1.3.1.1. Adipose derived stem cells (ADSCs)

ADSCs are multipotent mesenchymal stem cells, found in the adipose tissue. The mesoderm derived tissue is widespread in the body and therefore ADSCs can be viewed as an abundant cell source. The cells show a fibroblast-like shape and can reach a size of up to 40 µm in diameter with an average diameter of 22 µm (Figure 1.9A) (Ryu et al., 2013). These immature cells are capable of self-renewal. They further adhere to TCP in vitro where their morphology and mesenchymal phenotype
can be retained for many passages (Konno et al., 2013, Kim and Heo, 2014). The average doubling time on TCP is rapid and reported to be in the range of 4-5 days (Guilak et al., 2006).

ADSCs can be readily obtained via liposuction and abdominoplasty. These procedures are performed under local anaesthesia and are associated with minimal discomfort for patients when compared to the painful harvest of bone marrow derived mesenchymal stem cells (BMSCs) (Konno et al., 2013, Zuk, 2013). Furthermore, the fatty tissue from lipoaspirate or abdominoplasty is a by-product of cosmetic surgeries, therefore providing an easily available source for ADSC isolation (Tabit et al., 2012, Zuk, 2013). The isolation of ADSCs is based on collagenase digestion with subsequent centrifugation (Kim and Heo, 2014). From 1 g of adipose tissue it is possible to obtain approximately 5,000 ADSCs (Zuk et al., 2002, Philips et al., 2012, Tabit et al., 2012), which are capable of adipogenesis (Figure 1.9B). They share 90% of mesenchymal stem cell markers including the surface antigens CD90 and CD29 and are negative for hematopoietic lineage makers such as surface antigens CD45. In vitro culturing changes the ADSCs’ immunophenotype where surface antigens such as CD34 are known to decrease and CD13 is increased (Zuk et al., 2002, Tapp et al., 2009, Konno et al., 2013), although the mechanisms remain unclear as well as the proceeding search for an ADSCs specific surface antigen (Zuk, 2013).

ADSCs have the capability of differentiating along various lineages of mesodermal origin such as adipogenic (predisposition (Brayfield et al., 2010)), osteogenic, chondrogenic, myogenic and cardiomyogenic lineage. They also can differentiate into ectodermal and endodermal lineages: neuronal, hepatocytes, pancreatic islet cells, endothelial cells and epithelial cells through their multipotency (Ogawa, 2006, Choi et al., 2010a, Wong et al., 2010, Bauer-Kreisel et al., 2010, Nie et al., 2011, Tabit et al., 2012, Casadei et al., 2012, Philips et al., 2012, Konno et al., 2013). Due to the differentiation towards the three germ layers, ADSCs are even starting to be seen as a possible new source for pluripotent stem cells. However, the pluripotent stem cell character of ADSCs still needs to be confirmed (Zuk, 2013).
Through their ability to differentiate into smooth muscle and endothelial cells, ADSCs support the formation of new blood vessels during angiogenesis. Additionally, they contribute to and promote neovascularisation which is advantageous for fat grafts (Philips et al., 2012).

It has previously been shown that ADSCs can be differentiated towards the adipogenic lineage in diverse tissue engineered applications, under cultivation with adipogenic differentiation medium, in adipose ECM environments or even through the exposure of surface stiffness similar to the native adipose tissue (Zuk et al., 2001, Hong et al., 2005, Bunnell et al., 2008, Li et al., 2008, Brayfield et al., 2010, Lam and Longaker, 2012, Young et al., 2013). Zuk et al. cultured ADSCs in adipogenic differentiation medium containing isobutyl-methylxanthine, dexamethasone, insulin and indomethacin for 2 weeks. Lipid accumulation was confirmed through Oil-Red-O staining (Zuk et al., 2001). Similar results were shown in the work of Bunnell et al. (Bunnell et al., 2008). Hong et al. seeding ADSCs on a gelatin sponge and culturing in vitro in adipogenic differentiation medium before transplantation promoted adipogenesis (Hong et al., 2006). Cheung et al. showed that culturing of ADSCs within decellularized ECM induces adipogenesis (Cheung et al., 2014). Further, Young et al. induced adipogenesis in ADSCs through culture on surfaces simulating adipose tissue stiffness of 2 kPa (Young et al., 2013). These studies confirm the suitability of ADSCs for adipose tissue engineering applications due their ability to differentiate towards the adipogenic lineage.

Furthermore, ADSCs have been used for wound healing studies. Kim et al. demonstrated that ADSCs locally applied on wounds can promote fibroblast proliferation and migration, are involved in the secretion of collagen I and have an effect on wound size and skin re-epithelialisation in rodent models (Kim et al., 2007, Nie et al., 2011). Additionally, ADSCs secrete collagen, fibronectin and growth factors such as VEGF, HGF and FGF and contribute in this way to new vessel formation (Rehman et al., 2004, Kilroy et al., 2007, Vallée et al., 2009).
ADSCs have also been shown to be resistant to mechanical stress and ischaemia (Bauer-Kreisel et al., 2010, Choi et al., 2010a, Yoshimura et al., 2011, Philips et al., 2012). Here, Kilroy et al. displayed the capacity of ADSCs to release angiogenic HGF, thus contributing to regeneration in ischaemia and promoting tubule formation by vascular endothelial cells independent of VEGF (Kilroy et al., 2007). The work of Rehman and co-workers also confirmed the secretion of proangiogenic factors including VEGF, FGF and TGF-β. They further demonstrated that ischaemic hind limbs of mice treated with ADSCs show a better perfusion compared to mice, which were treated with culture medium (Rehman et al., 2004). This illustrates the advantage in the use of ADSCs and their ability to promote and enhance vascularisation.

ADSCs also display low immunogenicity due to low expression of human leukocyte antigen (HLA) (human version of the major histocompatibility complex (MHC)) class I and no expression of class II antigen (Casadei et al., 2012, Philips et al., 2012, Zuk, 2013). These genes encode peptides that are located on the cell surface and responsible for self or non-self-recognition of the immune system. The later can cause transplant rejection (Ayala García et al., 2012). Hence, the low expression and non-expression of HLA class I and II antigen on the surface of ADSC may allow allogenic or even xenogenic transplantation (Zuk, 2013).

The ADSC features described here underpin their present clinical use as a stem cell source to enhance fat grafts termed cell assisted lipotransfer (Yoshimura et al., 2011).
In conclusion, ADSCs are simple to isolate from lipoaspirate and abdominopalsties, which makes them an easily available stem cell source. They have a high proliferation rate, which is advantageous in cell culture expansion and regaining lost tissue volume. Furthermore, ADSCs can differentiate into cells of the mesodermal lineages, which make them an ideal source for tissue regeneration (Ji et al., 2013, Yan et al., 2014). Through their potency (differentiation ability towards the adipogenic lineage) as well as secretome (secretion of proangiogenic factors) ADSCs are a suitable cell source for adipose tissue engineering. Their capability to induce vascularisation and adipogenesis is favourable for thicker adipose tissue constructs. Here, the in vivo survival and differentiation of cells within bulk constructs is restricted by insufficient vascularisation of centre parts (Yoshimura et al., 2011, Dong et al., 2015). The use of ADSCs is known to improve the survival of fat grafts due to increased vessel infiltration as well as adipogenesis (Yoshimura et al., 2008a, Yoshimura et al., 2008b). This confirms their advantageous features for the employment in adipose tissue reconstruction. Further, their low immunogenicity even provides the possibility of allogenic clinical use. Hence, the physical properties, their abundance, low immunogenicity and wide accessibility make ADSCs an ideal cell source for adipose tissue engineering.

1.3.1.2. Microenvironment for adipose tissue engineering

The cell source is important for engineered tissue constructs. Through proliferation and differentiation into tissue specific cells they restore tissue volume and the cellular structure of the native tissue. Here, the adipose tissues own stem cells ADSCs feature a favourable cell type for adipose tissue reconstruction, as described in the previous section. However, if stem cells are implanted without any signalling that directs their fate, they are under high risk of migrating to undesirable areas (Wang et al., 2013b). Further, if they are exposed to unwanted extracellular signals they are under danger of differentiating into undesirable cell types such of the osteogenic or chondrogenic lineages. Therefore the guidance of stem cells is very important (Yoshimura et al., 2011). As such, the ECM, which surrounds the tissue specific cells and supports the tissue itself, plays an important role in influencing and guiding external forces, determining cell shape, cell differentiation and function (Mariman and Wang, 2010).
The importance of the adipose environment, which is made up of complex mixtures of molecules (described in section 1.2.1.4), for soft tissue reconstruction has been confirmed and well described (Piasecki et al., 2008, Yoshimura et al., 2008b, Stillaert et al., 2010). Yoshimura et al. demonstrated that the delivery of lipotransfer together with ADSCs has an improved positive outcome during surgical reconstruction of adipose tissue defects than the use of lipotransfer alone (Yoshimura et al., 2008b). Stillaert et al. observed the outgrowth of proliferating cells that are able to differentiate towards the adipogenic lineage out of graft biopsies when cultured in a 3D environment mimicking the extracellular matrix (Stillaert et al., 2010). Piasecki et al. also showed the importance of the use of the right microenvironment in adipose tissue reconstruction. The group investigated two different microenvironments in combination with purified adipose cells for adipose tissue reconstruction using a mouse model. The environments analysed were Matrigel which mimics the native ECM containing laminin, collagen IV and heparin sulphate compared to PuraMatrix, a protein hydrogel with unpurified fat grafts used as control. This study revealed minimal graft volume loss, low fibrosis and high vessel density after 3 months when adipose cells were implanted in combination with Matrigel. The unpurified fat and PuraMatrix hydrogel displayed significantly increased fat graft volume loss, high fibrosis formation and low vessel density (Piasecki et al., 2008). Hence, these studies underpin the relevance of an appropriate cell source (ADSCs) in combination with the right environment provided through the lipotransfer/Matrigel together resulting in positive effects on fat survival. This knowledge can be used in the process of designing a scaffold for adipose tissue reconstruction and emphasises the influence of the environment surrounding the stem cells.

The creation of an extracellular environment to provide the native surrounding in which tissue specific cells reside, to guide stem cell fate and to support adipose tissue regeneration has, to date, revealed a multitude of approaches. These are predominantly in the area of material design of scaffolds which mimic more closely the native microenvironment and such studies are described in detail in section 1.3.1.3. Other strategies comprise approaches in the application of growth factors, hormones, mechanical forces and the decellularisation of adipose tissue.
Hormones such as insulin and dexamethasone are constituents of differentiation medium (Zuk et al., 2001), which is applied to differentiate ADSCs within scaffolds prior to implantation to induce adipogenesis, and resulting in enhanced adipose tissue formation (Mauney et al., 2007, Wang et al., 2013b). Nevertheless, the differentiation medium cannot be delivered together with the ADSCs and scaffold to the defect site. Therefore, this method clearly lacks an appropriate delivery mechanism for the differentiating ADSCs with their microenvironment into the defect. Thus, the guidance of ADSCs towards the adipogenic lineage within the scaffold and defect site should be addressed. Furthermore, through culturing ADSCs in differentiation medium, the cells are guided to differentiate and the maintenance of their stem cell character cannot be guaranteed neither in vitro nor in vivo. Consequently, the further differentiation of the cells towards other lineages or their proliferation post transplantation is not ascertained and should be investigated.

The ECM is also a reservoir of growth factors (Kular et al., 2014). Hence, growth factors have been used to support adipose tissue regeneration through enhancing angiogenesis and adipogenesis of host tissue cells or an integrated cell source (Kimura et al., 2010, Lu et al., 2014, Ting et al., 2014). Growth factors such as adipogenic stimulating insulin like growth factor (ILGF) and FGF (promotes endothelial cell proliferation), VEGF (promoting endothelial cell proliferation) and PDGF (involved in vessel stabilisation) have been applied (Kimura et al., 2010, Ting et al., 2014, Lu et al., 2014). The strategy follows the binding of growth factors within ECM environments such as Matrigel or decellularised adipose tissue via integration of heparin. Heparin binds and releases growth factors to and out of the ECM components (Lu et al., 2014, Ting et al., 2014). Another strategy is of submerging the construct in growth factor dilutions as performed by Kimura et al. (Kimura et al., 2010). Here, after binding the growth factors within the ECM matrix, the constructs are implanted either with a cell source or alone. In vivo the constructs release the growth factors via diffusion and/or degradation in the surrounding host tissue to recruit cells (Hiraoka et al., 2006, Kimura et al., 2010, Lu et al., 2014) or influence the donor cells (Ting et al., 2014). Despite supporting adipose tissue formation as well as vascularisation these approaches reveal limitations. These include the fact that the growth factors suffer from a short halflife resulting in effects only in the first few weeks post implantation in animal models. Furthermore, the
integration of growth factors into matrixes such as commercially available ECM Matrigels, Myogel or PuraMatrix is not perfected yet (Ting et al., 2014). This system needs fine-tuning of the doses delivered and the growth factor release mechanism. The long term stability of the matrices is improved but still shows significant matrix loss after only six weeks and requires further research to be applicable on larger scale tissue defects (Lu et al., 2014).

Another approach in guiding the in vitro differentiation and influencing cells in tissue engineering is through the influence of the mechanical modification of the surroundings (Placzek et al., 2009). A way to control environmental factors such as oxygen tension, mechanical forces, hydrostatic pressure and temperature, which can modulate the microenvironment in vitro, is the use of bioreactors. Bioreactors are ex vivo systems engineered to maintain viability and support maturation of a bioengineered tissue through regulated control of environmental factors (Wong et al., 2010, Meng et al., 2014). Fischbach et al. showed that dynamic cultivation of cells within scaffolds in stirring bioreactors and stirring well plates enhanced adipogenesis (Fischbach et al., 2004).

However, despite the mentioned strategies there are limited approaches in the development of a native adipose matrix which can be integrated within the scaffold design and therefore transplanted in vivo to mimic an ADSC environment; a niche that supports adipogenesis which is essential for adipose tissue formation, volume retention and tissue integration.

1.3.1.2.1. Artificial ADSC stem cell niche

In the creation of an appropriate ADSC environment the obvious recreation of the native adipose ECM is the decellularisation of adipose tissue. The cellular free residues closely resemble the native adipose tissue surrounding. Hence, the use of the resident ECM for scaffolding or priming ADSCs towards the adipogenic lineage displays an increasing trend (Young et al., 2011, Yu et al., 2013, Cheung et al., 2014, Young et al., 2014). Cheung et al. manufactured scaffolds based on chitosan with ADSCs enclosed in decellularised adipose tissue. In a rodent model the construct displayed implant integration and adipogenesis. Despite the promising
results, decellularised adipose tissue lacks a standard methodology of isolation of the ECM and it is difficult to obtain a sufficient amount of decellularized tissue to treat bulk defects. Additionally, the degradation of the decellularised ECM is fast with full volume reduction after around eight and 12 weeks reported (Poon et al., 2013, Yu et al., 2013, Cheung et al., 2014). Also the amount of exact ECM components, growth factors and composition cannot be controlled and is dependent on technique, donor site and the health of the patient (Kular et al., 2014).

To control the ECM within the scaffold, specific components can be selected and incorporated within the scaffold as achieved by Handel et al. The group selected collagen hydrogels as an internal ECM within a polypropylene scaffold. ADSCs were delivered into the porous construct within the collagen hydrogel. The investigation revealed improved ADSC attachment, cell distribution within the scaffold structure and enhanced adipogenesis after induction with adipogenic differentiation medium (Handel et al., 2012). Furthermore, the influence of individual ECM components such as laminin and fibronectin on ADSC attachment, morphology and adipogenesis was proven before in two dimensional (2D) culture experiments (Spiegelman and Ginty, 1983, Lam and Longaker, 2012, Li et al., 2008) as described in section 1.2.1.4.2.

Knowledge of the exact ECM composition and recent studies with ECM components (Spiegelman and Ginty, 1983, Li et al., 2008, Lam and Longaker, 2012) are helpful in the selection of ECM constituents that can be used for the preparation of an artificial ECM. This artificial ECM should display an ADSC stem cell niche that supports adipogenesis of ADSCs for the regeneration of the lost tissue. At the same time this external environment should preserve an ADSC population for assisting angiogenesis and adipogenic differentiation post-transplantation. Furthermore, promoting lineage commitment of ADSCs prior to transplantation would support additional secretion of ECM components by ADSCs, thereby recreating the native environment for adipose tissue regeneration.
1.3.1.3. Scaffolds for adipose tissue engineering

Scaffolds designed for tissue regeneration applications should create a physiologically relevant microenvironment giving a support structure for cell anchoring, attachment, migration, proliferation, cell-cell and cell-matrix interactions (Choi et al., 2010a) and also provide boundaries for tissue shape (Patrick, 2001, Gomillion and Burg, 2006, Wong et al., 2010, Brayfield et al., 2010). Scaffolds can be alternatively described as an engineered matrix which re-creates the surrounding physical microenvironment (Wong et al., 2010) of the tissue and has effects on cell differentiation and rate of tissue formation (Patrick, 2001). Certain properties must be fulfilled if a scaffold is to be clinically useful. These include host compatibility, appropriate degradation time which allows the tissue to regenerate, mechanical characteristics, bioactivity, long term viability and minimal donor site morbidity (Choi et al., 2010a).

In detail, scaffolds for adipose tissue reconstruction should be easily adjustable in shape and volume to restore a multitude of defects with different dimensions. At the same time, the scaffold should resemble the rigidity and stiffness of adipose tissue to restore the smooth and soft feel of the native tissue. While fulfilling the cosmetic requirements the scaffold also should support cell infiltration, attachment, proliferation and differentiation to restore the lost tissue volume with tissue specific cells (Gomillion and Burg, 2006). To achieve these objectives and accompanying its use as a support structure, which is implantable and clinically suitable, the scaffold has a diverse spectrum of design features. These features can be adjusted to create the ideal structure. Within these elements are choice of material, porosity, pore size, mechanical properties and stability, bioactivity, biodegradability, hydrophobicity, toxicity, biocompatibility and low immogenicity (Placzek et al., 2009, Polak, 2010, Novosel et al., 2011, Illouz and Sterodimas, 2011). In relation to a scaffold for adipose tissue reconstruction, the optimal design features should therefore include:

1. Enough space for cell infiltration, attachment, proliferation and differentiation. During the differentiation process from stem cell to mature adipocytes cells increase dramatically in size form a few µm up to 190 µm (Skurk et al., 2007). Hence, the scaffold should provide pores with a size range from tens to several hundreds of
micrometres to allow cell infiltration, attachment, proliferation and differentiation. These pores should also be interconnected to allow the migration of cells and the diffusion of nutrient and waste products (von Heimburg et al., 2001, Bauer-Kreisel et al., 2010, Zhong et al., 2012).

2. The degradation time should allow the cellular regeneration process which has been shown to be about 12 months in the case of fat grafts (Figure 1.3) (Yoshimura et al., 2011).

3. The appropriate scaffold material should be non-toxic, soft with stiffness similar to adipose tissue and should take various shapes to be tailored to individual patients. The manufacturing of a scaffold with those properties would provide a physical structure for the use in adipose tissue reconstruction.

After fulfilment of these factors the scaffold can be seeded with cells of choice such as ADSCs isolated from abdominoplasty. Through modification of the internal scaffold microenvironment cell proliferation, migration and differentiation can facilitate tissue formation. The final construct can then be implanted in the recipient. The ideal scaffold would then be remodelled or absorbed in an appropriate time dependent on the degree of defect (Gomillion and Burg, 2006).

1.3.1.3.1. Porosity and pore size

Porosity and pore size (microarchitecture) (Placzek et al., 2009) are important criteria in the scaffold design (Zhong et al., 2012). They should promote and enhance the blood and oxygen supply of a scaffold. The strategy of a channel network or interconnected pores, which can support the flow of nutrients and metabolites and can lead to cell aggregation, has been shown to be successful by others (Placzek et al., 2009, Chang et al., 2013). Furthermore, maximal vascular ingrowth is essential for large and thick scaffolds especially for supplying the central areas with nutrients and facilitating gas exchange (Cao et al., 2006). In addition, the microarchitecture also influences the ratio of surface area to volume which has an impact on the cell attachment, cell spreading and expansion (Placzek et al., 2009). A large surface area is necessary which provides space and area in a small, limited
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frame and gives space for cell attachment, proliferation, differentiation and matrix deposition (Cao et al., 2006, Placzek et al., 2009, Zhou et al., 2011, Zhong et al., 2012). Even the shape and size of the pore plays an important role for permeability, filtration and mechanical properties (Zhong et al., 2012). Larger pores can decrease the surface area for cell attachment and weaken the mechanical properties of the scaffold in terms of toughness and stability. Very small pores would lower the permeability of the scaffold. Furthermore, if the pores are not connected with each other, the permeability and the support of cell infiltration as well as metabolite diffusion of the scaffold would decrease (Murphy and O’Brien, 2010, Zhong et al., 2012). Thick scaffolds in particular require a sufficient filtration ability and vascular structure as the diffusion limit of nutrients is 200 µm (Bauer-Kreisel et al., 2010). In adipose tissue reconstruction scaffolds with pore sizes up to 500 µm and the use of hernia meshes consisting of monofilaments with pore sizes of 1.5 mm have been reported (von Heimburg et al., 2001, Mauney et al., 2007, Bauer-Kreisel et al., 2010, Kimura et al., 2010, Lin et al., 2011, Grover et al., 2012, Handel et al., 2012, Wang et al., 2013b, Bellas et al., 2015).

In the case of soft tissue reconstruction a highly porous scaffold, which has micropores and macropores to maintain mechanical properties and support the vascularisation and flow of nutrients and oxygen while also mimicking the natural feel of adipose tissue, would be desirable.

1.3.1.3.2. Mechanical and degradation properties

The mechanical properties and stability of the scaffold should closely mimic those of the tissue that is to be reconstructed. Adipose tissue is a soft, loose, viscoelastic tissue which endures compression. It maintains a low stiffness which facilitates free movement of underlying muscle groups (Comley and Fleck, 2010). Through its viscoelastic properties, adipose tissue displays features of viscose substances that endure high stress and elastic materials that stretch and return to their original from. Thus, adipose tissue presents a non-linear stress strain curve when increased strain (up to 30%) is applied (Alkhouli et al., 2013). Different depots of human adipose tissue display different elastic moduli related to their function and location. The elastic modulus for breast tissue has been recorded between 3-24 kPa (Samani and
Plewes, 2004), for heel pad samples (protection of underlying bone) the moduli range is 22-175 kPa (Gefen et al., 2001), subcutaneous adipose tissue (main lipid storage site- needs to be expandable) showed values between 1.6-11.7 kPa and omental fat pads (belongs to the visceral fat and has a function in shock absorbance to protect underlying organs) range is 2.9-32 kPa (Alkhouli et al., 2013). Several research groups designing mechanical properties of materials (initial Young’s modulus of 2-4 kPa) similar to adipose tissue showed adipogenesis, angiogenesis and tissue integration of the scaffold in vivo (Yu et al., 2013, Young et al., 2013, Chang et al., 2013).

The engineered scaffold should be stable enough for transplantation into the host and biodegrade after a defined time. Post-transplantation of the cellularised engineered construct, there should be the possibility for the stem cells and differentiated cells to reconstruct the damaged tissue volume and to integrate with the host tissue. During this process the scaffold should degrade and leave space for the newly generated tissue. Thus, for the purpose of adipose tissue reconstruction, a scaffold should degrade slowly over a period of approximately 12 months (Yoshimura et al., 2011) while the cells can rebuild the lost volume and tissue.

1.3.1.3.3. Materials in use for scaffolds for adipose tissue engineering

The material used has a significant influence on the scaffold properties. It influences the stiffness, stability, biocompatibility and biodegradability of the scaffold. The material also provides an environment for cells to attach, to proliferate, to migrate and to differentiate (Bauer-Kreisel et al., 2010).

In the field of adipose tissue engineering, a range of scaffold materials have been reported in the literature. These include materials produced from both synthetic polymers like poly(lactic acid) (PLA) (Mauney et al., 2007), poly(ethylene glycol) (PEG) (Stosich et al., 2009, Moioli et al., 2010, Oliviero et al., 2012), poly(L-lactic-co-glycolic) PLLA (Gugerell et al., 2015), poly(lactic-co-glycolic) acid (PLGA) (Patrick et al., 2002, Cao et al., 2006) and natural polymers including gelatin (Hong et al., 2005, Hemmrich and von Heimburg, 2006, Gomillion and Burg, 2006, Lin et al., 2008, Placzek et al., 2009, Chiu et al., 2011) (Table 1.2). These scaffolds have
been produced in a number of physical forms including polymer disks, sponges and injectable microspheres (Figure 1.10) (Gomillion and Burg, 2006, Hong et al., 2006, Lin et al., 2008, Placzek et al., 2009, Chiu et al., 2011).

The synthetic polymer PLA has been shown to support adipogenesis in vitro and in vivo, therefore assisted adipose tissue regeneration. However, lipid accumulation was decreased when compared to other scaffolds based on natural materials such as collagen type I and silk. Further, PLA scaffolds display a fast degradation rate within 4 weeks in vivo which is undesirable when reconstructing larger defects (Mauney et al., 2007). PLLA scaffolds support adipogenesis when cultured in adipogenic differentiation medium in vitro but do not assist cell migration within the construct and dissolve in a short period of time (Gugerell et al., 2015). PLLA scaffolds would not therefore support host and donor cell migration into the construct and this is essential for the scaffold integration, vascularisation and adipose tissue regeneration within the native tissue. PLGA constructs are frequently used synthetic materials in the tissue engineering community as well as in adipose tissue reconstruction. Scaffolds based on PLGA display support of adipogenesis and tissue formation in vivo but reveal disadvantages in their fast degradation rate. In vivo studies demonstrated significant degradation (up to 90% of their original volume) within six weeks (Patrick et al., 2002, Cao et al., 2006, Oliviero et al., 2012, Wang et al., 2013b). In contrast, constructs based on PEG have a slow degradation rate. Nonetheless, they lack in support of cell migration and only assist in vivo adipogenesis if the cells used are pre-differentiated in adipogenic differentiation medium (Stosich et al., 2009, Moioli et al., 2010).

In conclusion, the main disadvantage in the use of these polyesters lies in their fast in vivo degradation rate and lack of supportive cell migration. A material with a slow degradation rate which provides sufficient time (about 12-18 month) for tissue to regenerate is favourable while providing cellular tissue regeneration (Yoshimura et al., 2011). Further, cell migration for tissue integration, vascularisation and adipose tissue formation is desirable. However, these synthetic biomaterials display a weak bioactivity (Placzek et al., 2009) and are therefore unfavourable material characteristics for the use in adipose tissue reconstruction.
1.3.1.3.4. Natural Biomaterials

In contrast to synthetic biomaterials, constructs based on natural biomaterials present inherent bioactivity and display favourable biocompatibility, mechanical and biological properties which mimic the *in vivo* environment (Placzek *et al.*, 2009). These desirable characteristics are reflected in the increasing application of natural derived materials for adipose tissue engineering. Widely used materials include chitosan, silk, fibrin, decellularised tissue along with adipose tissue, hyaluronic acid, collagen and gelatin (Table 1.2).

Chitosan is a natural polymer frequently used in the adipose tissue engineering area. The polymer structure is similar to GAGs that are contained in the natural ECM (Fan *et al.*, 2015). It is used as hydrogel or blend material for natural or synthetic materials to improve their bioactivity such as cell proliferation, attachment and differentiation towards the adipogenic lineage *in vitro* and *in vivo* (Mao *et al.*, 2003, Cheung *et al.*, 2014, Debnath *et al.*, 2015, Jaikumar *et al.*, 2015, Fan *et al.*, 2015). However, chitosan is not soluble in physiological solvents such as water and
degrades fast, therefore the material requires cross linking for stabilisation (Debnath et al., 2015). Here, alginate blends with chitosan derivatives displayed a fast degradation rate within 5 weeks in phosphate buffered saline (PBS) (Jaikumar et al., 2015). Fan et al. also confirmed the fast degradation related to chitosan when exposing composite chitosan hydrogels to degradation in PBS and enzymes. A severe volume decrease within 6 weeks was noted (Fan et al., 2015). Furthermore, the poor support of cell attachment is improved when another biomaterial such as fibrin is integrated (Jaikumar et al., 2015). The fast degradation rate related to chitosan and low cell attachment make this biomaterial less applicable for adipose tissue reconstruction which aims to restore contour defects over time through increasing cell mass. Thereby, the scaffold needs to provide a surface for cell attachment.

Silk from Bombyx mori silkworm cocoons is clinically used as suture material and is approved by the Food and Drug Administration (FDA) and has also been applied in the adipose tissue engineering area. This biomaterial has a slow degradation rate and low immunogenicity (Choi et al., 2010a). It was utilised in in vitro as well as in long term (18 months) in vivo studies (Bellas et al., 2013a, Bellas et al., 2013b), where it was demonstrated to support vasculature ingrowth and adipose tissue formation. However, in vitro experiments reveal the reduced cell attachment on silk, which was improved through laminin coating (Bellas et al., 2013a). Those in vitro experiments also showed a decrease of adipogenic gene expression, lipid and glycerol accumulation when culturing pre-differentiated ADSCs in porous silk sponges over a time course of 6 months (Bellas et al., 2013a). In vivo experiments that presented vascular ingrowth and adipose tissue formation were only performed on small and thin (2 mm thick and 5 mm diameter) silk scaffolds (Bellas et al., 2013b, Bellas et al., 2015), therefore neglecting the regeneration of larger contour defects. Another disadvantage of the use of silk in adipose tissue engineering is the rigid and robust nature of the material having elastic moduli above the values of adipose tissue (Bellas et al., 2015). It could be argued, therefore, that silk would not restore the soft and smooth feel of the lost adipose tissue. The stiffness also hinders the cocultivation with endothelial cells. Endothelial cells seeded in silk sponges to support vessel formation were shown to decrease in cell number during in vitro cultivation related to the stiff surface provided by the scaffold (Bellas et al., 2013a). Due to the
rigid nature and long term stability, silk is frequently used in other tissue engineering application such as bone and cartilage replacements (Yan et al., 2014).

Fibrin is another natural FDA approved polymer and is clinically used as a sealant and present naturally in blood clots. It can be isolated from donor blood and is biocompatible and as such is being investigated for potential use in adipose tissue reconstruction (Verseijden et al., 2012, Korurer et al., 2014, Chung et al., 2015). Fibrin hydrogels displayed support of vessel ingrowth as well as adipogenesis in vitro and in vivo (Aoyagi et al., 2012, Verseijden et al., 2012, Korurer et al., 2014, Chung et al., 2015). Nevertheless, the utilisation of fibrin is limited by its rapid degradation rate within days and weeks through fibrolysis and weak mechanical properties compared to collagen (Korurer et al., 2014, Chung et al., 2015). Aoyagi et al. showed that the shrinkage of fibrin within a few weeks is accompanied by a decrease in cell number (Aoyagi et al., 2012). Chung et al. revealed the severe degradation and collapse of fibrin gels cultured with ADSCs within 7 days. Furthermore, the group displayed the low storage modulus (low elasticity) of fibrin when compared to collagen scaffolds (Chung et al., 2015). In addition, the support of adipogenesis of ADSCs encapsulated in fibrin gel is lower when compared to gelatin based scaffolds (Korurer et al., 2014). Hence, the rapid degradation rate resulting in fast shape loss of the biomaterial make it less desirable when preparing a scaffold for adipose tissue engineering which aims to provide cosmetic improvement and a slow degradation rate to guide tissue regeneration.

To further resemble the native tissue environment, natural materials based on decellularised tissue such as placental matrix, Matrigel, Myogel or adipose tissue matrix are investigated as well. Decellularized placental matrix has been utilised in adipose tissue engineering and has been shown to enhance ADSC proliferation while displaying poor support of adipogenesis (Flynn et al., 2008). This biomaterial further undergoes a long and extensive decellularization time and its availability is restricted (Flynn et al., 2006). Matrigel, an extract from spontaneously occurring mouse sarcoma, including growth factors such as FGF 2 and VEGF was shown to induce neovascularisation and adipose tissue formation in rodents (Kawaguchi et al., 1998, Piasecki et al., 2008, Ting et al., 2014). However, the use of Matrigel is associated with concerns of tumour formation and adipogenesis has not been shown without
supplementing with growth factors. An alternative extract from skeletal muscle, Myogel with no described tumour risk, has also been revealed to support adipogenic differentiation and vascularisation when accompanied with fat grafts and growth factors in vivo (Ting et al., 2014). Nevertheless, Myogels showed reduced fat tissue formation and accumulation of cysts when compared to commercially available collagen gels and fat grafts (Ting et al., 2014). This material also has not been tested and characterised for use in clinical applications and therefore, its safety and suitability for this application remains unknown.

To create an environment closely resembling the native non-cellular mass of adipose tissue the obvious choice is the decellularisation of the fatty tissue. This process separates the non-cellular content mainly comprised of collagen I from cellular components and lipids. Hence, scaffolds based on decellularised adipose tissue (DAT), in which self-assembling to a gel is closely related to the collagen content and thus is pH and temperature dependent, have been increasingly investigated for its potential in adipose tissue reconstruction (Yu et al., 2013, Young et al., 2013, Poon et al., 2013, Wang et al., 2013a, Kochhar et al., 2014, Cheung et al., 2014, Lu et al., 2014, Sano et al., 2014, Young et al., 2014). DATs have mechanical properties similar to adipose tissue (Yu et al., 2013, Kochhar et al., 2014), are inducing and supporting adipogenesis in vitro and in vivo, are biocompatible and undergo tissue integration (Poon et al., 2013, Wang et al., 2013a). Yu et al. manufactured scaffolds measuring 10 x 10 x 5 mm from DAT in the form of foams and scaffolds produced as foam beads to gain microporosity and macroporosity. These scaffolds had mechanical properties similar to human adipose tissue. They illustrated adipogenesis and developing angiogenesis in vitro and in an in vivo rat model (Yu et al., 2013). Young et al. illustrated adipogenesis using thin extracellular matrix gels combined with ADSCs (Young et al., 2013). Despite the encouraging results connected with the use of DAT there are drawbacks in its utilisation. Through different decellularisation methods alternating protein contents are retained (Poon et al., 2013, Wang et al., 2013a, Sano et al., 2014). Poon et al. developed throughout their study a decellularisation protocol starting from the use of dispase alone then moving on to the dual use of dispase and pepsin. Comparative western blot analysis revealed varying protein contents (Poon et al., 2013). Sano et al. also displayed the quantity and size limitation of the decellularisation process with DAT comprising
30%-40% of the initial used adipose tissue volume in which the removal of cellular components was restricted to adipose tissue weight of 0.8 g (Sano et al., 2014). Furthermore, the degradation rate of DAT is fast, being within 6-8 weeks (Wang et al., 2013a, Lu et al., 2014). In vivo studies also revealed that DATs support of blood vessel and adipose tissue formation is decreased when compared directly with fat grafts (Wang et al., 2013a). The later drawbacks are currently addressed through augmenting DAT with growth factors (Lu et al., 2014) and cross linking (Young et al., 2014) for support of vessel formation and structure stabilisation. So far those approaches require optimisation of the growth factor release system and induction of adipogenesis (Lu et al., 2014, Young et al., 2014). Finally, through decellularisation of the adipose tissue the composition of the components cannot be controlled and are dependent on the individual patient as well as the donor site, health and age of the participant (Tottey et al., 2011, Poon et al., 2013). In conclusion, the size limiting decellularisation process and the fast degradation rate resulting in a severe lack of DAT studies with bulk scaffolds and long term in vivo evaluation, restricts its use in adipose tissue reconstruction.

In contrast, the use of defined, individual ECM components allows a more controlled approach to engineer a scaffold in adipose tissue engineering rather than whole matrices with unknown quantity and quality of components. Hence, hyaluronic acid (HA), collagen and its derivate gelatin are biomaterials being investigated in the adipose tissue engineering area. HA is a polymer disaccharide composed of glucosamine and glucuronic acid, belongs to the family of GAGs and is FDA approved (Fan et al., 2015). According to its natural origin HA based scaffolds display cell viability, proliferation, biocompatibility and also support adipogenesis (Flynn et al., 2008, Sarkanen et al., 2012, Korurer et al., 2014, Fan et al., 2015). However, they show a fast degradation rate. Fan et al. tested composite hydrogels containing chitosan and HA and revealed in vitro degradation within 5 weeks (Fan et al., 2015). To counteract the fast degradation HA can be cross linked. This can result in mechanical properties dissimilar to native tissue. Furthermore, the cross linking of the biomaterial can decreased cell number, cell viability and glucose consumption in comparison to decellularised placental matrix (Flynn et al., 2008, Kochhar et al., 2014). Additionally, in vivo acellular HA implantation did not support adipose tissue formation (Sarkanen et al., 2012). Hence, HAs disadvantages in degradation rate and
lack of adipose tissue formation makes it a less attractive material for adipose tissue reconstruction.

Collagen is widely used in clinical applications mainly as material in wound repair, abdominal wall and breast reconstruction (Werner et al., 2014). The structural protein has been shown to support adipogenesis, increase cell viability and lipid accumulation in vitro as well as in vivo (Lequeux et al., 2012, Frerich et al., 2012, Dinescu et al., 2013, Werner et al., 2014, Chung et al., 2015). The development of a vascularised system was reported with collagen gels in in vivo studies (Frerich et al., 2012, Lequeux et al., 2012). The use of collagen is advantageous due to its occurrence in native ECM. However, the disadvantage in the use of the biomaterial lies in its fast shape loss due to the short degradation time which can be prolonged through cross linking (Werner et al., 2014). Werner et al. demonstrated shape and volume loss of commercial collagen constructs within 14 days in vitro culture when compared to cross linked collagen (Werner et al., 2014). Similar degradation was observed in in vivo mouse and porcine studies were collagen particles significantly degraded over the course of 1 and 4 months respectively (Frerich et al., 2012, Lequeux et al., 2012).

The collagen derivate gelatin is FDA approved and a widely used naturally derived material in tissue engineering (Dainiak et al., 2010). For adipose tissue reconstruction it displays promise due to its soft natural feel, biocompatibility, non-toxicity, gel formation, and is simple to shape and inexpensive (Bigi et al., 2001, Lin et al., 2008, Yao et al., 2012, Grover et al., 2012, Kirsebom et al., 2013, Chang et al., 2013). The application of gelatin hydrogels mimic more closely the living tissue in relation to high water content, softness, flexible structure and low interfacial tension where water or biological fluids have been used (Yao et al., 2012). Indeed, the incorporation of gelatin with other biomaterials increased swelling behaviour as observed by Yao et al. The group combined gelatin with alginate to small microspheres (500 µm). These composite materials showed increased swelling behaviour, ADSC proliferation and adipogenesis resulting from improved cell attachment facilitated through gelatin when compared to microspheres containing only alginate (Yao et al., 2012). Therefore, the collagen derivate also supports adipogenesis, vascularisation and ADSC attachment, proliferation and migration.
(Lin et al., 2008, Korurer et al., 2014). Lin et al. used commercial gelatin sponges and demonstrated SVF attachment, adipogenesis and cell proliferation over 28 days conducted in in vitro studies (Lin et al., 2008). Korurer et al. described the fabrication of small (9.2 mm diameter) composite hydrogels consisting of HA, gelatin and fibrin. In 3 weeks in vitro studies with ADSCs cell attachment, viability and adipogenesis was increased when compared to pure fibrin hydrogels (Korurer et al., 2014). Furthermore, gelatin has already been applied in diverse in vivo studies and can be chemically modified for control of mechanical properties and degradation rate (Bigi et al., 2001, Hong et al., 2006, Lin et al., 2008, Kirsebom et al., 2013, Imani et al., 2013, Korurer et al., 2014). Hong et al. utilised small (3 x 3 x 3 mm) gelatin sponges seeded with ADSCs and demonstrated in vivo adipogenesis and vessel infiltration (Hong et al., 2006). Kimura et al. applied minced collagen I hydrogels and gelatin microspheres containing FGF in vivo on rabbits. They displayed increased adipose tissue formation and vascularisation when compared to collagen sponges (Kimura et al., 2010). This makes gelatin a promising material for soft tissue reconstruction. A few gelatin scaffolds have been developed for soft tissue reconstruction but also show limitations in size and are discussed in the following section.

1.3.2. Limitations of current engineered adipose tissue scaffolds

The scaffolds applied to reconstruct adipose tissue encompass injectable gels, liquids which gel within body temperature or pH, microspheres or solid pre-shaped scaffolds (Kimura et al., 2010, Chang et al., 2013, Young et al., 2014). They have been analysed in vitro as well as in vivo; predominantly in rodent models but also in porcine, rabbit or sheep animal models (Halberstadt et al., 2002, Kimura et al., 2010, Lequeux et al., 2012, Fan et al., 2015, Bellas et al., 2015). To date, those applications have not been shown to replace thick area defects.

Injectable gels were utilised with volumes ranging from a few µl up to a few ml (Hemmrich et al., 2008, Kimura et al., 2010, Sarkanen et al., 2012, Young et al., 2014). Sarkanen et al. injected rats with 100 µl of HA gels combined with adipose tissue extracts. Over a time period of 9 months they demonstrated adipose tissue formation (Sarkanen et al., 2012). Young et al. prepared cross linked DATs and
Table 1.2: Commonly applied synthetic and natural materials for adipose tissue reconstruction.

<table>
<thead>
<tr>
<th>Material</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synthetic materials</strong></td>
<td></td>
</tr>
<tr>
<td>poly(lactic acid) (PLA),</td>
<td>(Mauney et al., 2007)</td>
</tr>
<tr>
<td>poly(ethylene glycol) (PEG)</td>
<td>(Stosich et al., 2009, Moioli et al., 2010, Oliviero et al., 2012)</td>
</tr>
<tr>
<td>poly(L-lactic-co-glycolic) PLLA</td>
<td>(Gugerell et al., 2015)</td>
</tr>
<tr>
<td>poly(lactic-co-glycolic) acid (PLGA)</td>
<td>(Patrick et al., 2002, Cao et al., 2006, Mauney et al., 2007)</td>
</tr>
<tr>
<td><strong>Natural materials</strong></td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td>(Debnath et al., 2015, Jaikumar et al., 2015, Fan et al., 2015)</td>
</tr>
<tr>
<td>Silk</td>
<td>(Bellas et al., 2013a, Bellas et al., 2013b, Bellas et al., 2015)</td>
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<tr>
<td>Fibrin</td>
<td>(Verseijden et al., 2012, Aoyagi et al., 2012, Korurer et al., 2014, Chung et al., 2015)</td>
</tr>
<tr>
<td>Decellularized placenta</td>
<td>(Flynn et al., 2006, Flynn et al., 2008)</td>
</tr>
<tr>
<td>Matrigel</td>
<td>(Kawaguchi et al., 1998, Piasecki et al., 2008, Ting et al., 2014)</td>
</tr>
<tr>
<td>Myogel</td>
<td>(Ting et al., 2014)</td>
</tr>
<tr>
<td>Decellularised adipose tissue (DAT)</td>
<td>(Wang et al., 2013a, Yu et al., 2013, Kochhar et al., 2014, Lu et al., 2014, Young et al., 2014)</td>
</tr>
<tr>
<td>Hyaluronic acid (HA)</td>
<td>(Hemmrich et al., 2008, Sarkanen et al., 2012, Korurer et al., 2014)</td>
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<tr>
<td>Collagen</td>
<td>(Lequeux et al., 2012, Frerich et al., 2012, Dinescu et al., 2013, Werner et al., 2014)</td>
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<td>Gelatin</td>
<td>(Hong et al., 2006, Lin et al., 2008, Kimura et al., 2010, Lin et al., 2011, Zhou et al., 2011, Yao et al., 2012, Chang et al., 2013, Korurer et al., 2014)</td>
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injected 150-250 µl combined with ADSCs subcutaneously in mice. After 4 weeks blood vessel formation was seen but unsatisfying adipogenesis was noted (Young et al., 2014). Hemmrich et al. prepared small injectable hyaluronic acid based gels and injected 1 ml into porcine ears. The positive impact on adipose tissue formation was limited by an inflammatory host response (Hemmrich et al., 2008). Kimura et al. manufactured larger injectable scaffolds. They used a polypropylene cage 20 mm in diameter and 10 mm in thickness which was inserted in rabbits to confine the defect
size. To augment the created defect, they injected minced collagen I scaffolds (pore size 60-100 µm) mixed with gelatin microspheres containing FGF. After 4 weeks and multiple administration of the gel mix they could observe successful adipose tissue formation (Kimura et al., 2010). Taking into consideration the size of the animal, the augmented defect does not mimic large soft tissue loss as observed after traumatic injuries, where larger areas are lost. Furthermore, the application through multiple administrations does not differ from current surgical methods of liposculpture where small volumes are injected over a period of time to reconstruct small defects such as wrinkles (described in section 1.1.1). Overall, the augmentation of larger defects above 10 mm thickness through scaffold injection has not been proven yet.

In contrast, larger defect reconstruction has been attempted through the implantation of pre-shaped scaffolds. Sizes up to 25 cm² have been reported (Lequeux et al., 2012). Despite the larger area those scaffolds cover they are thin which makes their application for the replacement of bulk adipose tissue defects difficult (Hong et al., 2006, Moioli et al., 2010, Lin et al., 2011, Handel et al., 2012, Lequeux et al., 2012, Chang et al., 2013, Debels et al., 2015a). Chang et al. used gelatin/HA cryogel sheets 10 mm in diameter and 5 mm in depth with pores ranging from 100-400 µm seeded with ADSCs. The cell seeded construct was cultured for 3 weeks in adipogenic differentiation media prior to implantation in mouse and porcine models for 8 weeks. The in vivo studies demonstrated adipose tissue formation and some vessel infiltration. Therefore, it is clear that the vascularisation and adipogenesis of thicker scaffolds is missing in this study (Chang et al., 2013). Debels et al. manufactured DATs from porcine adipose tissue termed Adipogels. The square (1 cm x 1 cm) Adipogel was 0.6 cm thick. After 8 weeks in rats they noted adipose tissue and vasculature formation. Despite these encouraging results the scaffolds illustrated a fast resorption rate, partially due to degradation as well as fibrous tissue formation (Debels et al., 2015a). Moioli et al. manufactured a hybrid scaffold consisting of an inner poly(ethylene)glycol-diacylate (PEG-DA) acellular core covered with a thin cellular alginate sheet measuring a total size of 40 mm. In the rat model the thin alginate sheet displayed adipose tissue formation as well as vascularisation whereby severe degradation of alginate was noted. Nevertheless, the acellular core did not support adipose tissue formation or vascularisation which
leaves this approach unsuccessful in replacing large contour defects (Moioli et al., 2010). Lin et al. also applied scaffolds large in area but small in depth. The scaffolds used were collagen sponges with a size of 7 x 4 mm (10 - 300 µm pores) and gelatin sponges with 100 - 300 µm pores size and measurements of 3 x 10 x 10 mm. In in vitro studies they noted adipogenesis in both sponge types (Lin et al., 2011). Handel et al. utilised polypropylene hernia meshes with pores of 1.5 mm and nonwoven polypropylene scaffolds covering an area of 0.5 cm² in in vitro studies. Despite successful adipogenesis in vitro those scaffolds were not tested in vivo and lack in bulkiness (Handel et al., 2012). Lin and co-workers constructed a scaffold with a size of 0.5 x 1.0 x 1.5 cm which consisted of a polypropylene pocket filled with gelatin sponges and polyglycolic acid (PA) meshes. These were seeded with ADSCs and observed after 2, 4 and 6 months of transplantation in nude mice. The results showed that the ADSCs attached to all 3 materials used without any differences in attachment and proliferation. The authors also revealed vessel formation, adipocyte differentiation and resorption of gelatin and PA (Lin et al., 2008). In addition, the scaffold characterisation was missing in terms of cell compatibility and porosity. Furthermore, the use of collagen for long term implantation is unfavourable due to its short degradation time. Hong et al. applied a commercial gelatin sponge (3 x 3 x 3 mm) with a pore size of 200 - 400 µm seeded with ADSCs. They confirmed adipogenesis in vivo after 4 weeks of transplantation in a mouse model (Hong et al., 2006). Lequeux et al. utilised thin but large collagen I sheets covering an area of 25 cm². Removal from a porcine model after 12 weeks displayed matrix deposition and some vascularisation of the scaffold. However, the adipose tissue construction was unsuccessful due to fibrous tissue accumulation. Furthermore, scaffolds described for rodent models had measurements up to 4 cm (Moioli et al., 2010), therefore the applied scaffold of Lequeux et al. represents relative small measurements for the use in a porcine model (Lequeux et al., 2012).

These scaffolds are so far not suitable for bulk soft tissue reconstruction due to the lack of full characterisation of the material properties. No further results regarding vascularisation and adipogenesis in scaffolds with measurements above a depth of 1 cm (Kimura et al., 2010) were reported. Despite the thinness of pre-shaped scaffolds, they tended to cover larger areas when compared to injectable materials. The vascularisation while scaling up the size and dimensions of these scaffolds is still a
key challenge (Novosel et al., 2011). Therefore, current options are restricted to a few centimetres with regards to depth and dimension and therefore, are not suitable for larger contour defects.

1.4. Requirements for an advanced scaffold for adipose tissue reconstruction

The need for re-bulking adipose tissue defects, the limitations of present methods and the lack of current approaches make the requirement for a scaffold for adipose tissue engineering clear. This project focuses on bulk scaffolds made from gelatin and alginate. Both of these materials are natural polymers, widely used in medical applications, commonly available and are acquirable for low cost (Bigi et al., 2001, d'Ayala et al., 2008).

Gelatin is created through the hydrolysis of collagen. Through hydrolysis, the typical collagen triple helical structure is broken down leaving single linear polymer chains behind (Bigi et al., 2001, Singh et al., 2002, Farris et al., 2010). The polymer chains consist of positive and negative charged amino acids. Amino acids predominantly found in gelatin are lysine, glycine, proline and hydroxyproline (Singh et al., 2002, Li et al., 2005, Farris et al., 2010). Those amino acids form triple helical chains and in this way partially recover the initial collagen structure resulting in a gel when cooled (Bigi et al., 2001, Li et al., 2005, Farris et al., 2010). Gelatin is a superior material when compared to collagen due its absence of antigenicity in physiological conditions and lower cost (Bigi et al., 2001). However, like the majority of natural biomaterials, gelatin also demonstrates a fast degradation rate within weeks and poor mechanical properties (Bigi et al., 2001, Farris et al., 2010). Those can be improved through cross linking. A widely utilised, low cost effective cross linker is glutaraldehyde (Bigi et al., 2001, Dainiak et al., 2010, Imani et al., 2013). The cross linking mechanism is based on the linkage of the gelatin unprotonated amino groups of lysine and hydroxylysine with the aldehyde groups of the cross linker (Figure 1.11) (Bigi et al., 2001, Farris et al., 2010). Despite the extensive use of the aldehyde as cross linker there are concerns of toxicity. It is postulated that the release of non-reacted aldehydes or biodegradation may cause toxic effects (Imani et al., 2013). To
exclude those concerns it has been suggested that the aldehyde be applied in low concentrations (Bigi et al., 2001). Furthermore, the success of a variety of clinical applications using glutaraldehyde as cross linking agent contradicts those concerns (Jayakrishnan and Jameela, 1996, Chao and Torchiana, 2003, Imani et al., 2013). Nevertheless, gelatin is extensively used in the food and drug industry and in clinical applications such as vascular prosthesis, wound dressings or adhesive and absorbent pads as well as by the tissue engineering community (Bigi et al., 2001, Singh et al., 2002, Hemmrich and von Heimburg, 2006, Hong et al., 2006, Bauer-Kreisel et al., 2010). In adipose tissue engineering gelatin was shown to support vessel infiltration, adipogenesis in vivo and retains the produced shape after culturing with ADSCs (Hong et al., 2006, Lin et al., 2008, Grover et al., 2012).

Figure 1.11: Gelatin cross linking through glutaraldehyde. Gelatin is cross linked by nucleophilic addition reaction between the carboxyl (CHO) group of the aldehyde with the amino group (NH₂) of the gelatin amino acid lysine or hydroxylysine. The resulting covalent binding links gelatin polypeptide chains together (Farris et al., 2010). Figure is adapted from (Imani et al., 2013).

Additionally, gelatin has been displayed to be suitable for restoring small tissue defects (Hong et al., 2006). However, although this material has been represented to be applicable for adipose tissue engineering (Hong et al., 2006, Lin et al., 2008, Zhou et al., 2011), to date there is no evidence of large gelatin scaffolds for this purpose.

Alginate is a natural polymer present in the intracellular matrix of algae and typically extracted from brown seaweed. During the extraction process it is precipitated through sodium chloride to sodium alginate which is commercially available (Lee and Mooney, 2012). The polysaccharide is composed of 1,4’-linked β-D-1, 4’-linked
β mannuronic acid (M) and α-L-guluronic acid (G) residues organised in blocks of MM, GG or alternating GM (Figure 1.12). The G and M block content is source dependent (Wee and Gombotz, 1998, Lee and Mooney, 2012). This heteropolysaccharide and its hydrogels represent low immunogenicity and controllable biodegradability while the water soluble alginate chains being excreted by the kidney (Galateanu et al., 2012). Alginate therefore finds wide use as cell transplantation (used for ADSCs) vehicles for tissue reconstruction development and wound dressings (d'Ayala et al., 2008, Bidarra et al., 2014). Alginate gelation occurs according to the egg-box model. The most common divalent cation, which is used for alginate gelling is Ca$^{2+}$ (Lee and Mooney, 2012, Pawar and Edgar, 2012). The cation binds only at the G-blocks of alginate, they fit like eggs in a box (G-blocks) referred to as the egg-box model of cross linking. This creates a gel through linking G blocks of alginate chains together (d'Ayala et al., 2008, Lee and Mooney, 2012) (Figure 1.12). The cross link can be easily broken when exposed to an ionic exchanger with monovalent cations (sodium citrate) due to the higher affinity of Ca$^{2+}$ to the exchanger than the monovalent cation (d'Ayala et al., 2008, Lee and Mooney, 2012). In the tissue engineering area, alginate gelation and its reversibility has been used to prepare microspheres and manufacture porous scaffold structures (Tomei et al., 2009, Delaney et al., 2010, Phull et al., 2013, Calcagnile et al., 2014).

![Figure 1.12: Sodium alginate structure and gel formation.](image-url)

(A, C) Alginate is a block copolymer composed of 1,4'-linked β-D-1,4'-linked mannuronic acid (M) and α-L-gluluronic acid (G) residues organised in blocks of MM, GG or alternating GM blocks. (B, D) In the presence of divalent cations such as Ca$^{2+}$ gelling occurs according to the egg box model. The G-blocks providing the box were Ca$^{2+}$ ions fit as an egg to link G-blocks of alginate chains together to form a network structure resulting in gel formation (Lee and Mooney, 2012). Figure adapted from (Kashima and Imai, 2012).
Delaney et al. studied the application of two different systems of inkjet printing for the creation of porous scaffolds for tissue engineering applications. For the porous material preparation alginate beads were applied as a particulate leaching agent (Delaney et al., 2010). Calcagnile et al. utilised alginate beads to functionalise foams, enclosing particles within the alginate beads. The beads were then surrounded by a poly- (dimethylsiloxane) matrix. After reversing the alginate gelation the particles within the beads were freed and interacted with the pore surface (Calcagnile et al., 2014). Tomei et al. used the reversible gel behavior of alginate to create macroporous polyurethane structures in the application of a newly developed dynamic strain device for mechanobiological analysis of soft matrices (Tomei et al., 2009).

Our own group at the Blond McIndoe Research Foundation utilised sodium alginate beads as a sacrificing element to create a microporous macroporous gelatin sponge for application as a bulk scaffold in adipose tissue engineering. Specifically, the scaffold was prepared by particulate leaching of sodium alginate beads out of a solvent gelatin matrix leaving pores with the size of 2.08 ± 0.08 mm behind. These pores were termed macropores due to their large size. Furthermore, the resulting scaffold also represented pores within the gelatin walls separating the macropores. These pores are an artefact of the utilised cross linked 7% gelatin matrix and were classified as micropores. The microporous macroporous scaffold falls into the range of larger constructs with 2.75 mm in diameter and 0.73 mm in height. In vitro studies with ADSCs displayed cell proliferation and adipogenesis when directly seeding the cells or delivered within a fibrin matrix into the gelatin sponge and cultured in growth or adipogenic differentiation media (Phull et al., 2013). Despite the encouraging results of developing a clinically suitable bulk scaffold based on natural biomaterials for adipose tissue reconstruction the construct revealed significant limitations. The sponge preparation was time consuming and uncontrolled. For application in clinical settings the scaffold preparation should be efficient and reproducible. Furthermore, the macropore size was shown to be adjustable through the alginate beads neglecting the control of the micropores. The control over the micropore size would give an additional design element to construct a scaffold that can be modulated according to the demand of the used cells. The construct also illustrated insufficient characterisation such as porosity and swelling behaviour.
These features deliver information about the application in *in vitro* and *in vivo* settings. Furthermore, the delivery matrix used (fibrin) does not mimic the native adipose tissue surrounding and therefore needs significant development to produce a novel, effective matrix to allow cell proliferation and differentiation.

1.4.1. Aims

This project aimed to develop a range of novel scaffolds based on the method of particulate alginate bead leaching to produce a construct in an efficient and standardised way for clinical use. This included the creation of a scaffold internal microenvironment closely resembling the native adipose surrounding that supports adipogenesis, resulting in an advanced scaffold that underwent sufficient *in vitro* testing for further application in *in vivo* models. Hence, the specific project aims are listed below.

1. The development of an advanced scaffold based on the alginate bead leaching method to support and promote soft tissue reconstruction.

The manufacture of a microporous macroporous scaffold based on particulate leaching of alginate beads out of a solvent gelatin matrix (Phull *et al.*, 2013) was developed into a scaffold prepared through a standardised and efficient method where the features important for soft tissue engineering such as porosity and interconnectivity can be controlled and are able to be modified dependent on cellular demands. For this purpose, a selection of different scaffold types were created and physically characterised. The physical characterisation included pore size measurement, porosity, interconnectivity and liquid uptake. Out of the diverse scaffolds the types that showed the most suitable profile for cell based studies were selected. In the following cell-based study the selected scaffolds were further tested and characterised biologically with ADSCs for their support of cell seeding, infiltration, viability, proliferation and differentiation. From there the most promising scaffolds were chosen for further development of an artificial stem cell niche and a product appropriate for adipose tissue augmentation (aim 2 and 3).
2. To develop an artificial ADSC stem cell niche that can be utilised as a delivery matrix as well as support of adipogenic differentiation.

To fulfil this aim, a selection of ECM components was tested for support of ADSC differentiation. This study revealed components that were able to preserve ADSC stemness. At the same time components that influence ADSC differentiation towards the adipogenic lineage were explored. A combination of those components was picked to create a 3D environment functioning as cell delivery matrix for cellularisation of the scaffold as well as support of adipogenesis. This matrix mimicked the adipose surrounding, hence a niche harbouring ADSC and supporting their differentiation towards the adipogenic lineage. The chosen formula was tested with the selected scaffolds for support of adipogenesis (aim 3).

3. The combination of the selected scaffolds with the artificial ADSC stem cell niche to gain a product which displays a physical and biological profile suitable for adipose tissue reconstruction.

The third aim was reached through combining the selected scaffold (aim 1) with the designed ADSC stem cell niche (aim 2) to analyse the suitability for ADSC culture and support of adipogenic differentiation. The final construct displayed a cellularised scaffold which is further advanced, physically and biologically characterised and suitable for \textit{in vivo} adipose tissue reconstruction.

The original contribution to knowledge of this PhD emanated not only from the development of an advanced tissue engineering construct, presenting a novel scaffold, but also from the greater understanding of the mechanisms underlying adipose stem cell biology as applied to cell-material interactions.
Chapter 2: Scaffold preparation and characterisation

2.1. Introduction

Scaffolds are described as the shape and support element of a tissue engineered construct (Hemmrich and von Heimburg, 2006, Lawrence and Madihally, 2008). The earliest documentation of scaffold use is outlined in an ancient Indian text dating back 2600 years. The document reports nose replacement through the application of a hollow plant stem for shape and respiration support on which a skin flap taken from the forehead was sutured (Chakravorty, 1971). More recently, the development of porous scaffolds has followed the advances in the field of tissue engineering that was established in 1993 by Langer and Vacanti (Langer and Vacanti, 1993).

The scaffold used in tissue engineered constructs is a design element which can be shaped and modulated in a controlled and reproducible manner to replace the lost tissue component (Stella et al., 2010, Bellas et al., 2015). The general physical requirements for a successful adipose tissue scaffold are: 1. It should give stability and provide a morphology similar to the tissue that it is aimed to restore until replaced by mature adipose tissue and further degrade without inflammation (Hemmrich and von Heimburg, 2006, Ikada, 2006). 2. It should provide space for cell penetration, attachment, proliferation, differentiation and ECM deposition (Hollister et al., 2005, Hemmrich and von Heimburg, 2006, Ikada, 2006, Weigel et al., 2006, Stella et al., 2010). At the same time nutrient and waste diffusion has to be maintained as well as allowing vascular ingrowth. These features should be sustained by the scaffold while mechanical strength is maintained (Hemmrich and von Heimburg, 2006, Ikada, 2006, Weigel et al., 2006, Lawrence and Madihally, 2008).

Due to the functional requirements of the scaffold, it ideally consists of an interconnected porous network, which is one of the major design elements in
Chapter 2: Scaffold preparation and characterisation

scaffold development (Hollister et al., 2005, Hemmrich and von Heimburg, 2006, Ikada, 2006, Weigel et al., 2006, Annabi et al., 2010). A number of methods have been reported in the literature to create porous scaffolds including electrospinning (Courtney et al., 2006) and 3D printing (Pati et al., 2014) over gas foaming (Nazarov et al., 2004) and different freezing techniques with subsequent freeze drying (von Heimburg et al., 2001, Mao et al., 2003, Yu et al., 2013) to solvent casting (Cao et al., 2006). Particulate leaching, whereby particulate materials of appropriate size are added to the scaffold during some stage of the production and removed via an appropriate solvent to produce higher levels of porosity, has been widely reported as well (Weigel et al., 2006, Sobajo et al., 2008, Stella et al., 2010, Annabi et al., 2010, Zhong et al., 2012, Bellas et al., 2015).

In adipose tissue engineering the predominant cell type of the native tissue are adipocytes which are characterised by their ability to facilitate intracellular lipid storage and can reach a size up to 190 μm in diameter (Skurk et al., 2007). Here, porous constructs with pore diameter sizes up to 40 μm were shown to restrict the maturation of lipid accumulating adipocytes (von Heimburg et al., 2001). Therefore, porous constructs should offer pore diameters of at least 200 μm. Hemmrich et al. constructed hyaluronan benzyl ester based scaffolds with pore sizes ranging up to 400 μm and showed enhanced cell penetration and vessel formation (Hemmrich et al., 2005b). Bellas et al. analysed silk foams with pore sizes ranging from 300 μm up to 500 μm over 90 days in rats. During this study they noted tissue as well as vessel infiltration into the constructs (Bellas et al., 2015). With increased pore size, higher initial vessel density was produced as well (Druecke et al., 2004). Thus, high porosity scaffolds with larger pores sizes are favourable in adipose tissue reconstruction.

Recently our own group developed a microporous macroporous scaffold based on gelatin and alginate. Gelatin is biodegradable, biocompatible and can be purchased at a low cost (Farris et al., 2010). It is used in tissue engineering and clinically as plasma expander, wound dressing material and in adhesive and absorbent pads (Bigi et al., 2001, Hong et al., 2005, Huss et al., 2010). Gelatin hydrogels usually have poor mechanical properties and are water sensitive. Chemical cross linking is a widely used technique to improve those features (Farris et al., 2010).
Glutaraldehyde, a well-known collagen cross linker with low cost and high efficiency is commonly applied to chemically cross link gelatin. Glutaraldehyde is in clinical use and acceptable when applied in low concentration (Khor, 1997, Farris et al., 2010). Another popular biomaterial used by the tissue engineering community is the polysaccharide alginate (Rowley et al., 1999, Delaney et al., 2010, Bidarra et al., 2014). Alginate is an isolation product of algae and consists of mannuronic and guluronic acid. It forms a gel when diffused by divalent cations such as Ca$^{2+}$ (d’Ayala et al., 2008, Pawar and Edgar, 2012). The cross linking is achieved according the egg box model where the Ca$^{2+}$ links negative guluronic acid alginate chains together. This cross link can easily be broken by the addition of an ionic exchanger with higher affinity to Ca$^{2+}$ than alignate (Lee and Mooney, 2012). A commonly used example is trisodium citrate (Tomei et al., 2009).

Thus, our own group combined the reversible gelation behaviour of alginate into a solvent casting particulate leaching process to generate a porous gelatin sponge. Alginate beads were applied as a solid component in a gelatin matrix. After setting of the gelatin, the beads were dissolved through exposure of the construct to trisodium citrate (Figure 2.1). The resultant was a gelatin sponge consisting of alginate voids, termed macropores and micropores found within the surrounding gelatin walls itself (Phull et al., 2013). Despite promising results in in vitro studies, the possible clinical application was limited through the preparation of the porous scaffold structure. Thus, the process was uncontrolled in the alginate bead removal and took approximately 2 weeks. Further, the size of the macropores could be controlled through the dimensions of the sodium alginate beads. However, the micropore dimensions of the gelatin wall was depending on the biomaterial itself and accordingly, lacked a mechanism of control.

2.1.1 Aim of the chapter

Therefore, the aim of this chapter was to develop a novel version of a porous construct for adipose tissue reconstruction with optimised scaffold preparation, to create a mechanism of control over the microporous structure and to characterise the developed constructs.
Chapter 2: Scaffold preparation and characterisation

Alginate beads mixed with gelatin

Application of trisodium citrate

Dissolution of alginate beads

Figure 2.1: Initial scaffold preparation. The initial scaffold preparation consisted of 4 main steps: 1. Preparation of alginate beads (0.5% w/v sodium alginate cross linked in 0.5 % w/v CaCl₂), 2. alginate bead mixing with cross linked gelatin, 3. application of trisodium citrate (3% w/v) and 4. the dissolution of the alginate beads. (1) Alginate beads were prepared through cross linking alginate with CaCl₂, which linked the alginate chains together to form a gel. (2) The alginate beads were used as a template around which gelatin was cast that was cross linked with glutaraldehyde. (3) After the alginate bead gelatin mix set, the structure was transferred into trisodium citrate solution. (4) Trisodium citrate breaks the Ca²⁺ cross link of the alginate beads. The liquefied alginate solution leached from the gelatin matrix and left voids behind that created the macroporous structure of the scaffold. Structural formulas are modified from (Information, 2005, Kashima and Imai, 2012).
2.2. Materials and Methods

2.2.1 Materials

The plastic and consumables were purchased, if not stated otherwise, from Greiner Bio-One Ltd., UK.

2.2.2 Optimisation of the scaffold preparation

For the microporous macroporous scaffold preparation, gelatin was cast around cross linked alginate beads. The scaffolds were subsequently frozen and lyophilized to produce a range of scaffold materials using the following methods. The initial scaffold preparation illustrated in Figure 2.1 was based on casting macropores within a gelatin matrix by particulate leaching of alginate beads (Phull et al., 2013). The leaching process took over 2 weeks. The optimisation of the scaffold manufacture aimed to shorten this time consuming step to create an improved and efficient scaffold preparation technique.

2.2.2.1 Alginate bead preparation

A 0.5% w/v alginate acid sodium salt (alginate) in PBS (Sigma Aldrich, UK) solution was coloured with black food dye based on vegetable carbon (Silver spoon, UK) to provide contrast. The solution was dripped through a glass pipette caped with a 21 gauge needle into the cross linking solution (1.5% w/v CaCl₂ (Sigma Aldrich, UK)) to form calcium alginate beads (Figure 2.2). After a 10 minute incubation in the cross linker, the beads were washed twice in sterile water (H₂O). The manufactured alginate beads were stored in sterile H₂O till further use.
2.2.2 Optimising of alginate cross linker concentration/ time and dissolution solution concentration

To optimise the alginate dissolution from the gelatin matrix, the alginate bead preparation (see Figure 2.2) was adjusted. This included the investigation of different CaCl₂ cross linking concentrations, cross linking times and concentrations of trisodium citrate solutions (Sigma Aldrich, UK). Alginate beads with a weight of 0.5 g were exposed to different cross linking times (5 minutes, 10 minutes, 20 minutes, 30 minutes, 40 minutes), concentrations (0.5% (45 mM), 1% (90 mM), 1.5% (135 mM), 2% (180 mM), 2.5% (225 mM)) or trisodium citrate solution concentrations (1% (34 mM), 3% (100 mM), 5% (170 mM), 7% (240 mM), 10% (340 mM), 13% (442 mM)). The measurement of the dissolution time was taken by recording of the time starting from immersing the beads in 20 ml trisodium citrate solution until complete dissolution of the alginate beads. After breaking the cross link formed between Ca²⁺ ions and the G-blocks (explained in detail in Chapter 1, Figure 1.12A, B) of alginate chains, the solution was filtered through a 100 μm cell strainer to ascertain that the alginate beads were fully dissolved. The measured dissolution time
was compared to the dissolution time recorded from the application of the initial reagent concentration (0.5% CaCl₂, 3% trisodium citrate (Phull et al., 2013)).

### 2.2.2.3 Optimisation of the alginate dissolution technique

The initial technique of piercing the scaffold with a needle and immersing in the dissolution solution (Phull et al., 2013) that was changed every 2-3 days, was compared with two different techniques. (1) The non-pierced scaffold was immersed in 200 ml 10% (w/v) trisodium citrate solution (Sigma Aldrich, UK) (Tomei et al., 2009). (2) The 10% trisodium citrate solution was directly injected into the scaffold, to precisely target the alginate beads. After one day, the absorption of the dissolution solution was measured at 699 nm for vegetable carbon with the Sunrise™ Plate reader (Tecan Ltd., UK). The efficiency of the alginate beads leaching was calculated according to the alginate bead dissolution time standard curve (please see a detailed description of the calculation below).

#### 2.2.2.3.1 Quantification of dissolved alginate

To obtain a standard curve alginate beads were coloured with black food dye (Silver spoon, UK). Different numbers (50, 100, 200, 300) of alginate beads were dissolved, in 200 ml 10% (w/v) trisodium citrate solution and the absorption was measured at 699 nm for vegetable carbon with the Sunrise™ plate reader (Tecan Ltd., UK). The obtained standard curve was used to quantify alginate dissolution out of the scaffold.

### 2.2.3 Scaffold preparation

To prepare a microporous macroporous scaffold the method was optimised (as detailed in section 2.2.2) and carried out as follows. Alginate (0.5% alginate acid sodium salt (w/v in PBS) beads were prepared (section 2.2.2.1) and loosely packed (1 g) in a mould of choice (diameter 15.7 mm, height 16.5 mm; well of 24 well plate). Then glutaraldehyde (25% (w/v in H₂O) (Sigma Aldrich, UK) was mixed with 7% (w/v) warm gelatin Type B (Sigma Aldrich (Fluka), UK) in PBS in a ratio of 71: 1 (gelatin: glutaraldehyde). Immediately after the addition of the cross linker, 1.5 ml of the solution was mixed with the alginate beads. The mixture was left to cross link for 1h at room temperature. Followed by three washing steps with sterile H₂O to remove
residual cross linker and stopping the reaction with subsequent removal of the H$_2$O out of the device via filter paper. Next, the alginate beads were dissolved through direct injection of 10% w/v trisodium citrate solution with a needle into the scaffold. Macropores were formed through the removal of the beads. The micropore structure is given through the structure of gelatin by cross linking 7% w/v gelatin with glutardaldehyde. The prepared microporous macroporous scaffold was washed again with sterile H$_2$O to remove residual alginate and trisodium citrate. The final construct was sterilised through irradiation with the gammacell 1000 biological irradiator (Best Theratronics Ltd., Canada) for 16 h. After sterilization the scaffold was stored in sterile H$_2$O at 4°C until further use.

2.2.4 Construction and characterisation of different scaffold variations using ice as porogen

Different scaffolds were prepared using ice as porogen (Zhong et al., 2012). The obtained scaffolds were characterised according their morphology, micropore and macropore size, liquid uptake, porosity and interconnectivity.

2.2.4.1 Construction of different scaffold variations using ice as porogen

Freeze drying, or lyophilisation, is a process where materials are dried in a vacuum environment during which the frozen water sublimes directly into the gas form. Therefore, freeze drying removes ice out of the construct, leaving pores behind as well as dries and preserves the material (Christ, 2009).

Scaffolds were prepared as described in section 2.2.3. The scaffolds were either (1) not treated or frozen at (2) -20°C or (3) -80°C overnight prior to freeze drying or (4) directly freeze dried using a Christ Alpha 1-2 LD$plus$ Freeze-dryer (Martin Christ GmbH, Germany) (Zhu and Chen, 2013) with condenser temperature of -55°C.

2.2.4.2 Morphology Analysis

The dimension of the scaffold was measured after preparation. To do this a ruler was used to record the diameter and height in cm.
The analysis of the scaffold pores and structure were achieved with Scanning electron microscopy (SEM) (*Carl Zeiss Ltd., UK*) and fluorescent microscopy applying an Axio scope A1 (*Carl Zeiss Ltd., UK*) using the auto fluorescence of gelatin or light microscopy with a Nikon Eclipse TS100 (*Nikon, UK*).

### 2.2.4.2.1 Analysis of Surface and Topography Structure - Scanning Electron Microscopy (SEM)

SEM is used for analysis of surface topography and structure of materials at high magnification. This type of microscopy scans the surface of a material point by point with a single electron beam created by an electron gun. The gun consists of a heated filament which creates electrons that are attracted by an anode. This electron beam is filtered through lenses which forms a single electron beam. When this single beam hits the material/sample the electrons are decelerated into secondary electrons, backscattered electrons, diffracted backscattered electrons, photons, heat and light. The secondary electrons are used for the SEM imaging of surfaces and topography (*Bhardwaj and Gupta, 2013*).

The gelatin scaffolds were prepared as described in section 2.2.4.1, dried (*Alpha Christ Freeze dryer, Carl Zeiss, Germany*), cut at a centre, top and bottom part of the scaffold and mounted on sticky carbon pads adhered to aluminium sample stubs followed by coating of a 6-8 μm layer of palladium.

### 2.2.4.3 Micropore and macropore size analysis

The macropores and micropores of the different scaffolds were analysed using histological analysis.

#### 2.2.4.3.1 Histological analysis

The microporous macroporous scaffolds were submerged in OCT Embedding Matrix solution (*Thermo Scientific, UK*) and left for at least 30 minutes to absorb the mixture to ensure even freezing throughout the scaffolds. The submerged scaffolds were frozen over liquid nitrogen and stored at -20˚C until further use.
2.2.4.3.1 Haematoxylin and Eosin (H + E) staining

Sections (30 µm) of the scaffolds were cut using a Cryostat (Bright Instrument Co Ltd., UK). The obtained sections were left for 30 minutes to dry on a drying platform. To analyse and quantify the porous structure of the scaffold, H + E staining was performed.

Haematoxylin is a basic dye that contains the dye hematein in combination with aluminium ions (Al\(^{3+}\)). During the staining the aluminium salt binds to the negatively charged components and haematoxylin to the salt. Usually the method is used to colour cell nuclei but it also stains other negatively charged components including the polyampholyte gelatin (Farris et al., 2010). Eosin is a pink acidic dye and stains positively charged/ basic structures red or pink like extra cellular matrix component including the collagen derivate gelatin. Gelatin consists of positive as well as negatively charged amino acids. Hence, the staining of gelatin either with haematoxylin or eosin can be carried out and is pH dependent. To stain the entire gelatin structure of the scaffold both dyes were applied.

Haematoxylin was double filtered through Whatman filter paper (Ø 270mm, Whatman Ltd., UK) and the slides (Fisher Scientific Ltd., UK) with the sections were submerged for 30–60 seconds. Followed by dipping the slides in tap water for 15 minutes (progressive stain) or acid wash (0.3 % hydrochloric acid (HCl) in 70% industrial methylated spirit (IMS) (VWR International Ltd., UK). After the removal of non specific binding, the pH of the sections was neutralised by dipping them in tap water. Eosin counter stain followed by immersing the slides for 4 minutes in eosin (Sigma Aldrich, UK) (1% aqueous solution). The eosin stained sections were dehydrated by increasing concentrations of alcohol (70%, 100%). Finally, the sections were washed for 4 and 6 minutes with xylene to remove the alcohol and to allow xylene suffusion for clear imaging of the sectioned material. After drying, the sections were mounted in DPX mounting solution (BDH\(^{®}\) Laboratory supplies, UK).
2.2.4.3.2 Macropore characterisation

The alginate beads used as template for the macroporous structure of the scaffolds were characterised using the Nikon SMZ800 Steromicroscope (Nikon, UK). Therefore, pictures were taken from 10 individual beads. Three diameters of the beads were measured to define the bead dimensions applying the Axivison 4.8 release software (Zeiss Ltd., UK).

The diameter of the macropores in the scaffold created by the leaching of alginate beads was characterised using a Nikon Eclipse TS200 Microscope (Nikon, UK) with Axiovision 4.8 release program (Zeiss Ltd., UK).

Specifically, the quantification of the macropore size was carried out on 30 µm sections stained with H + E. The sections were taken from the centre area of each scaffold type as detailed above (2.2.4.3.1.1). In the stained part of the scaffold the diameter of the macropore was measured using Axiovision release 4.8. Images were taken of at least 10 random macropores from each of the 4 scaffolds with Nikon Eclipse TS200 Microscope (Nikon, UK) at magnification 2x.

2.2.4.3.3 Micropore characterisation

The micropore size was analysed using 30 µm thick H + E stained sections. Scaffolds were sectioned in 4 areas, 0.1 mm apart. From each area 4 random images were taken using an Axio scope A1 (Zeiss Ltd., UK) with magnification 20x. The micropore measurement was carried out for each section on 10 pores whereby area and diameter was recorded using Axiovision release 4.8 (Zeiss Ltd., UK).

Furthermore, the reproducibility of the micropore structure was analysed using H + E stained sections (30 µm) taken from the centre of the different scaffolds. In total 3 scaffolds of each type were analysed. The investigation was as described above.

2.2.4.4 Scaffold liquid uptake, porosity and interconnectivity

The quantification of the liquid uptake, porosity and interconnectivity of the scaffolds were analysed by swelling the constructs as well as applying the liquid
replacement method (Hsu et al., 1997, Grimm and Williams, 1997, Nazarov et al., 2004, Kasoju et al., 2009).

2.2.4.4.1 Liquid uptake

To measure the uptake of liquid into the scaffold ($V_L$), it was immersed into a Falcon containing 5 ml H$_2$O ($V_1$). After 5, 10, 20 and 30 minutes the scaffold was removed and the remaining liquid was recorded ($V_2$). The liquid uptake was calculated as followed.

$$V_L = V_1 - V_2 \quad \text{(Equation 1)}$$

Where: $V_1$ start volume, $V_2$= volume of H$_2$O – scaffold, $V_L$= volume uptake

2.2.4.4.2 Liquid replacement method

To measure the porosity, hence the porosity that is accessible for the cells, the liquid replacement method with H$_2$O as replacement liquid was utilised (Hsu et al., 1997, Nazarov et al., 2004, Kasoju et al., 2009). Water was chosen because it easily penetrates the scaffold and does not dissolve the biomaterial. Hence, a 10 ml syringe was filled with 5 ml H$_2$O. Three constructs of each type were investigated. Each scaffold was added to 5 ml H$_2$O and left 10 minutes to swell and then removed from the syringe. The volume changes were recorded and the porosity was calculated as followed:

Porosity (%) = $\left(\frac{V_1-V_3}{V_2-V_3}\right) \times 100 \quad \text{(Equation 2)}$

Where: $V_1$= start Volume; $V_2$= volume H$_2$O + Scaffold; $V_3$= volume water-scaffold

2.2.4.4.3 Interconnectivity

The interconnectivity of the scaffolds was measured by the ability of liquid passing through the porous environment of the scaffold. For measurement of the interconnectivity all 4 scaffolds were prepared as described in 2.2.4.1.

Three scaffolds of each type were covered in cling film around the perimeter to create a tube that tightly enclosed the biomaterial to ensure the liquid flowed through
the construct. The enclosed scaffold was loaded into a 5 ml syringe to give stability to the tube formation. The syringe with the scaffold was fixed with a stand over a measuring cylinder. Water with an initial volume of 5 ml was placed in the top of the syringe above and in contact with the scaffold. The time was taken of the first \( (T_1) \) and last \( (T_2) \) drop of water leaving the syringe and scaffold for calculation of the diffusion time \( (T_D) \) (Grimm and Williams, 1997).

\[
T_D = T_1 - T_2 \tag{Equation 4}
\]

\( T_D \) = time of scaffold holding liquid, \( T_1 \) = time of first drop appearance; \( T_2 \) = time of last drop appearance;

### 2.2.5 Statistical Analysis

All experiments were performed at least 3 times to ensure repeatability. Significance was calculated for \( p \leq 0.05 \).

To identify the optimal conditions for the scaffold preparation the two sample unequal variance student’s T-test was used to compare the initial to tested conditions.

To characterise the macropores and their template the alginate beads, the single factor analysis of variance (ANOVA) with the post hoc one tailored, two sample unequal variance students T-test was applied to investigate differences in size.

To evaluate the liquid uptake, porosity and diffusion ability of the different scaffolds the single factor ANOVA with the post hoc one tailored, two sample unequal variance students T-test was applied as well.

All calculations were performed using Microsoft office Excel 2007 (Microsoft, UK).

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2.3. Results

The aim (described in section 1.4.1) of the scaffold study was the development of an microporous macroporous scaffold based on an pre-designed alginate bead leaching method (Phull et al., 2013) to produce a characterised construct suitable for cell seeding, proliferation and differentiation accordingly for soft tissue reconstruction. The scaffold was designed to have micropores and macropores. The microporous structure of the scaffold was created by the gelatin itself. The macropores were produced through the dissolution of alginate beads within the cast gelatin structure.

The first goal was to develop a relatively rapid, reproducible and standardised method for the preparation of the microporous macroporous materials to move forward into the cell based study.

Further, previous materials used to create a soft tissue scaffold (Phull et al. 2013) included no mechanism to control the microporosity. Hence, with the advancement of the scaffold’s microporosity, a mechanism of dimensional control was required.

The scaffolds developed required characterisation of micropore and macropore size, also of porosity created through the microporosity and macroporosity, as well as liquid uptake and diffusion ability. This would allow detailed information to be collected about the potential for nutrient and cell infiltration, waste removal and the ability of the scaffold to allow cell and liquid uptake. Furthermore, this characterisation of the different scaffolds would yield information about the variations between scaffolds which showed a suitable physical profile for a scaffold intended for adipose tissue reconstruction.

2.3.1 Optimisation of the scaffold preparation

The preparation technique of the microporous macroporous scaffold described by Phull et al. (Phull et al., 2013) took approximately 2 weeks of processing. The most time consuming step was the manufacture of macropores through the dissolution of alginate beads out of the gelatin matrix (Figure 2.1). The initial method was based on piercing the scaffold with a needle and immersing it in the dissolution solution.
Chapter 2: Scaffold preparation and characterisation

(trisodium citrate) (Phull et al., 2013) which slowly infiltrated the scaffold (obtained through stirring the scaffold-solution mix). Trisodium citrate breaks the alginate crosslink through $\text{Ca}^{2+}$ ionic exchange reaction. When the cross link was broken, the bead structure was dissolved and the alginate was able to drop out of the gelatin matrix, leaving pores behind with the size of the former beads, identified in this thesis by the term macropores. Consequently, the process was dependent on the slow penetration of gelatin by the dissolution solution. The applied process was not manually controlled and did not directly target the alginate beads. Consequently, this leaching method took over 2 weeks and was inefficient in the removal of the beads. To gain a more controllable and efficient way of dissolving the alginate beads in an appropriate time frame, the scaffold production technique was optimised by adjusting the reagents used and the alginate dissolution technique itself.

2.3.1.1 Optimising of alginate cross linker concentration/ time and dissolution solution concentration

To obtain an efficient technique of dissolving the alginate beads out of the gelatin scaffold to gain a macroporous structure, the alginate bead formation was analysed first (Figure 2.3A).

The effect of the cross linker ($\text{CaCl}_2$) concentration on the dissolution time of the alginate beads was tested first to ensure even cross linking of the alginate beads resulting in homogenous bead morphology. Therefore, 0.5 g of alginate beads were immersed in $\text{CaCl}_2$ varying concentrations (0.5%-2.5%). The dissolution time and bead formation was noted (detailed in section 2.2.2.2) and compared to beads cross linked in the initial used $\text{CaCl}_2$ concentration of 0.5%.

It was demonstrated that the use of different cross linking concentrations (0.5%, 1%, 1.5%, 2.0% and 2.5% w/v $\text{CaCl}_2$) had a significant influence on the dissolution of alginate beads when cross linked in 1.5% (p= 0.007), 2 % (p= 0.011) and 2.5% (p= 0.003) $\text{CaCl}_2$ (Figure 2.3A). Compared to the initial applied concentration the dissolution time increased to 4.26 minutes ($\pm$ 0.01 minutes), 4.40 minutes ($\pm$ 0.08 minutes) and 4.24 minutes ($\pm$ 0.05 minutes) under named conditions. In the initial concentration of 0.5% alginate beads dissolved within 3 minutes ($\pm$ 0.14 minutes).
At a concentration of 1% the alginate cross link was broken in a slightly longer time of 3.8 minutes when compared to the initial concentration.

However, a difference in the bead formation was observed (Table 2.1). A rapid and uniform formation of the beads with 1.5% - 2.5% \( \text{w/v} \) \( \text{CaCl}_2 \) concentration was achieved. Visually, the beads all appeared to be the same size and they formed immediately after dropping into \( \text{CaCl}_2 \). Adherent beads that clumped together were obtained using 0.5% and 1% \( \text{CaCl}_2 \). Therefore, for further studies, despite the significant increase of the dissolution time of 1 minute the alginate bead formation was carried out with 1.5% (\(^{\text{w/v}}\)) \( \text{CaCl}_2 \) as cross linker.

**Table 2.1: Effect of different cross linking concentrations on alginate bead morphology**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Note/ Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50%</td>
<td>Formation slow, accumulating beads, uniform spherical shape</td>
</tr>
<tr>
<td>1.00%</td>
<td>Accumulating beads, uniform spherical shape</td>
</tr>
<tr>
<td>1.50%</td>
<td>Formation fast, not sticky, uniform spherical shape</td>
</tr>
<tr>
<td>2.00%</td>
<td>Formation fast, not sticky, uniform spherical shape</td>
</tr>
<tr>
<td>2.50%</td>
<td>Formation fast, uniform spherical shape</td>
</tr>
</tbody>
</table>

After analysing the effect of the cross linker concentration on alginate bead formation and dissolution, the influence of the cross linking time on the alginate bead dissolution was tested. To gain the most adequate use of the cross linking time, alginate beads were cross linked in 1.5% \( \text{CaCl}_2 \) for different time intervals (5 minutes, 10 minutes, 20 minutes, 30 minutes, 40 minutes). Beads (0.5 g) were dissolved in 3% trisodium citrate and the dissolution time was recorded and compared to the initial cross link time of 30 minutes (described in paragraph 2.2.2.2).

The tested cross linking times did not show any significant differences in the time taken for dissolution of the alginate beads with the dissolution time being between 3-4 minutes in each sample (Figure 2.3B). Therefore, the bead production was continued using 1.5% (\(^{\text{w/v}}\)) \( \text{CaCl}_2 \) as the cross linking concentration and minimised cross linking time of 10 minutes to ensure an even cross link of the alginate beads.
after the last bead was dropped into the cross linking solution (outlined in section 2.2.2.1).

Finally, the dissolution solution was tested at different concentrations of trisodium citrate and its effect on the dissolution time of the beads was measured. This ensured the most efficient use of the reagent in terms of time. In detail, 0.5 g of alginate beads were dissolved in trisodium citrate solution with different concentrations of 1%, 3%, 5%, 7%, 10%, 13% \(^{\text{w/v}}\) and compared to the original concentration of 3% (complete description see section 2.2.2.2).

Higher concentrations of trisodium citrate resulted in greater cation exchange of Na\(^+\) against the Ca\(^{2+}\) ions from the cross link due to the higher affinity of Ca\(^{2+}\) to citrate then Na\(^+\) (Bienaimé \textit{et al.}, 2003, d'Ayala \textit{et al.}, 2008, Lee and Mooney, 2012) (Figure 2.3C). The 0.5 g alginate beads (0.5% \(^{\text{w/v}}\) alginate, cross linked in 1.5% CaCl\(_2\)) were dissolved significantly faster ≤1 minutes in 7%, 10% and 13% \(^{\text{w/v}}\) (p= 0.003, p= 0.0001, p= 0.0001) trisodium citrate. The longest dissolution time was observed in 1% \(^{\text{w/v}}\) trisodium citrate solution within 11 minutes. The greater the trisodium citrate concentration the faster was the dissolution of beads. The scaffold production process with 10% \(^{\text{w/v}}\) trisodium citrate solution was selected for further studies due to the faster dissolution time of alginate beads, when compared to the initial applied concentration of 3%. Further, no significant increase of the reverse gelation time with the highest tested dissolution concentration of 13% \(^{\text{w/v}}\) was observed.

Overall, in the analysis of the optimising of alginate cross linker concentration/ time and dissolution solution concentration lead to an adjustment of the reagents used in the alginate bead preparation and dissolution. The alginate beads were prepared from this point onwards with 1.5% CaCl\(_2\) cross linker solution and a cross linking time of 10 minutes. The dissolution solution concentration was increased from initially 3% to 10% \(^{\text{w/v}}\) trisodium citrate. This adjusted use of reagents during the scaffold manufacturing aimed to optimise the alginate dissolution from the scaffold due to homogenous bead production and higher ratio trisodium citrate to Ca\(^{2+}\) ions within the alginate bead structure.
Figure 2.3: Optimisation of alginate bead formation and dissolution. (A) Different cross linking concentrations (CaCl₂), (B) cross linking times and (C) trisodium citrate concentrations at constant pH of 8.3-8.5 were tested to optimise alginate bead dissolution. Therefore, the time needed to completely dissolve the alginate beads was recorded. All experiments were carried out with 0.5 g alginate beads (0.5% alginate) and repeated 3 times (n=3). Initial reagent concentrations and time to cross link and reagent concentration to dissolve the beads of 30 minutes cross linking in 0.5% CaCl₂ and dissolution in 3% trisodium citrate were compared to tested concentrations. Error bars indicate standard deviation. Significance is given by *p ≤ 0.05.
2.3.1.2 Optimisation of the alginate dissolution technique

The reagent optimisation studies undertaken in the previous section investigated the dissolution of the alginate beads alone. The application of the trisodium citrate concentration on removal of the alginate beads held within the gelatin scaffold provides other challenges. The gelatin matrix encloses the alginate beads and acts as a diffusion barrier for the dissolution solution. Therefore, the technique of applying trisodium citrate more efficiently into the scaffold was optimised.

Specifically, the trisodium citrate solution was applied using different techniques to the prepared scaffold with the same amount of alginate beads (1 g). (1) The initial method of piercing the scaffold with a needle and immersion in 200 ml trisodium citrate (Phull et al., 2013) was compared to (2) the unpierced scaffold immersed in 200 ml trisodium citrate (Tomei et al., 2009) and (3) the direct injection of the dissolution solution into the scaffold with a syringe. Quantification of dissolved beads was based on a standard curve (portrayed in section 2.2.2.3).

After applying each of the techniques to the alginate bead containing scaffolds, the direct injection of the trisodium citrate solution into the scaffold showed the most efficient removal of alginate beads, followed by the pierced and unpierced technique (Figure 2.4A). The quantification of the dissolved bead confirmed this (Figure 2.4B). Applying the dissolution solution directly into the scaffold dissolved an average of 300 beads. An average of 150 beads was dissolved though the other techniques, after one day of submerging in 200 ml dissolution solution (Figure 2.4B).

The most effective way of reversing the alginate bead gelation out of the gelatin matrix was the direct injection of trisodium citrate into the scaffold where the majority of the beads were dissolved from the scaffold in one day. From this point on piercing the scaffold and immersing into the trisodium citrate solution was replaced with the direct injection of it into the materials by syringe.
Figure 2.4: Optimisation of the alginate bead dissolution technique. (A) The structure of the gelatin scaffold and (B) the amount of dissolved beads after applying different dissolution techniques. The scaffold was not treated (unpierced) or pierced with a needle and submerged in 10% w/v trisodium citrate (200 µl) for 1 day. The final utilised technique was the injection of small amounts of the 10% w/v trisodium citrate solution into the scaffold with a syringe. (C) With the help of a standard curve of the dissolution of alginate beads, the dissolved amount of beads x was quantified. Therefore, the pictured formula was determined through plotting a trend line with a coefficient of regression $R^2 = 0.9852$. All tested scaffolds were produced with 1 g alginate beads and treated with 10% w/v trisodium citrate solution. Error bars indicate standard deviation. The technical replicate was n=3. Scale bars are 2 mm.
2.3.1.3 Morphological analysis of the optimised scaffold preparation

To analyse the optimised scaffold production for retaining an interconnected porous structure which is an essential feature for supporting nutrient exchange as well as cell infiltration, a scaffold was produced according the improved method, as described in section 2.2.3. The scaffold was manufactured using 10% trisodium citrate direct injections and analysed under the SEM (outlined under 2.2.4.2).

The microscopy revealed micropores in a visible size up to several hundred µm (Figure 2.5). The micropores were covered with a thin layer of material, as illustrated at the higher and lower magnification SEM image (Figure 2.5A, C). The layer is most likely alginate due to the appearance of the film mainly in the void left by the alginate beads. Hence, free pores are visible on the groove between two macropores (Figure 2.5A). This layer blocked the connection between the pores thus potentially limiting the interconnectivity required for the passage of fluids, nutrients and waste products. To obtain complete interconnectivity additional washing steps with water were added to the scaffold manufacture which acted in the removal of the layer (Figure 2.5B, D). The resulting optimised scaffold preparation method is displayed in Figure 2.6.
Figure 2.5: Enhancement of interconnectivity through washing. (A, C) A thin layer which
blocked the micropores is visible in the void left by the alginate beads (white arrow) as well as in
higher magnification. Scale bars are 200 µm and 10 µm. (B, D) The layer was removed (white arrow)
through adding of 3 washes with water. Scale bars represent 200 µm and 10 µm. (-) = without
washing; (+) with washing.
Figure 2.6: Initial and optimised scaffold preparation. (A) The initial time consuming scaffold manufacture. (A1) Alginate beads were mixed with gelatin containing cross linker. (A2) After the mixture set, the construct was immersed in (3%) trisodium citrate solution to break the alginate cross link, to remove the alginate out of the gelatin structure to produce macropores. (B) Displays the optimised scaffold production. (B1) The first step stayed unchanged from the initial method, but (B2) after setting of the alginate gelatin mixture a washing step (3x washing with sterile H₂O) was included to remove residual cross linker. (B3) Afterwards, a syringe with 10% (W/V) trisodium citrate was utilised to apply the dissolution solution directly into the scaffold and (B4) dissolve the alginate cross link. (B5) A final wash step was added to erase residual alginate and trisodium citrate. (C) The chemical structural formulas and the key that was used to illustrate it. Structural formulas are modified from (Information, 2005, Kashima and Imai, 2012).
2.3.2 Construction and characterisation of different scaffold variations using ice as a porogen

The scaffold described above (section 2.3.1.3) is similar to the constructs produced by Phull et al. that displayed microporosity with pore size diameters of 20-200µm and macroporosity with a pores size range of 2.01 mm ± 0.8 mm. The macroporosity can be adjusted according the size and number of alginate beads used. The microporosity, however, was produced through the cross linked gelatin (Phull et al., 2013). To further obtain controllability of the microporosity, different freezing regimes were applied. In this way ice crystals were used as porogen whereby the lower the freezing temperatures the smaller the formation of ice crystals, hence the smaller the pores that formed (Mao et al., 2003).

Therefore, the scaffolds were prepared as described in 2.2.3 and directly freeze dried or pre-frozen either at -20°C or -80°C and subsequently freeze dried which removes the ice crystals out of the porous gelatin structure (outlined in section 2.2.4). The scaffolds obtained (Table 2.2) were named scaffold I-IV. Furthermore, they were characterised regarding their size, microporous macroporous morphology, pore size range, porosity, interconnectivity and liquid uptake as detailed in 2.2.4. This provided detailed information about the potential ability of the scaffolds to be infiltrated by nutrients, cells, waste removal and the feature of habiting cells which attach, proliferate and differentiate. Through this characterisation the scaffold variations with a suitable physical profile for adipose tissue reconstruction can be selected for further cell based studies.

Table 2.2: Different scaffolds gained through the application of ice as porogen.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Pre-freezing</th>
<th>Freeze dried</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaffold I</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Scaffold II</td>
<td>-20 °C</td>
<td>√</td>
</tr>
<tr>
<td>Scaffold III</td>
<td>-80 °C</td>
<td>√</td>
</tr>
<tr>
<td>Scaffold IV</td>
<td>/</td>
<td>√</td>
</tr>
</tbody>
</table>
2.3.2.1 Morphology of microporous macroporous structure

To characterise the scaffolds a macroscopic analysis was carried out to gain information about scaffold size and appearance. Further, a microscopic investigation through fluorescence microscopy exploring the auto fluorescence of the gelatin materials as well as SEM was conducted to obtain knowledge about the porous scaffold structure (outlined in section 2.2.4.2).

The 4 different scaffolds are represented in Figure 2.7 with similar size and visible differences in the physical appearance (Figure 2.7A). Scaffold I measured 1.24 cm (± 0.13 cm) in diameter and 1.06 cm (± 0.11 cm) in height. Scaffold II and III displayed 1.1 cm (0.08 cm) and 1.02 cm (± 0.04 cm) in diameter with a height of 0.97 cm (± 0.17 cm) and 0.98 cm (± 0.04 cm). Scaffold IV represented an average diameter of 1.17 cm (± 0.08 cm) and average height of 1.1 cm (± 0.15 cm). Further, the initial scaffold I produced by solvent casting (2.2.3) demonstrated a wet and gel-like appearance.

However, the frozen and freeze dried scaffolds (II-IV) showed a different morphology. Scaffold III demonstrated a finer surface structure when compared to scaffold II and scaffold IV (Figure 2.7A). Here, scaffold IV visibly appeared to have a greater surface rugosity.

Importantly, all 4 scaffolds retained a microporous macroporous morphology with different sizes of micropores represented in Figure 2.7B. The macropores (≥ 1 mm) are visible separated by the porous gelatin walls containing micropores (< 160 µm) in all scaffolds. The microporous macroporous structure was analysed in detail by using SEM. This revealed a microporous macroporous structure for the 4 scaffolds (Figure 2.7C, D). In Figure 2.7C single macropores were observed in a range ≥ 1 mm. This underpins the size detected in the florescent pictures (Figure 2.7B). The void left behind by alginate beads was clearly present in all produced scaffolds. In addition, the obtained microporous gelatin walls were detected in each produced scaffold as well (Figure 2.7D). The micropore size was similar to observed size in the florescent images ≤ 1 mm.
Figure 2.7: Different scaffold morphology through the use of ice as porogen. (A) Overview pictures of the different prepared scaffolds with their corresponding size, scaffold I (initial scaffold), scaffold II and III (−20°C and −80°C pre-frozen scaffolds) and scaffold IV (freeze dried scaffold). Scale bar represents 1 cm. stdev = standard deviation. (B) The microporous macroporous structure of the different scaffolds is visible. Scale bar is 200 µm. green = auto fluorescent gelatin. (C, D) SEM pictures of macropores and micropores of the scaffolds. The macropores were produced via particulate leaching of alginate beads out of the scaffold structure. The alginate beads that are leaving upon dissolution macropores are marked with white arrows. Scale bars are 20 µm and 100 µm for macropores and micropores of scaffold I. Scale bar are 200 µm for the porous structure of scaffold II. The scale bar of the represented macropores and micropores of scaffold III are 200 µm and 100 µm. Scale bars of the macroporous and microporous structure of scaffold IV are 100 µm and 10 µm.
2.3.2.2 Micropore and macropore size analysis

To reveal information about the pore size found in the scaffolds, detailed measurements of the micropores and macropores were determined. This information was important for assessment of the suitability of the scaffold design for further cell infiltration, attachment, proliferation and differentiation and therefore cell based studies. Here, ADSCs displaying an average size of 22 µm and maturing adipocytes a size of up to 190 µm in diameter (Skurk et al., 2007, Ryu et al., 2013).

2.3.2.2.1 Macropores

For the macropores the alginate beads which were used as templates were characterised in diameter (Figure 2.8). Therefore, 10 alginate beads were measured in three diameter according to section 2.2.4.3.2 to confirm their shape.

This revealed that the cast alginate beads are spherical with an average diameter of 1.83 mm (± 0.02 mm).

The next step was to analyse the macropores cast by the alginate beads in the scaffolds to underpin the use of alginate beads as macropore template. Specifically, 3 scaffolds of each type were investigated. The centre sections of the scaffolds were H + E stained and the macropore size was analysed in diameter with Axiovision release 4.8 (detailed in section 2.2.4.3.2) (Figure 2.9).

The macropores were clearly visible in the scaffold structure (Figure 2.9A-D). In all the different scaffolds, the macropore size displayed a high variability due to sectioning resulting in different parts of the pores and therefore varying size ranges. Scaffold I represented an average macropore diameter of 1.6 mm (± 0.59 mm) with a maximum and minimum pore diameter of 2.6 mm and 0.4 mm. Macropores with an average diameter of 1.8 mm (± 0.74 mm) ranging from a minimum diameter of 0.82 mm up to a maximum diameter of 3.09 mm were found in scaffold II. The pore diameter measured in scaffold III was an average 1.7 mm (± 0.43 mm) ranging from a minimum size of 0.81 mm up to 2.59 mm. The macropore size of scaffold IV also did not significantly differ from the other
scaffolds. An average diameter of 1.61 mm (± 0.53) with minimum and maximum pore diameter found at 0.12 mm and 2.18 mm (Figure 2.9 A).

Overall, the measured macropore diameter did not significantly differ among the scaffolds. The macroporous structure was in the size range of the alginate beads used. This consolidates the observation that the alginate beads created similar macropores in the different scaffolds according their size. Higher variability was created resulting from the compression of the beads through the gelatin matrix as well as from the area sectioned. However, the analysed size of the macropores provides a niche with enough space for ADSC attachment, proliferation and differentiation.
Figure 2.8: Alginate bead characterisation. (A) The alginate beads were measured in (B) three diameter (a, b, c). The sample number is n=10. Alginate beads were obtained by cross linking 0.5% \( w/v \) alginate with 1.5% \( w/v \) CaCl\(_2\). Error bars indicate standard deviation. Scale bar represents 1 mm. a= diameter 1, b= diameter 2, c= diameter 3
Figure 2.9: Macropore size range of scaffold variations. (A-D) Representative H+E stains (magnification 2x) of each fabrication technique used to analyse the macropore structure (outlined in white dash line) created by alginate beads. Scale bars are 1 mm. (E) The diameter in mm of scaffold macropores left by the dissolution of alginate beads is presented. Sample number was n=15. Scaffold I= initial scaffold; Scaffold II= -20°C pre-freezing; Scaffold III= -80°C pre-freezing. Scaffold IV= freeze dried.
2.3.2.2.2 Micropores

The size range of the micropores in the scaffold types and their repeatability was investigated using H + E staining (detailed description in section 2.3.2.2.2). This gives information about the suitability of the micropore size to support cell infiltration and nutrient and waste diffusion. Hence, the pore size was measured with Axiovision release 4.8 software. To analyse the size distribution throughout the 4 produced constructs one scaffold of each type was sectioned from the centre towards the periphery every 1000 μm. This gained 4 areas for the investigation (outlined in detail in section 2.2.4.3.3).

An alteration of the micropore size through the application of different freezing techniques and freeze drying of the initial scaffold I was observed (Figure 2.10). A wider pore size range was noted for scaffold I, III and IV. A narrow pore size distribution and fewer pores were observed for scaffold II (Figure 2.11). In detail, the initial scaffold I displayed a pore sizes distribution from 25 μm to 200 μm, with a maximum pore size of 150.09 μm. The majority of pores represented a size range of 50 μm (Figure 2.11A). Scaffold II was pre-frozen at -20˚C and illustrated a narrow size range. Furthermore, due to the decreased pore diameter, the pores appeared to be smaller numbered in thicker gelatin walls when visually compared to the other versions (Figure 2.10). The pore range measured was from 3.55 μm to 30.96 μm. The mean pore size was 10.03 μm (Figure 2.11B). The -80˚C pre-frozen scaffold III produced a wider pore size range from 12.9 μm - 159.18 μm. The mean pore size was 55.9 μm (Figure 2.11C). The freeze dried scaffold IV represented a wide pore size distribution from 11.1 μm to 121.84 μm, with a mean pore size of 26.21 μm (Figure 2.11D).
Figure 2.10: Microporous structure of the different scaffolds. (A) The scaffolds were sectioned every 1000 μm to obtain four areas and stained with H + E to reveal the microporous structure of the gelatin matrix. (B) The application of pre-freezing at -20°C, -80°C with subsequent freeze drying and freeze drying alone altered the microporous structure of the gelatin walls of the constructs when compared to the non-frozen material (scaffold I). Scaffold II showed smaller pores in thicker gelatin walls when compared to scaffold I. Scaffold III and IV revealed smaller pores in the centre and larger pores in the periphery when compared to the non-frozen biomaterial. Scale bars 50 μm. Scaffold I = initial Scaffold, Scaffold II = -20°C pre-freezing, Scaffold III = -80°C pre-freezing, Scaffold IV = freeze dried.
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Figure 2.11: Micropore size range of the different scaffolds. The scaffold was sectioned every 1000 μm to obtain four areas. The pore diameter was measured to analyse the pore size distribution throughout the scaffolds gained by preparation (A) without freezing, with pre-freezing at (B) -20°C, (C) - 80°C with subsequent freeze drying and freeze drying alone (D). Scaffold I = initial Scaffold, Scaffold II = -20°C pre-freezing, Scaffold III = -80°C pre-freezing, Scaffold IV = freeze dried.
To investigate the repeatability of the alteration of the micropore size, the different scaffolds were sectioned in the centre and haematoxylin staining was performed (described in section 2.2.4.3.3). This was repeated 3 times for each different scaffold type.

In scaffold I produced without freezing, the micropore size ranged from 12 µm - 192 µm, with a mean size of 65 µm (Figure 2.12A). Freeze drying of the initial scaffold resulted in a mean micropore size of 131 µm, ranging from 36 µm - 382 µm, pre-freezing at -20°C lead to a mean size of 31 µm, ranging from 9 µm - 69 µm, and pre-freezing at -80°C produced a mean size of 83 µm, ranging from 20 µm - 230 µm (Figure 2.12B-D). Freeze drying and pre-freezing, therefore altered the micropore size compared to the initial scaffold I. A wide size range (36 µm - 382 µm) was obtained with scaffold IV, whereas a narrower range (9 µm - 69 µm) was gained with pre-freezing at -20°C. Due to the narrow and small size range of the pores in -20°C pre-frozen scaffold II the pores appear fewer compared to the other scaffold versions. Thus, the alteration produced reproducible size ranges within the samples tested.

In conclusion, the use of ice crystals as a porogen altered the micropore size of the scaffold. Hence, three further scaffold types were created differing in micropore size from the initial scaffold and among each other. The manufactures pore size range was proven to be reproducible. Therefore, freezing can be used for controlled alteration of the micropore size.
Figure 2.12: Reproducible micropore size range of the scaffold variations. The scaffolds were sections in the central area and the pore diameter was measured to analyse the pore size distribution of the micropores in (A) scaffold I, of the (B) pre-frozen scaffolds II at -20°C, and (C) III - 80°C and (D) freeze dried scaffold IV. (E-H) Show examples of the H + E stain (magnification 20x) used to analyse the micropore size. Scale bars 100 μm. Error bars indicating standard deviation by a sample number of n=30. Scaffold I = initial scaffold, Scaffold II= -20°C pre-freezing, Scaffold III= -80°C pre-freezing, Scaffold IV= dried.


2.3.2.3 Liquid uptake, porosity and interconnectivity of the scaffolds

The porosity (= void volume) of the scaffold is one of the major features to ensure interconnectivity that will allow cell infiltration. Pores also provide the space for cell differentiation, migration and proliferation. Furthermore, they help to maintain nutrition flow and waste removal. After analysing the microporous macroporous structure of the different scaffold variations produced through different freezing temperatures, the liquid that can be taken up by the pores, the porosity and the pore interconnectivity were evaluated.

2.3.2.3.1 Liquid uptake

Firstly, the liquid uptake of the scaffolds was determined over time. This gives information about the time and the exact volume that it takes to infiltrate the scaffolds with liquid. Thus, this investigation delivers information about the time and volume that is needed to infiltrate the constructs with a cell-media mix and consequently is important for subsequent cell based studies. Therefore, the scaffolds were swollen in water and the uptake of the liquid was monitored at time points 5, 10, 20 and 30 minutes as specifically outlined in section 2.2.4.4.1.

All scaffold variations showed a maximum of liquid uptake at 5 minutes (Figure 2.13A). The liquid uptake of scaffold I was 0.4 ml, scaffold II and III showed a liquid uptake of 0.45 ml and of 0.5 ml at 5 minutes. Scaffold IV reached a liquid uptake of 0.7 ml at 5 minutes in solution. Scaffold II was the only construct type that absorbed more liquid after 5 minutes. Hence, at the 10 minute time point this variation absorbed an average liquid of 0.25 ml. The volume uptake of liquid at 10 minutes is less than at 5 minutes. Furthermore, the wide error bar indicates a high variability of the result. Thus, it can be assumed that scaffold II takes most of the liquid up at 5 minutes.

Overall, an average of 0.5 ml water can be taken up by all of the scaffolds within 5 minutes of contact with the liquid.
2.3.2.3.2 Porosity

The porosity of biomaterials gives information about the designed space for cell infiltration, migration and ability to be diffused by metabolites. Consequently, a high porosity in the design of scaffolds is desirable (Zhong et al., 2012). Therefore, the liquid accessibility of the pores was determined through the liquid displacement method (Hsu et al., 1997, Nazarov et al., 2004, Kasoju et al., 2009). The volume that can be held within the scaffolds was calculated according to section 2.3.2.3.2. The initial scaffold and the 3 different freezing techniques were compared; -20°C and -80°C pre-freezing with subsequent freeze drying and freeze drying to gain knowledge about the voids that are assessable for cells and diffusion products.

All 3 freezing techniques showed a significant increase in porosity when compared to the initial scaffold I porosity of 29.85% (±3.06) (Figure 2.13B). An increase in porosity to 65.42% (±2.54, p = 0.0071) was demonstrated with the freeze drying method. The freezing of the scaffold at -80°C showed a porosity of 52.77% (±3.20, p = 0.0006) and freezing at -20°C a 48.88% (±2.48, p = 0.0006) porous scaffold.

In general all applied freezing techniques displayed an improvement of the porosity compared to the non-dried scaffold I. Furthermore, the freeze drying of the scaffold lead to a significantly higher porosity compared to the initial scaffold I.

2.3.2.3.3 Interconnectivity of the scaffolds

To gain knowledge about the interconnectivity and therefore ability of the scaffold design to be diffused by metabolites and subsequent use in cell based studies, all 4 scaffold types were diffused by liquid. Followed by recording of the time needed for the liquid to pass through the gelatin sponges (detailed in paragraph 2.3.2.3.3).

The interconnectivity displayed through the liquid flow through the scaffold was in all constructs given (Figure 2.13C). The liquid diffused the scaffolds in average time of 15 seconds. Therefore, all the scaffolds tested demonstrated interconnectivity and consequently, have the potential to be used in 3D culture and are with the ability to allow nutrient/cell infiltration and waste removal.
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Figure 2.13: Liquid uptake, porosity and interconnectivity of the scaffolds. (A) The liquid uptake over time (30 min) of the different scaffold variations is represented. The constructs were immered in H₂O and showed a maximum uptake of liquid after 5 min which was stable over the analysed time interval. (B) The porosity of the different scaffolds was determined through the liquid replacement method. It is shown that an increase of the porosity was gained through the application of different freezing techniques. (C) The diffusion time needed by liquid to pass through the constructs was not altered through the utilisation of different freezing protocols. Error bars indicate standard deviation. The sample number was n= 3. Significance is given by *p≤0.05. Scaffold I= initial scaffold; Scaffold II= -20°C pre-frozen scaffold; Scaffold III= -80°C pre-frozen scaffold; Scaffold IV= dried scaffold.
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2.4. Discussion

The loss of adipose tissue has a severe impact on patients, not just physically but also emotionally. The current main treatment methods for the restoration of contour defects including lipofilling, fat grafting and the application of synthetic fillers are often regarded as unsatisfactory. This is mainly due to the high resorption rate of the fat used in the procedures and allergenic effects with unpredictable outcomes (detailed in section 1.1). Adipose tissue engineering provides a strategy for the development of an alternative treatment method (outlined in section 1.3). The tissue engineering strategy can be divided into 3 aspects. The first aspect is the scaffold which gives shape and the mechanical properties of the lost tissue. The second aspect is the cells used to promote tissue regeneration. The final aspect is the additional incorporation of extra cellular matrix components to guide the tissue regeneration and reconstruction via cells and scaffolds (Gomillion and Burg, 2006, Bauer-Kreisel et al., 2010).

This chapter deals with the physical properties of the first component of the tissue engineering strategy – a novel scaffold for adipose tissue reconstruction. The basic design was based on that of Phull et al. (Phull et al., 2013). This scaffold represented potential both in terms of biological as well as physical efficiency for use in adipose tissue reconstruction. The physical features were described as a soft adipose tissue-like feel and a characteristic microporous macroporous structure. The microporous structure was gained by cross linking 7% (w/v) aqueous gelatin type B. The macroporous structure was obtained by particulate leaching of alginate beads out of the gelatin matrix. The size range of the resultant macropores was 2.01 ± 0.08 mm (Phull et al., 2013). However, the scaffold preparation was time consuming. The scaffold was poorly characterised and the microporosity was dependent on the gelatin and cross linker concentration.

Therefore, the aim of this chapter was to further develop this microporous macroporous scaffold for soft tissue reconstruction by approaching each of the shortcomings to develop a new generation of scaffold designed specifically for adipose tissue reconstruction. Through further developing and re-designing the initial
biomaterial different scaffolds were produced with varying properties. Furthermore, a characterisation of micropore and macropore size range, liquid uptake, void volume and interconnectivity of the newly designed scaffolds were performed. The characterisation informed the selection of a scaffold with physical features appropriate for adipose tissue reconstruction.

2.4.1 Optimisation of the scaffold preparation

Firstly, to gain a controlled, relatively rapid, reproducible and standardised method to prepare the pre-designed material the manufacture process was optimised. In addition, the optimised method delivered a platform for further scaffold design approaches. Through the optimisation of the alginate dissolution solution concentration and the dissolution technique the scaffold production time was reduced from weeks to days. A more efficient production process and a refined characterisation of the alginate bead cross linker concentration and time was also achieved.

2.4.1.1 Dissolution solution concentration, alginate bead cross linker concentration/time

To improve the time consuming manufacture of macropores through particulate leaching the scaffold constituents were analysed and optimised. This involved the optimisation of a standardised alginate bead production protocol, followed by adjustment of the methodology of the subsequent manufacturing.

Firstly, different CaCl$_2$ concentrations were tested to adjust the alginate bead formation. CaCl$_2$ acts as a divalent ionic cross linker for ionotrophic gel formation of alginate. Hence, the Ca$^{2+}$ ions bind on the polysaccharide chains G-blocks (guluronic acid blocks) of alginate that stacks the biomaterial chains together in a gel according the egg box model (Braccini and Pérez, 2001, Imai, 2012, Lee and Mooney, 2012). It was shown that alginate cross linking with lower CaCl$_2$ concentrations of 0.5% and 1% slowly formed uniform shaped coalesced beads which dissolved in 3-4 minutes. Cross linking with higher CaCl$_2$ concentrations up to 2.5% resulted in uniform spherical beads which dissolved in increased time when compared to the lower cross
link concentration. Bienaimé et al. analysed the structure of alginate beads with varying alginate: CaCl₂ ratio by using different charged staining molecules. This study delivered detailed insights into the alginate bead formation and structure. They illustrated that alginate bead formation begins from the outside and processes towards the bead core which leaves an outer strong gelled crust and inner weak gelled zone behind. Thus, using lower CaCl₂ concentration makes the outer zone more distinguished from the weaker inner zone still resulting in uniform tear drop bead morphology (Bienaimé et al., 2003). However, fewer Ca²⁺ ions are incorporated in the alginate chains resulting in quicker breakage of the cross link as observed in our study. Further, the outer surface of the construct would be weakly cross linked, with weak inner gelled zone when compared to cross linking with higher CaCl₂ concentration. Hence, in present study the application of lower CaCl₂ concentration resulted in less Ca²⁺ ions in the alginate structure displayed in fast dissolution time when compared to the application of higher CaCl₂ concentration. Consequently, the later resulted in longer dissolution time. Furthermore, the application of a reduced cross linking concentration creates a heterogeneous distribution of positive and negative charges which cause the beads to aggregate to each other, hence gives the observed coalescence in Bienaime et al. as well as in present study (Bienaimé et al., 2003).

In the current study, the increased cross link concentrations displayed significantly longer dissolution times compared to the initial used 0.5% CaCl₂. The dissolution time of the alginate beads prepared with the increased CaCl₂ concentrations did not differ among each other. Thus, the beads reached saturation, as a consequence the alginate was completely diffused by calcium ions. Because of the complete diffusion, a homogeneous cross linking throughout the bead occurred, resulting in a similar dissolution time. Furthermore, the bead formation was rapid and gave uniform spherical bead morphology. The later indicates as well the formation of the outer strongly gelled zone. Therefore, a more homogenous cross linked bead is obtained, the longer it is exposed to the cross linking solution (CaCl₂) (Bienaimé et al., 2003).
In summary, to create a homogeneously cross linked alginate bead with uniform shape which can be used as template for the macroporous structure of the scaffold, the beads have to be cast with a CaCl$_2$ concentration, ≥ 1% (w/v) when maintaining a low alginate concentration of 0.5% (w/v). Consequently, the dissolution time increases with the saturation of the cross link. Despite the slight increase of the dissolution time, the CaCl$_2$ concentration was increased from 0.5% to 1.5% to gain homogenous, non-coalesced beads and therefore the potential for homogeneous macropores.

However, the various CaCl$_2$ cross linking times (5, 10, 20, 30, 40 minutes) of the alginate beads did not have a significant influence on the dissolution time of the beads. Thus, Bienaimé et al. also showed that the higher the CaCl$_2$ and alginate concentration the faster a saturated cross link is reached. Specifically, they displayed that a 1.5 mm radius calcium alginate bead fully cross links after 4.1 minutes in 1.5 M CaCl$_2$ solution using 1% alginate solution. The same radius sized bead took longer to fully cross link (16.5 minutes) by decreased CaCl$_2$ concentration (0.1 M) and increased alginate concentration (1.5%) (Bienaimé et al., 2003). Hence, the alginate beads for the presented gelatin sponge were cast out of 0.5% alginate solution and left at least 10 minutes in the cross link solution (0.135 M CaCl$_2$). The resulting similar dissolution times lead to the assumption that all beads contained similar amounts of cross linked Ca$^{2+}$. Consequently, the beads were fully diffused by Ca$^{2+}$. An incubation time of 10 minutes in CaCl$_2$ after casting the beads was applied in the subsequent bead preparation to ensure even cross linking throughout the alginate bead.

Furthermore, our study revealed that the higher the trisodium citrate concentration the faster was the dissolution of alginate beads. Other studies already confirmed that with an increased concentration of the ionic exchanger, in this case citrate with mobile bound sodium (Na$^+$), the faster the alginate crosslink is broken (d’Ayala et al., 2008, Lee and Mooney, 2012). The higher affinity of Ca$^{2+}$ results from the increased attraction of trisodium citrate to the cation compared to alginate (Wee and Gombotz, 1998). Hence, the increased trisodium citrate concentration (≥ 7%) led to a higher availability of citrate for the ionic exchange mechanism. This was illustrated
in the significantly faster alginate dissolution time. Here, 2 Na\(^+\) is exchanged for 1 Ca\(^{2+}\) due to the higher affinity of Ca\(^{2+}\) to citrate. The sodium ions are bound to alginate and as they are monovalent a cross link between the alginate G-blocks cannot be formed (Wee and Gombotz, 1998, Bienaimé et al., 2003, Lee and Mooney, 2012, Pawar and Edgar, 2012). Consequently, the beads are dissolved. Further, the two highest tested concentrations represented the same dissolution time of 1 minute suggesting citrate saturation.

This analysis resulted in a better understanding of the alginate cross linking concentration limit and time. In addition, 10% (w/v) trisodium citrate was chosen for further scaffold production. The higher concentrated dissolution (10% w/v) solution had a pH of 8 which did not affect gelatin (Jones, 2004, America, 2012). Trisodium citrate components do not influence the cross linked gelatin matrix chemical properties. Hence, the increased dissolution concentration was further tested within the gelatin scaffold.

### 2.4.1.2 Optimisation of the alginate dissolution from gelatin scaffold

The solvent casting and particulate leaching method recently published by our group (Phull et al., 2013) was repeated to gain a higher efficiency in the removal of the alginate beads which are surrounded by the gelatin matrix. The initial method was carried out through immersion of the gelatin/alginate bead mix in trisodium citrate. The trisodium citrate solution penetrated the gelatin matrix slowly. Hence, the Ca\(^{2+}\) ions which link the G-blocks of the alginate chains together to form a gel are reversed. The trisodium citrate structure incorporates 3 Na\(^+\) which are exchanged due to Ca\(^{2+}\) due to its higher affinity to citrate compared to the sodium ions. Thus, the alginate beads were liquefied and leached out of the matrix leaving macropores behind (Phull et al., 2013).

The slow penetration of the solution into and throughout the scaffold made this leaching step uncontrolled and time consuming, especially due to the large size of the scaffold. However, others who applied alginate beads to gain a macroporous structure within a surrounding matrix displayed similar dissolution strategies and limitations. Tomei et al. used sodium citrate as leaching agent but on a much smaller
sized scaffold with the measurements 10 mm x 20 mm x 5 mm (Tomei et al., 2009). As a result, the penetration of the solution was faster compared to our scaffold that is double in size. Delaney et al. applied ethylenediaminetetraacetic acid (EDTA) as calcium coordinating ligand for the dissolution of alginate beads. However, the utilisation was also performed on thin (15 µm) scaffolds (Delaney et al., 2010). Hence, the application of a calcium ligand into a larger scale of scaffolds such as the microporous macroporous gelatin sponge needed addressing.

To approach this problem the delivery of the dissolution solution into the scaffold to prevent the slow penetration was adjusted. Different strategies from the initial method of piercing the scaffold were used, comparing the original methodology (Phull et al., 2013) with non-piercing (Tomei et al., 2009) and direct injection of the solution into the scaffold. An improvement was reached by direct injection with a syringe of the adjusted 10% trisodium citrate concentration of the solution into the scaffold. The fact that the dissolution solution directly targeted the alginate beads improved the breaking of the crosslink. This also meant that the solution was renewed upon each injection preventing a reduction in the ionic exchange equilibrium. In the other methods tested, the solution had to slowly penetrate the scaffold from the outside to the inside which depends on the swelling and the porosity of gelatin (Grover et al., 2012). This resulted in reduced dissolution of the alginate beads from the scaffolds. The complete removal of the beads took 2 weeks in total. In contrast, the bead removal with the direct injection of the dissolution solution was manually controlled and took just minutes. However, recently Calcagnile et al. published a method to remove alginate beads out of larger scaffolds with sizes that reached centimetres in diameter. These authors applied high temperature (70°C) under vacuum conditions to shrink the beads within a poly(dimethylsiloxane) foam, followed by removal of resident materials through water flow (Calcagnile et al., 2014). This is similar to our approach where residual material removal was assisted by injections with a syringe and additional washing steps. The high temperature of 70°C is difficult to apply on our gelatin matrix due to risk of melting the biomaterial (Bigi et al., 2001, Singh et al., 2002).
The direct injection of the dissolution solution into the scaffold was chosen to use from this point on for further scaffold production. The injection holes created through this elected method did not influence the structure and shape of the scaffold but probably assisted the diffusion of the alginate beads out of the gelatin matrix. Thus, the initial method was successfully optimised for use in the production of scaffolds for adipose tissue reconstruction. Furthermore, the direct injection of the trisodium citrate solution delivers a new approach of removal of alginate beads out of larger scale scaffolds. The method is also adjustable according to the dissolution solution used such as EDTA (Delaney et al., 2010).

### 2.4.1.3 Morphological analysis of optimised scaffold preparation

To analyse the porous structure created through the optimised scaffold preparation SEM analysis was performed on the non-frozen construct representing the initial scaffold of Phull et al. (Phull et al., 2013). Hereby, a thin layer was revealed covering the walls of the macropores, at the interface of the alginate beads and gelatin. This layer blocked the micropores and therefore limited the interconnectivity of the whole scaffold. The thin layer was likely produced whilst mixing the alginate beads with the gelatin containing the crosslinker, glutaraldehyde.

The cross linker was used to obtain a long-term stable shape for adipose tissue regeneration (Bigi et al., 2001, Korurer et al., 2014). Despite the known toxicity after release via degradation of the cross linked structure glutaraldehyde is a widely used collagen cross linker. Furthermore, it is already used in clinical settings in bioprostheses such as heart valves, vascular grafts and elastic cartilage (Jayakrishnan and Jameela, 1996). It has been shown to be important to remove free residuals which can be achieved through washing as done in present study and by others (Imani et al., 2013) and the use of reduced glutaraldehyde concentrations (Bigi et al., 2001). Bigi et al. demonstrated in in vitro studies that the lower the applied glutaraldehyde concentration, the less free residuals are found in the cross linked gelatin films. They presented that gelatin films cross linked with glutaraldehyde concentration ≤ 2% do not show any release of the cross linker over 2 weeks stored in buffer solution (Bigi et al., 2001). Imani et al. tested fibroblast viability on gelatin sponges cross linked with varying concentration of glutaraldehyde (0.5% -1.5%).
They removed non-reacted cross linker residuals through washing with double distilled water after gelation of the gelatin sponge at room temperature. In subsequent cytotoxic studies with fibroblasts they did not observe differences in toxicity compared to TCP (Imani et al., 2013).

However, similar layer formation has been seen previously (Calcagnile et al., 2014). In the study of Calcagnile et al. poly (dimethylsiloxane) solution was cased around alginate beads with subsequent bead removal. Analysis of the porous structure using SEM revealed a thin layer in areas of the beads which did not perturb the interconnectivity of the porous structure (Calcagnile et al., 2014). Nonetheless, an interesting observation was that the layer disappeared after we included a number of additional wash steps in the scaffold preparation process. After mixing the alginate beads within the gelatin and the setting of the biomaterial it was copiously washed, resulting in the removal of the thin layer. Therefore, the interconnectivity of the micropores structure was re-established which would potentially permit waste removal, nutrient and cell infiltration.

In summary, the scaffold preparation was optimised through the adjustment of each step of the scaffold production. This included bead formation, inclusion of beads within the gelatin matrix and subsequent removal by dissolution of the alginate beads to form macropores within microporous walls. Through the adjustment of each step, a standardised and reproducible method was developed. In addition, the method is flexible enough to allow further optimisation if required as dictated the outcome of any future cell seeding experiments. Furthermore, understanding of the development of alginate bead formation is gained, adding important methodological advances to previous studies (Bienaimé et al., 2003). Additionally, this newly developed and optimised, time efficient method provides advantages for any future clinical use, with the potential to rapidly produce any shape of scaffold for subsequent cultivation with cells prior to implantation. The improved technique represents a novel methodology for the creation of macropores in a gelatin matrix for the application in adipose tissue reconstruction.
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2.4.2 Construction and characterisation of different scaffold variations using ice as a porogen

Another limitation of originally designed scaffolds has been the lack of control over the microporosity within the cross linked gelatin that forms the structure of the construct (Phull et al). Therefore, the subsequent aim was to develop a method to control the micropore size range throughout the scaffold allowing a range of microporosities to be produced suitable for scaffolds designed for adipose tissue reconstruction. In addition, a detailed characterisation of the resultant porosity and pores should support the selection process.

Pores are one of the most important criteria in the scaffold design (Zhong et al., 2012). They are essential for cell and nutrient infiltration. The proliferation and migration of the cells also is dependent on the pore size as well as ECM secretion (Kang et al., 1999, Annabi et al., 2010). For this purpose a combination of larger and smaller pores within a scaffold combines the idea of pores which should be wide enough for cell infiltration, with those that contribute to facilitate nutrient supply and waste removal (Novosel et al., 2011). Larger pores should provide a structure to allow cell infiltration, proliferation and differentiation (Bauer-Kreisel et al., 2010, Zhong et al., 2012) whereas smaller pores should provide the porous structure with space for cell infiltration, metabolite diffusion as well as mechanical stability. In this study a scaffold was to contain both micropores and macropores. The macropores are created through the use of alginate beads as templates and their subsequent removal out of a solvent gelatin matrix. Micropores are found within the gelatin matrix strongly dependent on gelatin and the concentration of the cross linking reagent.

The average size of ADSCs is 22 µm which increases during maturation up to 190 µm (Skurk et al., 2007, Ryu et al., 2013). Recent approaches in adipose tissue engineering showed successful adipogenesis and therefore ADSC infiltration and differentiation with pore sizes between 100 µm - 500 µm and meshes with pores even reaching 1.5 mm in diameter (Hemmrich et al., 2005b, Hong et al., 2005, Bauer-Kreisel et al., 2010, Handel et al., 2012, Chang et al., 2013, Bellas et al., 2015). Through the rapid increase of differentiating ADSCs up to 190 µm the pore
size should be at least 200 µm to provide space for differentiating ADSCs (Hemmrich et al., 2005b, Illouz and Terodimas, 2011). In addition, Hemmrich et al. report that the mixture of smaller and larger pores would support adipogenesis (Hemmrich et al., 2005b). Thus, pores with a size up to 2000 µm would provide enough space for ADSCs to attach, proliferate and also differentiate. In addition, the space would be wide enough to locate several mature adipocytes and therefore creates a place for tissue regeneration, starting in the macropores, inside the scaffold. Therefore, the optimal pore size range should be at least within the above mentioned dimensions. Furthermore, the combination of micropores and macropores would also potentially allow vascularisation to occur within the structure.

Thus, a further important feature for an implanted scaffold intended for soft tissue reconstruction is the ability to allow ingrowth of blood vessels. Druecke et al. reported larger blood vessel ingrowths in scaffolds with pores greater than 250 µm (Druecke et al., 2004). Further, Hemmrich et al. showed advanced vessel formation with HA scaffolds containing pores of 400 µm in size (Hemmrich et al., 2005b). Besides, the pores should be connected for nutrient and waste diffusion, cell migration and vascularisation (Rouwkema et al., 2008). Cao et al. also demonstrate that larger pores ≥ 300 µm represent increased vessel infiltration and tissue ingrowth. They compared two scaffolds based on PLGA with different pore sizes. The smaller pore sized scaffold displayed pore diameters less than 50 µm up to 250 µm. The construct with larger pores included pore dimensions between 300 µm - 417 µm in diameter. In vivo studies where the constructs were implanted in an ateriovenous loop chamber for 8 weeks in rats displayed predominantly tissue and vessel ingrowth into pores ranging from 300 - 400 µm and tissue death in the smaller pores. The later resulted from blockage of the smaller sized pores through host cell infiltration resulting in less vessel ingrowth (Cao et al., 2006). Therefore the concept of micropores and macropores provides the scaffolds with features to allow vessel and cell infiltration, cell proliferation and differentiation hence, adipose tissue regeneration. Thus, the control over the pores is an important feature to control in the scaffold design process.
The previous attempt to produce microporous macroporous adipose tissue scaffolds has lacked control of the microporosity (Phull et al., 2013). Pores can be manufactured in scaffold materials through different methods. Among those methods are electrospinning, solvent casting and particle leaching, gas foaming and the use of ice as a porogen with subsequent freeze drying (Annabi et al., 2010).

Here, the application of different freezing temperatures with subsequent freeze drying was used to construct different scaffolds with varying micropore sizes and constant macropore dimensions. The production of pores by the use of ice crystals is commonly used and presents a bio-clean method without the use of additives or organic solvents (Kang et al., 1999, Mao et al., 2003, Nazarov et al., 2004, Imani et al., 2013, Lloyd et al., 2014). Further, through the subsequent freeze drying step the pores created through frozen ice nuclei are maintained by sublimation of the ice without thawing (Kang et al., 1999). Hence, the porous structure gained through freezing is conserved. The freezing temperatures applied to create different scaffolds with varying micropore size range were pre-freezing at -20°C, -80°C with subsequent freeze drying and freeze drying under vacuum with a condenser temperature of -55°C (Christ, 2013).

The visual analysis of the freeze dried scaffolds revealed their bulk dimensions and confirmed the effect of the application of different freezing temperature and freeze drying (Figure 2.7). All scaffold types are similar in size with around 1 cm in diameter and 1 cm in height. This size ranks the prepared scaffolds into the range of thicker constructs for adipose tissue reconstruction (Hemmrich et al., 2008, Kimura et al., 2010, Lequeux et al., 2012, Chang et al., 2013, Debels et al., 2015a). Further, as expected the frozen and dried scaffold types appeared solid in their dehydrated form. In contrast, the initial scaffold I presented wet and gel-like manifestation due to the retained water content. Hence, the varying surface structures indicate the different production process.

2.4.2.1 Macropores

Despite the varying freezing protocols the macroporous structure was maintained in all scaffolds. The size of the macropores corresponds with the size of the alginate
beads (Figure 2.8, 2.9). In addition, the alginate beads were removed before freezing and freeze drying. This left pores behind and therefore a structure without water content. Thus, the freezing did not have an effect on the macropore size. Hence, it was confirmed that alginate beads can be used as templates for the creation of macropores in the gelatin scaffold as demonstrated by others (Tomei et al., 2009, Delaney et al., 2010, Calcagnile et al., 2014). Tomei et al. constructed a composite matrix out of polyurethane in connection with a strain device to measure mechanical properties in porous structures. They manufactured large pores with diameters of 50 \( \mu m \) – 500 \( \mu m \) using alginate beads as template (Tomei et al., 2009). Delaney et al. created porous polyethylene glycol dimethacrylate scaffolds with pore diameters of 48 \( \mu m \) ± 4 \( \mu m \) and 248 \( \mu m \) ± 4 \( \mu m \). For the pore manufacture they used particle leaching of alginate beads (Delaney et al., 2010). Calcagnile et al. fabricated poly(dimethylsiloxane) foams with pore diameters ranging from 309 \( \mu m \) up to 1.56 mm using alginate beads (Calcagnile et al., 2014). However, the microporous macroporous scaffolds showed a macropore diameter up to 3.09 mm where the beads were compressed through the surrounding matrix or more than one bead created a macropore. Hence, the method of using alginate beads was utilised before but has firstly been described for adipose tissue engineering and in connection with gelatin by our group (Phull et al., 2013).

The manufactured size of the macropores in the different frozen materials developed in this study with a pore diameter up to 3.09 mm have the potential size to be able to accommodate cells and provides enough space for differentiation into mature adipocytes (Hemmrich and von Heimburg, 2006). Furthermore, cell–cell interaction and ECM deposition would also be possible.

2.4.2.2 Micropores

The macroporous structure was confirmed to be maintained but at the same time the microporosity was visibly altered by controlled freezing (Figure 2.10, 2.12). Other groups already demonstrated that the alteration of pore size is possible by using different freezing temperatures (Kang et al., 1999, Mao et al., 2003, Nazarov et al., 2004, Lai et al., 2013, Imani et al., 2013, Lloyd et al., 2014). Kang et al. prepared cross linked gelatin hydrogels and applied -20°C, -80°C or freezing with liquid
nitrogen combined with subsequent freeze drying. The results revealed that smaller pore diameters (85 µm ± 35 µm) were obtained following freezing at -80°C and larger pore diameters due to larger ice crystals when samples were frozen at -20°C (250 µm ± 120 µm). Hence, small ice crystals formed at faster freezing rates resulting in the formation of smaller pores (Kang et al., 1999). Similar results were obtained in our study. Scaffold I without freezing demonstrated a micropore diameter range of 25 µm - 200 µm. The freeze drying gained a similar micropore diameter range in scaffold IV (25 µm to 150 µm). However, the broader pore diameter range observed is due to the fact that the water content present in 7% gelatin matrix sublimes quickly during the freeze drying process and leaves pores behind. It would appear that the gelatin underwent dehydration. Therefore, the boarder pore size range possibly results from single small micropores as well as from large pore formations arising from merging of small neighbouring pores into bigger openings.

The -20°C pre-freezing of scaffold II created pores in a smaller range (3.55 µm to 30.96 µm in diameter) compared to the initial scaffold I. The slow freezing of the water containing gelatin matrix results in small and more uniform pores (Kang et al., 1999, Mao et al., 2003, Lai et al., 2013). Additionally, the gelatin walls of scaffold II seem thicker compared to walls of the other scaffold types. This was observed before by Imani et al.. These authors tested different freezing temperatures on gelatin sponges and revealed thicker gelatin walls resulting from higher tested freezing temperatures such as -10°C and -25°C (Imani et al., 2013). The other scaffolds I, III and IV have larger pores in visually thinner gelatin walls. Here, the gelatin is more extended due to the larger pores resulting in thinner but wider material walls. Through the -80°C pre-freezing technique in scaffold III pores with a diameter range of 12.9 µm - 159.18 µm were created. Although the colder temperature freezes the water in the gelatin matrix more quickly which results in smaller pores, the pores seen in the samples frozen at -80°C demonstrated a wider size range than those at -20°C. This may be due to the fact that the smaller pores merged with each other and created bigger pores. This is evident in the wider range of pores starting from small pores of 12.9 µm up to larger pores with measurements up to 159.18 µm in diameter.
Furthermore, scaffold III and IV revealed visible larger pore sizes in the periphery areas compared to pores located in the centre. This confirms the rule that the freezing starts from the outside and proceeds to the inside. Here, ice nuclei formed quicker on the outside layers resulting in smaller pores which are likely to merge into larger pores resulting in larger outer pores and smaller central pores. Others successfully applied constructs in vitro and in vivo studies with larger outer and smaller inner pores (Bellas et al., 2015, Korurer et al., 2014). Consequently, the constructed inner pores were wide enough to contribute to cell and nutrient infiltration as well as waste removal.

Throughout the whole study, it has to be considered that the size measurement of the pores which are 3D structures, was investigated on 2D images (displayed under 2.3.2.2.2). Therefore, it cannot be excluded that the area sectioned might have affected the measurement to some extent. Here, a smaller pore diameter could have been recorded when the pore was sectioned at the outer surface compared to larger size noted when the image would have been gained from central areas of the pore. However, this error was minimised through the measurement of several pores in each sectioned area and performed serial sections resulting in pore size distribution with average pore size values (Mao et al., 2003, Lai et al., 2013, Korurer et al., 2014, Bellas et al., 2015).

However, the different frozen scaffolds were freeze dried under vacuum. This final step sublimes the ice crystals gained through different pre-freezing temperatures within the porous structure of the cross linked gelatin matrix and resulting in a dehydrated construct (Kang et al., 1999, Christ, 2009). With the use of different freezing techniques different micropore sizes were obtained that were within the ideal pore size range of 100 µm - 400 µm (Hong et al., 2005, Bauer-Kreisel et al., 2010, Chang et al., 2013). This pore size range has also been suggested to have the potential to result in enhanced vascularisation (Druecke et al., 2004, Cao et al., 2006, Chang et al., 2013).

In conclusion, this analysis resulted in an understanding about the micropore size range in the different scaffolds. The results confirm other recent studies (Kang et al.,
All freezing techniques created a different range of sizes of micropores. These ranges displayed all suitable sizes for metabolic waste removal, nutrient and cell infiltration and vascular penetration (Hemmrich and von Heimburg, 2006). Furthermore, the different techniques deliver a degree of controllability over the micropore size and can be used for design of the scaffold. The macropore size was confirmed to be consistent throughout the different freezing techniques and related directly to the size of alginate beads used. The chosen macropore size provides space for cell differentiation and proliferation. Further controllability of the macropore size can be gained through modulation of the alginate bead dimensions. Thus, these biomaterials provide a platform for advanced studies to find an optimal construct for adipose tissue reconstruction.

### 2.4.3 Scaffold porosity and interconnectivity

The porosity can be defined as the percentage of the void volume in a solid material (Wang et al., 2010). A high porosity is desirable to provide space for the cells within the scaffold (Zhong et al., 2012). Further, the porosity as well as the interconnectivity of the pores is important for cell infiltration, migration, and diffusion of nutrients and waste. Current approaches in adipose tissue engineering scaffolds were described with porosity ranging from 63% up to ≥ 90% which showed positive results in adipose tissue regeneration studies (Kimura et al., 2010, Grover et al., 2012, Wang et al., 2013b, Chang et al., 2013, Korurer et al., 2014). Thus, information about scaffold porosity and interconnectivity mark important features in adipose tissue reconstruction.

To quantify the porosity, obtained through the microporous macroporous structure of the scaffolds, the liquid displacement method was used (detailed in section 2.2.4.4.2) (Hsu et al., 1997, Nazarov et al., 2004). With the application of this method the liquid uptake was analysed as well. The liquid uptake gives information of swelling ability and quantity of liquid that the scaffolds can retain over time and are essential for metabolite exchange during cell culture and also as a method of delivery of cells to the porous construct (Liu and Ma, 2009, Kasoju et al., 2009, Grover et al., 2012, Fan et al., 2015). It was seen that all scaffolds swelled within 5 minutes, taking an
average of 0.5 ml of liquid and are stable within 30 minutes. Hence, it compares with other studies of swelling of cross linked gelatin (Chang et al., 2013).

However, all three freezing methods showed significantly enhanced porosity compared to the initial scaffold I. The enhanced porosity corresponds to the drying of the scaffold. Thus, more liquid can be taken up in the frozen scaffolds as in the hydrated scaffold I. Scaffold IV represented the highest porosity of 65.42%. Through lyophilisation the water is removed out of the biomaterials resulting in freed space that is more accessible for liquid and a high porosity is gained. Hence, elevated liquid can be retained reflecting the increase of porosity seen in re-hydrated constructs such as in scaffold IV.

Overall, as expected, a significant increase in porosity was achieved through the different freezing techniques suggesting that these scaffolds with high porosity are suitable for soft tissue reconstruction when compared to the initial scaffold I (Kimura et al., 2010, Grover et al., 2012, Wang et al., 2013b, Chang et al., 2013, Korurer et al., 2014). Therefore, it was decided to exclude scaffold I from further cell based studies. It has to be mentioned that the measured porosity of the gelatin sponges lies at the lower limit of the ideal porosity range for scaffolds for adipose tissue engineering. These ideal porosities were mostly theoretical determined through analysing micrographs or predetermined in the use of commercially available constructs. Specifically, the porosity calculation was performed by calculating the fraction of the total area occupied by pores per image in 2D (Grover et al., 2012). Liu and Ma calculated the porosity through the density ratio. As such, the density of the applied material is divided through the density of resulting biomaterial and size (Liu and Ma, 2009). The theoretical porosity analysed in these methods described cannot be used to distinguish pores within open pores, open pores with dead ends or closed pores. Thus, the accessible porosity might be lower than that calculated. Furthermore, it gives no information about the space in 3D that actually can be occupied by liquid like the liquid replacement method does.

Traditionally, porosity has been measured using techniques such as mercury porosimetry. This method is based on the capillary flow whereby the fluid applied is
pressed into the material via pressure. The pressure used is higher as the capillary force of the largest pore (Wang et al., 2010, Zhong et al., 2012). With the mercury based porosimetry method pores larger than 500 μm cannot be measured. To measure large pores the applied pressure has to be low which could distort the structure of flexible gels (Chang et al., 2013). This method is also limited to a cylindric pore shape. Therefore, through the application of pressure or the analysis of micrograph as in the traditional and the theoretical porosity evaluation higher porosities are calculated when compared to the liquid replacement method. In the liquid replacement method, the construct is immersed in liquid and the volume retained in the biomaterials gives information about the porosity that can be accessed by liquid, representing possible cell infiltration. Thus, no pressure is applied as well as only open pores are diffused by liquid resulting in possible lower porosity values when compared to the traditional methods. Chang et al. used the liquid replacement method to calculate the porosity (of > 90%) combined with sonication for 5 mm thick and 10 mm in diameter gelatin hyaluronic acid scaffolds for adipose tissue engineering. As a replacement liquid ethanol was used, as it does not dissolve the used biomaterial. These workers also revealed that with increasing gelatin concentration the porosity declined (Chang et al., 2013) as seen by others (Imani et al., 2013). Thus, the application of a different liquid replacement solution, sonication, smaller scaffold with low gelatin concentration explained the higher determined porosity.

However, in present study the pre-frozen and freeze dried gelatin sponges showed a porosity < 90 % (48-65%, respectively). This porosity is lower when compared to other studies (Chang et al., 2013) and can be traced back to the different methods used for the investigation of the voids left by the pores. Through the application of the liquid replacement method a differentiation between open and closed pores for liquid was done. This cannot be made with porosity that is theoretically calculated. Further, no pressure was applied to force the liquid into the pores, therefore the measured values give information of the voids of the scaffold that can be seeded with cells, hence the cell assessable porosity. To obtain complete understanding of the cell and liquid access of the scaffold design, and the interconnectivity of the pores, the interconnectivity of the pores was investigated. Therefore, the scaffold was held in
place and liquid was passed through. The time was taken of the appearance of the first and last drop (Grimm and Williams, 1997). It was shown that the scaffolds support the flow through of liquid suggesting interconnectivity. Thus, the scaffolds maintain the requirement for diffusion by nutrients and waste removal. In addition, the feature to support the passing of liquid underpins the ability for the constructs utilisation in cell culture as well as in adipose tissue reconstruction.

Overall, the scaffolds designed were prepared with an optimised solvent casting particulate leaching method. This process was based on the method previously developed from our own group (Phull et al., 2013) and adjusted for the efficient application on bulk constructs. The further utilisation of different freezing systems emerged into three novel scaffolds (II-IV) which compared to the non-frozen construct (scaffold I), resembling the original Phull et al. biomaterial, showed similar macropore size, interconnectivity but different micropore dimensions and increased porosity (Figure 2.14).

The macropore size range would hypothetically assist the differentiation of ADSCs over preadipocytes towards lipid laden adipocytes of up to 190 µm (Skurk et al., 2007). This has to be confirmed in further cell based studies. The micropore size range was demonstrated to be dependent on the freezing temperature applied and therefore displays an element of control over the initial scaffold I (Kang et al., 1999, Mao et al., 2003, Lloyd et al., 2014, Imani et al., 2013). The range of gelatin sponges with different micropore size distribution represents a panel of bulk constructs that show size ranges which theoretically support cell infiltration as well as metabolite diffusion (Bellas et al., 2015, Korurer et al., 2014). The demonstrated interconnectivity of all constructs is an essential requirement for cellularised tissue engineered products. Respectively, through a connected porous network cell infiltration, migration throughout the constructs as well as metabolite diffusion is gained. A high porosity is desirable for cell infiltration, attachment, proliferation, differentiation and ECM deposition (Zhong et al., 2012).
Chapter 2: Scaffold preparation and characterisation

**Gelatin**

**Literature:** mechanical support, shape is adjustable, natural feel, bioactive, biocompatible, clinically applied, utilised in in vitro and in vivo investigations for adipose tissue reconstruction

**Macropore**

**Average size:** 1.8 mm, 1.7 mm, 1.61 mm in scaffold II, III and IV represented through histological analysis

**Designed:** to create space for cell infiltration (ADSCS, 22 µm), attachment, proliferation and differentiation (mature adipocytes, 190 µm), as well as assisting of angiogenesis

**Micropore**

**Different size distribution:** in scaffold II-IV gained through diverse freezing regimes (pre-freezing -20 °C, -80 °C with subsequent freeze drying or freeze drying -55 °C)

**Pore diameter:** of 3.55 µm up to 159 µm, confirmed through serial sections combined with histological investigations

**Designed for:** support of mechanical stability as well as cell infiltration (ADSCS, 22 µm), interconnectivity, metabolite diffusion

**Porosity**

**Porosity:** up to 65% of the scaffold volume investigated through the liquid replacement method and prepared through the microporous macroporous biomaterial structure via particulate leaching, different freezing regimes and subsequent freeze drying

**Designed:** to support cell seeding as well as cell infiltration, migration, attachment, proliferation, differentiation and metabolite diffusion

**Liquid uptake**

**Average:** 0.5 ml of liquid can be taken up by the scaffolds, determined through biomaterial swelling

**Designed:** to support cell-media uptake and therefore to assist cell seeding.

**Interconnectivity**

**Liquid:** is able to pass through the constructs, confirmed through water passing the biomaterial

**Designed:** for support of cell infiltration, migration, metabolite diffusion

**Figure 2.14:** Overview of investigated design and structural features of the microporous macroporous scaffolds. The scaffolds are made of gelatin which is a widely investigated biomaterial in the tissue engineering society as well as for adipose tissue reconstruction due to its biomaterial characteristics. The constructs are characterised through their micropores and macropores which are designed to support cell infiltration, attachment, proliferation, differentiation and to provide mechanical support and interconnectivity for metabolite diffusion. In the present study, those structures were histologically investigated. Further, the interconnectivity as well as the manufactured porosity were confirmed through liquid diffusion as well as the liquid replacement method. These features are essential for cell infiltration, proliferation, differentiation and metabolite diffusion. The liquid uptake was determined via swelling properties of the gelatin constructs and was an important constant to be determined for later use in cell based studies, especially for cellular seeding abilities.
Chapter 2: Scaffold preparation and characterisation

The support of these features through a tissue engineered biomaterial are necessary to reconstruct the volume and cellular components of lost tissue. Therefore, scaffold II-IV are selected for the application in further cell based studies, over the initial scaffold I. Thus, these scaffolds display novel constructs, advanced in the degree of porosity as well as microporosity size distribution, when compared to the initial scaffold I. Consequently, they can be investigated within cell based studies for the selection of the most promising scaffold for adipose tissue reconstruction.

2.4.4 Conclusion

In this study a novel range of bulk microporous macroporous scaffolds for subsequent application in adipose tissue reconstruction were produced. The macropores are made by inclusion of alginate beads within a gelatin matrix and subsequent leaching, leaving large pores behind up to 3.09 mm in diameter. The micropores imparted by a process of freezing and freeze drying to provide pores with diameters in the region of 3.55 μm up to 159.18 μm. This has been achieved by following:

1. The optimisation of the scaffold preparation. The alginate bead formation was analysed and optimised (0.5% (w/v) alginate cross linked with 1.5% (w/v) CaCl$_2$). The dissolution solution of the alginate bead was adjusted to 10% (w/v) trisodium citrate. The application method of the dissolution solution into the scaffold to remove the alginate beads was also tailored. From now on the direct injection of the trisodium citrate solution with a syringe into the scaffold was applied. Additional washing steps were added to the scaffold preparation to maintain interconnectivity. Through the optimisation process a controlled, time saving and efficient scaffold production method was developed.

2. Different microporous macroporous scaffold variations were created for the first time using ice as porogen to gain controllability of the scaffold porosity. The total 4 different new scaffolds were produced and characterised – initial scaffold I, -20°C and -80°C pre-frozen scaffold II and III and the freeze dried version scaffold IV of the initial scaffold. The constructs were characterised, in micropore and macropore size range, liquid uptake, porosity and interconnectivity. Hereby, scaffold II-IV
Chapter 2: Scaffold preparation and characterisation

revealed increased porosity as well as liquid uptake when compared to the initial scaffold I. High porosity as well as liquid uptake is advantageous in the uptake of cells and diffusion with nutrients. Therefore, the initial scaffold will be excluded in further cell based studies.

3. This study confirmed that the produced scaffolds show physical attributes such as high porosity, increased liquid uptake, diffusion ability and pore size to potentially act as cell based scaffolds for adipose tissue reconstruction.

The observed micropore size range of 3.55 μm up to 159.18 μm has the potential to allow the infiltration of ADSCs (22 μm). Furthermore, ADSCs would be able to migrate through interconnectivity into the macropores which have the possibility to produce a niche for cell proliferation and differentiation towards adipocytes (190 μm) if used in conjunction with a ECM delivery system. Waste and nutrient removal and infiltration would be possible as well. Further, the repeatability of the pore size range alteration through different freezing temperatures is also present. The physical properties fullfill the ideal requirements for the physical scaffold design for adipose tissue reconstruction. However, their biological suitability has to be tested to confirm acquired knowledge and confirm the scaffold design. Furthermore, long term incubation with cells would provide some initial insights into the degradation behaviour of the scaffolds. Therefore, the following chapter deals with the second aspect of tissue engineering; the cell source and its encounter with the scaffold design.
Chapter 3: Scaffold design and its interaction with adipose derived stem cells (ADSCs)

3.1 Introduction

The existence of stem cells in adipose tissue was first described by Zuk and co-workers in 2001 (Zuk et al., 2001). The group isolated cells from lipoaspirate termed processed lipoaspirate cells (PLA) and revealed their MSCs properties of proliferation and differentiation. A year later, Zuk et al. reported that PLA cells express a immunophenotype similar to MSCs (Zuk et al., 2002). Henceforth, those cells became recognised as MSCs of the adipose tissue. In 2004 at a meeting of the International Fat Applied Technology Society a common nomenclature was agreed to refer to the stem cells of the adipose tissue as ASCs or ADSCs (Gimble et al., 2007). To date ADSCs are a popular cell source for clinical and tissue engineering use (Zuk, 2013). The first clinical use was described in 2004 by the group including Lendeckel. Lendeckel et al. applied ADSCs in combination with fibrin glue to augment a calvarial defect in a 7 year old girl with satisfactory outcome (Lendeckel et al., 2004). ADSCs are also utilised clinically in cell enriched lipotransfer which improves clinical results as described in section 1.1.1 (Yoshimura et al., 2008a).

In the tissue engineering field ADSCs are used, amongst other cell types, in the development of novel methods for bone, cartilage and adipose tissue reconstruction (Zuk, 2013). As ADSCs are abundant cells found in the adipose tissue and can be isolated with minimal invasive procedures, they are considered a remarkable cell source for tissue engineering (Zuk et al., 2002, Bunnell et al., 2008, Zuk, 2013, Bourin et al., 2013). Furthermore, ADSCs are known to differentiate towards the adipogenic lineage, are easy to culture and can contribute to angiogenesis, which
makes them an attractive cell source for adipose tissue engineering specifically (Brayfield et al., 2010, Zuk, 2013).

In adipose tissue engineering, as generally in tissue engineering, there must be a coordination between the cell type and the engineered scaffold (Gomillion and Burg, 2006) including the choice of cell and the designing of a scaffold component appropriate to the demand of the cell source to facilitate tissue regeneration. Therefore, the scaffold should replace the lost tissue in terms of shape and mechanical properties (Gomillion and Burg, 2006). Additionally, the scaffold should provide a platform for cells to remain viable and proliferative to restore lost tissue volume (Gomillion and Burg, 2006, Placzek et al., 2009, Wong et al., 2010). The scaffold design should also support cell infiltration and even distribution throughout to ensure homogenous tissue formation (Carrier et al., 1999, Martin et al., 2004, Hemmrich et al., 2005b, Hong et al., 2006, Weinand et al., 2009). Carrier et al. compared engineered cardiac tissue formation using neonatal rat heart cells seeded on polyglycolic acid scaffolds with cardiac tissue. Through the use of bioreactors a homogenous cell distribution was achieved. It was revealed that the engineered tissue had similar ultrastructural characteristics as the native cardiac tissue (Carrier et al., 1999). Weinand et al. demonstrated good tissue integration when chondrocytes were observed to be distributed through an engineered scaffold via application of different seeding techniques combined either with a cartilaginous or a PLGA scaffold (Weinand et al., 2009). In the field of adipose tissue regeneration Hemmrich et al. assessed ADSC uptake throughout a hyaluronic acid based scaffold. In an in vivo mouse model this scaffold displayed vessel formation in all layers of the construct as well as cell penetration and distribution (Hemmrich et al., 2005b). Hong et al. illustrated homogenous adipose tissue formation after implantation of a gelatin scaffold seeded throughout with ADSCs (Hong et al., 2006). Therefore, essential features of successful scaffold design include appropriate cell infiltration and distribution throughout the construct, maintenance of viability, proliferation and when stem cells are used the preservation of stemness.

Another important feature in adipose tissue regeneration of bulk defects is the slow degradation of the scaffold while the donor and host cells reconstruct the lost tissue components. Cellular regeneration of fat grafts was displayed to take at least 12
months (Yoshimura et al., 2011). Therefore, constructs for adipose tissue reconstruction should display a slow degradation rate in vitro. A slow in vitro degradation rate would indicate a slow in vivo degradation rate. This would, therefore, commensurate with in vivo adipose tissue regeneration (Wang et al., 2013b, Chang et al., 2013, Bellas et al., 2015). Chang et al. investigated gelatin/hyaluran scaffolds for the use in adipose tissue reconstruction. They showed that the constructs support ADSC proliferation and viability in in vitro studies, performed for 21 and 28 days. Furthermore, the scaffolds also displayed a slow degradation rate with 10% degradation within 12 weeks when subjected to PBS. Subsequent in vivo studies in mouse and porcine models showed that the constructs supported tissue integration, adipogenesis as well as angiogenesis within 8 weeks. In the analysed time frame the construct structure was still recognisable (Chang et al., 2013). The group around Bellas et al. analysed silk foams as a material for adipose tissue regeneration. The constructs showed a slow degradation over 90 days when subjected to collagenase with a degradation rate ≤ 40%. Follow up in vivo studies in rats confirmed the slow degradation of the biomaterials while cell and tissue remodel of the construct was observed within 90 days (Bellas et al., 2015). Contrastingly, Wang et al. prepared PLGA/chitosan scaffolds which displayed a fast degradation rate within 14 days in in vivo mouse model. Despite success of the biomaterial to support adipogenesis and vascular infiltration, it could not assist in the restoration of volume due to material shrinkage, resembling the rapid degradation of the scaffold (Wang et al., 2013b). Accordingly, a scaffold for adipose tissue regeneration should ideally have a slow degradation rate while supporting cell proliferation and viability to assist corresponding cellular regeneration of the lost tissue.

### 3.1.1 Aim of the chapter

In the present study, scaffold II, III and IV revealed favourable pore size, porosity, interconnectivity as well as liquid uptake (detailed in Chapter 2). To assess the potential of the scaffolds for application in adipose tissue reconstruction, the investigation of features such as cell viability, proliferation, distribution, preservation of the ADSC phenotype and the construct degradation behaviour are the aim of this chapter. The aim of this chapter was, therefore, 1. The isolation and characterisation
of ADSC from abdominoplasty. 2. The assessment of combining the cells with the designed biomaterials.

Thus, ADSCs were seeded onto the scaffold materials as well as into the scaffold to assess cell viability through metabolic and live/dead assays. Cell proliferation was further investigated histologically. Through histological analysis, cell infiltration and distribution throughout the scaffolds was analysed. The preservation of ADSC phenotype when cultured within the scaffolds was evaluated by gene expression analysis. From this cell based study the most promising scaffold versions were subjected to a long term *in vitro* degradation study, to analyse the degradation behaviour of the selected biomaterials. Therefore, the constructs were seeded with cells and cultured *in vitro* for a period of 2 months. Here, the physical as well as morphological changes of the sponges were evaluated through histological analysis.


Chapter 3: Scaffold design and its interaction with ADSCs

3.2 Materials and Methods

3.2.1 Materials

The plastic and consumables were purchased from Greiner Ltd., UK unless stated otherwise.

3.2.2 ADSCs

3.2.2.1 Isolation and culture

Human subcutaneous adipose tissue was obtained with patient consent and full approval from the National Regional Ethics Service (REC: 06/Q1907/81) from discarded tissue during routine surgical procedures in the Queen Victoria Hospital and The McIndoe Centre, East Grinstead, UK.

The isolation of ADSCs was carried out as previously described in Zuk et al. and Bunnell et al. (Zuk et al., 2001, Bunnell et al., 2008). An excised skin sample was placed in a large petri dish (Thermo Fisher Scientific, UK) with the fat uppermost. Sterile scalpels were used to harvest the required amount of adipose tissue (1-2 cm$^2$) which was placed in a small petri dish with Hank’s Balanced Salt Solution (HBSS) (Life Technologies Ltd., UK). After 3 times washing of the sample by submerging and shaking it in Falcon tubes filled with HBSS with 5% v/v penicillin/streptomycin (Pen/Strep) (Life Technologies Ltd., UK), the adipose tissue was finely minced and incubated for 40 minutes in digestion buffer (DB) (0.075% w/v collagenase type 1 (Sigma Aldrich, UK), 2% Pen/Strep, in HBSS) at 37°C.

The digestion of the extra cellular matrix was stopped with 10 ml Dulbecco's Modified Eagle Medium (DMEM) (Gibco, UK) with 10% fetal calf serum (FCS) (Life Technologies Ltd., UK) and followed by straining the fluid through a 100 µm cell strainer (BD Bioscience, UK) into a 50 ml Falcon tube. A further 10 ml of medium was added to the digested material and strained through the cell strainer. The filtrate was centrifuged for 5 minutes at 2000 rpm to obtain a cell pellet consisting of ADSCs and other SVC. Due to their low density, mature adipocytes remained in the supernatant.
To mechanically separate ADSCs and the SVC from each other the cell pellet was disrupted by vigorous shaking. In this way, the remaining cell-cell contacts were broken and a mixed cell solution was obtained. Through a further centrifuge step for 5 minutes at 2000 rpm a cell pellet consisting of SVC, ADSCs cells and a supernatant containing floating low density adipocytes was obtained. The pellet was re-suspended in 3 ml DMEM 10% FCS medium with 1% v/v Pen/Strep and filtered through a 100 μm cell strainer (*BD Bioscience, UK*) into a sterile 50 ml Falcon to retain the larger SVF and cell debris in the strainer. The process additionally separates smaller sized ADSCs and mature red blood cells into the Falcon. The Falcon was washed with a further 2 ml medium, which was also strained into the clean tube. The contents of the Falcon were transferred into a 25 cm² culture flask. After 2 days the flask containing adhered cells was washed with HBSS to remove free-floating red blood cells as these do not adhere to TCP. This resulted in a population of adherent ADSCs in the flask. The medium was changed every 2 to 3 days. The ADSCs were maintained at 37°C and 5% CO₂. When 80-90% confluent, the cells were sub-cultured.

### 3.2.2.2 Sub-culturing

ADSCs were sub-cultured when they reached 80-90% confluence. The medium was aspirated and the cells were washed once with HBSS to remove remaining growth medium. The cells were detached through the addition of 2 ml of 0.05% Trypsin-EDTA (*Life Technologies Ltd., UK*) and incubating for 6 minutes at 37°C, 5% CO₂. After detachment of the cells, 3 ml medium was added to neutralise the trypsin. The trypsin medium mix was removed though centrifugation for 5 minutes at 1500 rpm. The cells were then re-suspended in 5 ml medium and counted (described in section 3.2.2.3). The suspension was split in half and added to two 75 cm² flasks and cultured at 37°C and 5% CO₂.

### 3.2.2.3 Cell count

After detaching of the cells from TCP as described above, cells were counted using a TC²⁰™ Automated cell counter (*BioRad Laboratories Ltd., UK*). In detail, 10 μl of the cell solution was transferred into counting slides provided by the manufacturer.
(BioRad Laboratories Ltd., UK). The slide was inserted into the TC20™ cell counter and the total amount of cells was counted within the allocated gate of 5 µm - 40 µm, the size range of ADSCs (Ryu et al., 2013).

3.2.3 Fibroblasts

3.2.3.1 Isolation and culture

Human skin was obtained with patient consent and full approval from the National Regional Ethics Service (REC: 06/Q1907/81) from discarded tissue during routine surgical procedures in the Queen Victoria Hospital and The McIndoe Centre, East Grinstead, UK.

Primary human fibroblast were isolated and cultured as described in (Harris et al., 2009). In detail, the skin was sterilised through wiping once with iodine solution (EcoLab, UK) and twice with 70% IMS (VWR International Ltd., UK). Thin skin was shaven with a dermatome (DeSoutter Medical Ltd., UK) to produce thin samples containing epidermis and dermis. The samples were washed 3 times by transferring them to a Falcon tube containing HBSS (5% Pen/Strep) and shaken vigorously. The washed samples were placed into dispase (4 mg/ml (Invitrogen, UK)) for 30 minutes to separate the epidermal from the dermal layer. The separation was completed by shaking the skin vigorously which mechanical facilitates the detachment of the layers. The resulting dermal layer was washed in HBSS (5% Pen/Strep) and transferred to a Falcon tube containing 0.5% collagenase (Sigma Aldrich, UK) for 2-3 hours at 37°C. The separation of fibroblasts from the surrounding dermal matrix was stopped by adding of DMEM containing 10% FCS. The media cell mix was strained through 50 µm strainer into a Falcon tube to separate fibroblasts from cell debris by size and centrifuged for 3 minutes at 1500 rpm. The DMEM collagenase mix was aspirated and the resultant cell pellet was re-suspended in DMEM (10% FCS). The cell medium mix was transferred into a cell culture flask and cultured at 37°C, 5% CO₂ with media change every 2-3 days. At confluence the cells were harvested and counted as described in 3.2.2.2 and 3.2.2.3.
3.2.4 Morphological analysis

Images of cells on TCP were taken utilising an inverted light microscope Nikon Eclipse TS 200 (Nikon, UK) applying the NIS-Elements F 2.20 software (Nikon, UK).

3.2.5 Flow cytometry

Cytometry is the analysis of chemical and physical characteristics of biological cells. Flow cytometry measures cell characteristics while cells flow in a stream of fluid through the instrument (Mandy et al., 1995, Saphiro, 2003). The characterisation is based on deflection and excitation of light. The light is deflected by the cells in different ways, which produces a forward scatter and side scatter. The forward scatter displays the light that is directly reflected and measures the shape and size of the cells. The side scatter reflects the granularity of the cells. In addition, cell specific antigens can be detected through labelling with an antibody conjugated to a fluorophore. The conjugated antibody binds to a cell specific protein. When light of particular wavelength is shone on the fluorophore it is excited and fluorescences. The fluorescent can be detected by a fluorescence detector (Haynes, 1988).

In order to prepare and label the ADSCs for flow cytometry, cells were harvested and counted (as detailed in section 3.2.2.2 and 3.2.2.3). After washing once in PBS to remove excess medium, cells were fixed in 4% v/v PFA for 10 minutes at room temperature, followed by blocking in 0.5% w/v Bovine Serum Albumin Fraction V (BSA) (Roche Diagnostic Ltd., UK) in PBS for at least 5 minutes at room temperature. The samples were re-suspended in staining buffer in PBS (0.1% w/v sodium azide (Sigma Aldrich, UK), 2% w/v BSA) and the concentration was adjusted to 3.5 x 10^5 cells per 80 µl for labelling with CD90 and CD29 and 90 µl for labelling with CD45 antibodies. From the cell suspension 80 µl or 90 µl was added to a 15 ml Falcon tube and mixed with 20 µl CD90 and CD29 or 10 µl CD45 fluorochrome-conjugated monoclonal antibody (Table 3.1) resulting in a total volume of 100 µl according to the manufacturer’s instructions (BD Pharmingen™, UK; Invitrogen, UK). The mixture was incubated on ice for 30–45 minutes and then washed with 2 ml wash buffer in PBS (0.1% sodium azide) and re-suspended in 500 µl PBS. The
analysis was carried out with an Accuri C6 ® Flow cytometer (BD Biosciences, UK) using the BD Accuri C6 software (BD Biosciences, UK). In all samples 20000 events were counted. The unlabeled control samples were visualised in the emission channel of the fluorochrome and gates were set on the emission border. The gates were applied on the labelled samples. Data values obtained were plotted in a bar chart using Microsoft Excel 2007 (Microsoft, UK).

Table 3.1: Flow cytometry antibodies used for the immunophenotype characterisation of ADSCs.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Dilution (concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC mouse Anti-Human CD29</td>
<td>BD Pharmingen ™, UK</td>
<td>Undiluted (0.05 mg/ml)</td>
</tr>
<tr>
<td>PE Mouse Anti-Human CD90</td>
<td>BD Pharmingen ™, UK</td>
<td>1:150 (0.2 mg/ml)</td>
</tr>
<tr>
<td>Human CD45 Alexa Fluor® 700</td>
<td>Invitrogen, UK</td>
<td>Undiluted (1.000 mg/ml)</td>
</tr>
</tbody>
</table>

3.2.6 Differentiation potential

The differentiation capacity of ADSCs and fibroblasts was assessed through cultivation in lineage specific differentiation media: adipogenic media, osteogenic media and chondrogenic media, followed by staining for the presence of lineage specific markers.

3.2.6.1 Adipogenic differentiation

The adipogenic differentiation medium contained 0.5 mM isobutylmethylxanthine, 50 μM indomethacin, 0.5 μM dexamethasone in stromal medium (DMEM, 10% FCS) (Life Technologies Ltd., UK) (Zuk et al., 2001, Bunnell et al., 2008). The medium was applied for 10 days to ADSCs passage 3 and fibroblast with medium exchange every 2-3 days.

3.2.6.2 Chondrogenic differentiation

For chondrogenic differentiation, 5 ml stromal medium was supplemented with 6.25 μg/ml insulin, 10 ng/ml TGF-β1, 50 μg/ml ascorbate-2 phosphate, 1% Pen/Strep and 10% FCS (Life Technologies Ltd., UK) (Zuk et al., 2001, Bunnell et al.,
2008). ADSCs passage 3 and fibroblasts were exposed to differentiation medium for 20 days. The medium was changed every 2-3 days.

### 3.2.6.3 Osteogenic differentiation

Osteogenic differentiation was carried out on ADSCs passage 3 and fibroblasts for 30 days using the StemPro® Osteogenesis Differentiation Kit (Life Technologies Ltd., UK). The medium was supplemented prior to application with the osteogenesis supplement solution as per the manufacturer’s instructions. Medium was changed every 2-3 days.

### 3.2.7 Confirmation of multilineage differentiation

Passage 3 cells were cultured in differentiation media until morphological differences could be observed microscopically (accordingly to section 3.2.4), followed by staining with specific markers. Oil-Red-O stain was used to stain the lipid droplets of maturing adipocytes (Ramírez-Zacarías et al., 1992). Alcian blue stain was employed to stain acidic polysaccharides present in chondrocytes (Zuk et al., 2001). Alkaline phosphatase stain was utilised to illustrate activated phosphatase in osteoblasts using Sigma Aldrich (UK) Leukocyte Alkaline Phosphatase Kit.

### 3.2.7.1 Oil-Red-O staining

A 1% (w/v) Oil-Red-O solution was prepared with 0.35 g Oil Red O (Sigma Aldrich, UK) in 100 ml isopropanol and left to dissolve overnight in a fume hood. The precipitate was filtered and the clear solution was mixed with 0.5 volume of distilled H₂O. Before use, the solution was filtered again, to fully remove any Oil-Red-O particles which had not dissolved. After 10 days the differentiation medium (described in section 3.2.6.1) was aspirated from the cells and replaced with PBS. Following the wash step, cells were fixed in 4% v/v PFA (Sigma Aldrich, UK) for 1 hour and subsequently rinsed once in PBS and twice in H₂O. The 1% Oil-Red-O solution was added for 1–2 h to the cells until intracellular lipid droplets were visible. After washing three times in water to remove excess dye the cells were viewed under the light microscope (Nikon Eclipse TS1000).
3.2.7.2 Alcian blue staining

Cells were cultured up to 20 days in chondrocyte media (outlined in section 3.2.6.2) until the characteristic morphological cell shape of chondrocytes was visible. The cells were fixed in 4% v/v PFA for 15 minutes at room temperature, followed by staining with alcian blue (Sigma Aldrich, UK) by addition of 1% alcian blue in 0.1 M HCl (pH 1.0) for 30 minutes. The excess stain was removed with 0.1 M HCl (Sigma Aldrich, UK). To show the cell specific morphology, a counter stain with H + E was performed (detailed in section 3.2.7.2.1) and cells visualised via light microscopy (Nikon Eclipse TS100).

3.2.7.2.1 Haematoxylin and eosin counter staining

Cells were submerged in filtered Haematoxylin (Sigma Aldrich, UK) for 30–60 seconds. The Haematoxylin was removed through washing with H2O before staining with 1% aqueous Eosin solution (Sigma Aldrich, UK) for 4-6 minutes. Cells were then dehydrated through rinsing in increasing alcohol levels and washing in Xylene (Sigma Aldrich, UK) for 4 and 6 minutes to remove the remaining alcohol and to gain clear imaging. The stained cells were analysed using light microscopy (Nikon Eclipse TS100).

3.2.7.3 Alkaline phosphatase staining

To stain for alkaline phosphatase, a Leukocyte Alkaline Phosphatase Kit (Sigma Aldrich, UK) was used according to the manufacturer’s instructions. The cells were fixed with citrate-acetone-formaldehyde solution, washed with water and then immersed in alkaline dye mixture and incubated for 15 minutes at room temperature. After the removal of the dye mixture a counter stain with haematoxylin solution Gill No3 included in the kit followed. The cells were analysed by light microscopy (Nikon Eclipse TS100).
3.2.8 ADSC seeding on scaffold, culture and harvest

3.2.8.1 ADSCs seeding and culture

ADSCs Passage 3 were used for all scaffold studies. The cells were harvested and counted according to section 3.2.2. A dilution of 300,000 cells/ml was mixed and transferred into a 15 ml Falcon. The mix was centrifuged at 1500 rpm for 5 minutes to obtain a cell pellet containing 300,000 cells. The medium was aspirated and the cell pellet was re-suspended in 500 µl medium. This applied volume of 500 µl resembles the average liquid that can be taken up by the scaffolds (detailed in Chapter 2, section 2.3.2.3.1). The cell solution was applied to the scaffolds in 12 well plates by dripping the dilution on the top of the scaffolds. The scaffolds were incubated for 30 minutes at 37°C and 5% CO₂, to allow uptake and attachment of cells. Afterwards, the scaffolds containing ADSCs were removed to cell culture spinner bottles (Integra, UK) containing 50 ml media at 37°C and 5% CO₂. The spinner bottles were set on CELLSPIN spinner platforms (Integra, UK) containing a drive united (the platform) located inside the incubator and an external control unit. The control unit was set on continuous rotation at 15 rpm for the time of cultivation. Every 2-3 days 25% of the medium was changed.

3.2.8.2 Scaffold harvest

The scaffolds seeded with cells were removed after 10 days from the spinner bottles and transferred to 12 well plates, for easier handling. The medium was aspirated and the scaffolds washed with PBS. The scaffolds were cut in half with a scalpel. One half was fixed with 4% PFA for histological analysis while the other half was processed for RNA extraction.

3.2.9 ADSC infiltration and distribution throughout the different scaffolds

3.2.9.1 Direct immunohistochemistry

Immunohistochemistry is an antibody-based method to detect specific molecules in tissues. The direct method is the use of labelled antibodies that bind specifically to the molecule or protein of interest. These antibodies can be applied on frozen and
fixed sections and observed microscopically through immunofluorescent microscopy (Kroese, 2001).

The scaffolds were frozen (described in section 2.2.4.3.1) and cut into 30 µm sections using a Bright Cryostat (Bright Instrument Co. Ltd., UK). The sections were allowed to dry 30 minutes at room temperature and 30 minutes at 37°C. Fixation was performed with acetone (Sigma Aldrich, UK) at 4°C for 10 minutes, followed by three washes with PBS for 3 minutes, then blocked with horse serum (Vector laboratories Ltd., UK) for 30 minutes prior to the incubation in ActinRed™ 555 ReadyProbes® Reagent Kit (Life Technologies Ltd., UK). The ActinRed™ 555 ReadyProbes® Reagent Kit contains F-actin antibodies conjugated red-orange fluorescent dye tetramethylrhodamine. The antibody solution was diluted (2 drops in 1 ml 1% BSA), prior to application. The sections were washed for 3 minutes in PBS followed by H₂O to remove unbound antibody. The slides were dried and mounted with ProLong® Gold Antifade Mountant with DAPI (Life Technologies Ltd., UK). The stained slides were left to dry over night in the dark and viewed with the Axio Scope A1 fluorescent microscope (Carl Zeiss Ltd., UK) using Axiovison 4.8 software (Carl Zeiss Ltd., UK).

3.2.9.2 Cell infiltration studies

The infiltration of cells was investigated through histological analysis. ADSCs were seeded overnight in the different scaffolds as described in 3.2.8.1. Serial sections were cut from the centre of each scaffold moving out towards the periphery with sections taken at 1000 µm intervals, to produce cuts from at least 4 areas of each construct. Cells were identified by nuclear staining through mounting in ProLong® Gold Antifade Mountant with DAPI (Life Technologies Ltd., UK). At least 4 images per area were taken with the Axio Scope A1 fluorescence microscope (Carl Zeiss Ltd., UK).

3.2.9.3 Cell distribution in micropores and macropores

The distribution of the ADSCs cultured in the micropores and macropores of the different scaffolds was histological investigated. Therefore, sections were cut from
the central area of each scaffold and stained with Actin Red™ Ready Probes® Reagent (*Life Technologies Ltd., UK*) (specified in section 3.2.9.1). At least 4 random images per scaffold were taken with the fluorescent microscope Axio scope A1 (*Carl Zeiss Ltd., UK*) at a magnification of 20x. The location was allocated as macropore or micropore according the pore size (macropores ≥ 1mm (outlined in Chapter 2)).

3.2.10 ADSCs viability and proliferation studies

3.2.10.1 ADSCs viability on scaffold materials

Tissue culture plates were coated with the scaffold materials and cell viability was tested after 1 day of cell culture.

3.2.10.1.1 Scaffold material coated plates

The materials used to produce the scaffold (gelatin, cross linked gelatin) were used for coating 24 well plates. Wells were coated with 1 ml 7% w/v gelatin (*Sigma Aldrich, UK*) in PBS to mimic the scaffold gelatin. To test for the viability of ADSCs in the cross linked biomaterial, wells were coated with 1 ml 7% w/v gelatin in PBS cross linked with 0.014 µl/ml glutaraldehyde (25% w/v) (*Sigma Aldrich, UK*). The coated wells were sterilised through washing with 70% ethanol, followed by rinsing in sterile water to remove remaining ethanol and air dried over night. The total coating volume was 300 µl for each well. The plates were seeded with ADSCs and viability assessed compared to TCP as described in following sections.

3.2.10.1.2 ADSC viability

To test the ADSC viability the cell counting kit -8 (CCK-8) (*Fluka, UK*) was applied. The Kit is based on water soluble tetrazolium salt (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2 H-tetrazolium, monosodium salt, WST-8) which is reduced by dehydrogenases of cells to formazan dye that appears yellowish in colour. Therefore, the formazan dye is proportional to the number of living cells (*Berridge et al.*, 2005).
The coated plates were seeded with 1 ml cell medium mix containing 10,000 cells/ml and left overnight to allow cell attachment and proliferation. The analysis followed the next day with the CCK-8. In detail, the medium was removed from the cells and replaced with 1 ml fresh medium followed by the addition of 100 µl of CCK-8 solution and incubated for 4 hours at 37°C, 5% CO₂. Afterwards, 100 µl of CCK-8 media mix from each well was transferred to a corresponding well in a 96 well plate. The absorption was measured at 450 nm with the reference wavelength 650 nm using the Sunrise™ plate reader (Tecan, UK). The absorption values were compared to a standard curve as outlined below.

### 3.2.10.1.3 Standard curve evaluation

To obtain a standard curve covering lower and higher cell numbers, cell dilutions were prepared with known cell numbers of 0, 1000, 3000, 8000, 10000, 12000, 15000, 20000, 50000 and 90000. The dilutions were transferred in 24 well plates and left to attach overnight at 37°C, 5% CO₂, prior to adding the reagent. The next day the medium in each well was replaced with 1 ml of fresh medium and 100 µl CCK-8 solution. After 4 hours of incubation at 37°C, 5% CO₂, 100 µl of the CCK-8–media mix was transferred to a 96 well plate and the absorption was measured at 450 nm with a reference wavelength of 650 nm using Sunrise™ plate reader (Tecan, UK). A standard curve was plotted and a trend curve was applied using Microsoft Excel 2007 (Microsoft, UK). The formula obtained was applied for quantifying cell numbers.

### 3.2.10.2 Live/Dead assay of ADSCs

The viability of ADSCs cultured in the different scaffold variations was evaluated with the Live/Dead® Cell Imaging Kit (488/570) (Life Technologies Ltd., UK) (Chumbler et al., 2012). The Kit is based on 2 dyes, a cell-permeable dye (calcein AM) to stain living cells in green and a cell-impermeable dye which stains dead cells red. Calcein AM can enter the cells where it is converted by the intracellular esterase to green fluorescent calcein. The cell-impermeable dye cannot enter living cells but can access dying cells with a compromised cell membrane the dye than binds to the
DNA and fluoresces in red (Cheung *et al.*, 2014, Lin *et al.*, 2014). Hence, living cells can be observed with green fluorescence and dead cells fluorescent in red.

To utilise the assay for this study, the different scaffolds were seeded with 0.5 ml of 500,000 cells/scaffold and cultured in spinner flasks for 10 days (detailed description in section 3.2.8.1). The scaffolds were cut into 3 pieces to obtain a middle, a bottom and a top sample. The three samples of each scaffold variation were treated with the Live/Dead Assay according the manufacturer’s instructions (*Life Technologies Ltd.*, UK). Specifically, after thawing the live (green) and (red) dead dye at room temperature the live green solution was mixed with the dead red reagent to create a 2x stock. The stock was applied to the scaffold ADSC culture within 2 hours of preparation, followed by 15-20 minutes incubation at room temperature. Images were taken with the Nikon Eclipse TS200 using a fluorescent light source combined with a green emission fluorescein isothiocyanate (FITC) filter and a red emission rhodamine filter (*Nikon, UK*) to visualise the amount of live green and dead red cells.

### 3.2.10.3 Cell proliferation

To analyse the proliferation ability of ADSCs cultured in the different scaffold variations, cell proliferation assays such as CCK-8 and Alamar blue (*Life Technologies Ltd.*, UK) could not be used. The formazan dye and reduced resazurin dye were absorbed and trapped inside the scaffolds resulting in false read outs. Instead cells were counted using Image J (Schneider *et al.*, 2012).

The number of nuclei of ADSCs in the different scaffolds was counted and compared between culture for 1 day and 10 days. Specifically, ADSCs were cultured in the different scaffold variations over night or for 10 days and harvested according to section 3.2.8.

Images stained as outlined in section 3.2.9.1 of the central area of the different scaffolds were analysed. The pictures were investigated using Image J. In detail, the background of the DAPI nuclear stain channel images was removed, to produce a clear picture of the nuclei (Figure 3.1A). To obtain a higher contrast and clearer visualisation of the nuclei the threshold was further adjusted. This converted the
image into a black and white format. The nuclei were illustrated in black (Figure 3.1B). Dense areas of nuclei were separated by processing them in a binary modus and applying watersheds. Watershed is a function of Image J where borders of neighbouring particles are made clearer. The nuclei were counted through the particle analysis function of the Image J using a pixel range of 80-300 depending on the nuclei size (Figure 3.1C). The size of visualised individual nuclei can be affected by the plane of focus in which the image is acquired. Therefore, nuclei of cells that are located sideways in the section appear smaller in the images compared to cell nuclei that are frontal positioned. The number of nuclei obtained was plotted using Microsoft Excel 2007 (Microsoft, UK).

![Figure 3.1: Cell count analyses of cells seeded for (A) 1 day and (B) 10 days in the scaffolds by Image J. For the cell count analysis black and white images of the cell nuclei were analysed. The background of the images was removed through the threshold function of Images J to gain a clear contrast picture with black nuclei on white background. On this adjusted Images the particle count function of the program was applied which automatically counted the black dots (nuclei). The counted nuclei of (A) were compared with the nuclei counted in (B) to determine the cell proliferation over 10 days in the scaffolds. Scale bars represent 100 µm. Figure was created after (Allan et al. 2009).](image)

### 3.2.11 Quantitative real time analysis (qRT-PCR)

To analyse the differentiation potential of ADSCs towards the adipogenic, the chondrogenic and the osteogenic lineage in the different scaffolds, quantitative real time polymerase chain reaction (qRT-PCR) analysis was performed. The expression of the cluster of differentiation 90 (CD90), peroxisome proliferator activated receptor gamma (PPARG), glucose transporter type 4 (GLUT 4), transcription factor SOX 9 (SOX 9), runt related transcription factor 2 (Runx 2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were analysed. CD90 is expressed in stem cells and is commonly used as a stem cell marker for ADSCs (Tapp et al., 2009). PPARG expression occurs during early adipogenesis, in pre-
adipocytes, and is therefore used in this study as a pre-adipocyte marker (Choi et al., 2010b, Bellas et al., 2013b). Mature adipocytes express GLUT 4, therefore it is used as a late adipogenesis marker (Bellas et al., 2013b). SOX 9 and Runx 2 are key transcription factors in the early chondrocyte and osteoblast differentiation (Komori 2006, Hino et al. 2014). GAPDH expression is constant in ADSCs, in early as well as in the late phase of adipogenesis, therefore it is utilised as reference gene (Yu et al., 2013). The expression of these genes was analysed to gain an understanding of the effect of the different scaffold versions on spontaneous differentiation on ADSCs (Choi et al., 2010a, Yu et al., 2013) and is described below.

3.2.11.1 RNA Isolation

RNA was isolated from cells in the different scaffolds after 10 days in culture according to the manufacturer’s protocol of the RNA isolation Kit - RNeasy Mini Kit (Qiagen Ltd., UK). In detail, scaffolds were cut in half and washed in PBS, prior to lysing the cells directly inside half of each scaffold. The other half was used for histological analysis and fixed in 4% PFA (section 3.2.8.2). The other half was treated with lysis buffer to lyse the cells located inside the scaffold. The lysate was directly pipetted into the QiAshredder spin column system and centrifuged. After homogenisation the lysate was transferred to a silica spin column system where the RNA binds to the silica membrane. The RNA was eluted in 30 µl RNase free water after 3 washing steps with wash buffers. The concentration of RNA was measured with the Nanodrop 2000 (Thermo Scientific, UK).

3.2.11.2 cDNA synthesis (reverse Transcription)

The cDNA synthesis was performed with the Iscript™ cDNA Kit (BioRad Laboratories Ltd., UK) with 100 ng of RNA template using the Mini Opticom MJ Mini™ Personal Thermal cycler (BioRad Laboratories Ltd., UK). The reaction mix and program are shown in Table 3.2 and Table 3.3. The concentration of cDNA was measured to confirm successful synthesis using a Nanodrop 2000 (Thermo Scientific, UK).
Table 3.2: Reaction mix Iscript™ cDNA Kit

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcriptase Mix</td>
<td>4 µl</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>1 µl</td>
</tr>
<tr>
<td>Water</td>
<td>adjustable µl</td>
</tr>
<tr>
<td>RNA</td>
<td>100 ng</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20µl</strong></td>
</tr>
</tbody>
</table>

Table 3.3: Cycling parameters cDNA synthesis

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>5min</td>
</tr>
<tr>
<td>42°C</td>
<td>30min</td>
</tr>
<tr>
<td>85°C</td>
<td>5min</td>
</tr>
</tbody>
</table>

**hold 45 minutes at 4°C**

3.2.11.3 Quantitative PCR (qPCR) reaction

The cDNA obtained by reverse transcription was used as a template for the quantitative PCR (qPCR) reaction. The SsoAdvanced™ Universal SYBR® Green Supermix (BioRad Laboratories Ltd., UK) with the fluorochrome SYBR Green was employed for the PCR. When the fluorochrome binds to double-stranded DNA the fluorescence is amplified by 1000 times above the signal of the unbound version. This signal was measured at a wavelength of 480 nm (Zipper et al., 2004). Pre-validated primer sets from BioRad Laboratories Ltd. (UK) were utilised (Table 3.4) to detect CD90, GLUT 4, PPARG and GAPDH gene expression. The method was carried out according to the manufactures protocol with the master mix as displayed in Table 3.5. The procedure was also performed according to the minimum information for publication of qRT- PCR experiments (MIQE guidelines) (Bustin et al., 2009).
Table 3.4: List of qRT-PCR primers used.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrimePCR assay CD90 (THY-1)</td>
<td>BioRad Laboratories Ltd., UK</td>
</tr>
<tr>
<td>PrimePCR assay PPARG</td>
<td>BioRad Laboratories Ltd., UK</td>
</tr>
<tr>
<td>PrimePCR assay GLUT 4 (SLC2A4)</td>
<td>BioRad Laboratories Ltd., UK</td>
</tr>
<tr>
<td>PrimePCR assay GAPDH</td>
<td>BioRad Laboratories Ltd., UK</td>
</tr>
<tr>
<td>PrimePCR assay SOX 9</td>
<td>BioRad Laboratories Ltd., UK</td>
</tr>
<tr>
<td>PrimePCR assay RUNX 2</td>
<td>BioRad Laboratories Ltd., UK</td>
</tr>
</tbody>
</table>

Table 3.5: Master mix protocol using SsoAdvanced™ Universal SYBR® Green Supermix (BioRad Laboratories Ltd., UK).

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SsOAdvanced™ Universal Supermix</td>
<td>5µl</td>
</tr>
<tr>
<td>PrimePCR assay</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>3.5 µl</td>
</tr>
<tr>
<td>cDNA sample</td>
<td>1µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10 µl</strong></td>
</tr>
</tbody>
</table>

The CFX 96™ Real time System C1000 Touch™ Thermal cycler (BioRad Laboratories Ltd., UK) was used for all qPCR reactions with following program (Table 3.6):

Table 3.6: Thermal cycler programme used for qPCR reaction (adapted from BioRad Laboratories Ltd. manual).

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>95°C</td>
<td>2 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>5 seconds</td>
<td>40</td>
</tr>
<tr>
<td>Annealing/extension</td>
<td>60°C</td>
<td>30 seconds</td>
<td>40</td>
</tr>
<tr>
<td>Melt curve</td>
<td>65°C - 95°C</td>
<td>5 seconds/step</td>
<td>1</td>
</tr>
</tbody>
</table>

3.2.11.4 Melting curve analysis

The limitation in the use of the SYBR Green fluorochrome is that it can bind to nonspecific DNA double strands such as primer dimers. Therefore, melting curve analysis followed after the amplification step which is achieved by continually increasing the temperature from 65°C up to 95°C. In this way, nonspecific binding is visible at lower melting temperatures, which is indicated by additional peaks at
lower temperatures. A peak at a higher temperature occurs in the case of specific binding. Using this method, it was confirmed that the primers used in this study were specific.

### 3.2.11.5 Quantification of gene expression using quantitative PCR (qPCR)

The qPCR is based on exponential amplification. The first few cycles (3-15) serve for threshold calculation, resulting from the background fluorescence. The signal that exceeds this threshold is called the threshold cycle (Ct) and characterised through an exponential increase. After this exponential increase the fluorescent signal transits to a plateau phase due to the exhaustion of nucleotides and polymerase contained in the reaction mix. According to the Ct-values measured during the exponential phase the relative expression of the gene of interest (Relative expression (RQ)) can be calculated as follows:

First, the Ct value of the sample (Ct\text{sample}) was normalized through subtraction of the Reference-Gene Ct (Ct\text{REF}) value.

\[
\text{Delta Ct} = \text{Ct}_{\text{sample}} - \text{Ct}_{\text{REF}} \quad \text{(Equation 8)}
\]

To obtain the ratio of expression, the difference in Ct values was calculated between the normalised sample and the normalised control sample (cells grown on TCP). This is called delta delta Ct. The fold change was then calculated as relative difference in expression between the cells grown in the different scaffold versions and cells grown on TCP. The calculation was performed as followed after (Livak and Schmittgen, 2001):

\[
\text{Delta Delta Ct} = \text{Delta Ct}_{\text{sample}} - \text{Delta Ct}_{\text{TCP}} \quad \text{(Equation 9)}
\]
\[
\text{RQ Delta Delta Ct} = 2^{\Delta \text{Ct}} \quad \text{(Equation 10)}
\]

Data values were plotted using using SigmaPlot® 10 (Systat, USA).
3.2.12 Long term \textit{in vitro} study

The long term \textit{in vitro} study was conducted to investigate the cellular degradation of selected scaffold variations within 54 days of culture.

3.2.12.1 Scaffold seeding, culture and harvest

Scaffolds were seeded with $3 \times 10^5$ cells/scaffold. The decreased cell number when compared to the studies described in 3.2.10.2, is due to the restricted availability of cells after harvest and subculture. The seeded scaffolds were cultured in growth medium for 2 months and harvested as outlined in 3.2.8.

3.2.12.2 Histological analysis

Subsequent direct immunohistochemistry studies for the actin cytoskeleton were conducted as described in 3.2.9.1.

3.2.13 Statistical analysis

All experiments were performed at least 3 times with cells isolated from different donors to account for biological variation. A p-value of $p \leq 0.05$ was set as the threshold for statistical significance and calculated applying the 2-tailed and unpaired student’s t-test using Microsoft Office Excel 2007 (\textit{Microsoft, UK}).
3.3 Results

3.3.1 ADSCs

ADSCs are described as fibroblast-like cells, which show the two main characteristics of multipotent stem cells - self-renewal and the ability to differentiate towards multiple lineages of the same germ layer (Zuk et al., 2001, Zuk et al., 2002, Bourin et al., 2013). ADSCs are of mesodermal origin and therefore capable of differentiating towards the osteogenic, chondrogenic and adipogenic lineages (Zuk et al., 2001, Bunnell et al., 2008). To verify the phenotype of cells used in this study their morphology was assessed. Furthermore, flow cytometry was conducted to determine the expression of cell surface antigens, which are characteristic for ADSCs. Additionally, the ability of the cells to differentiate towards the adipogenic, chondrogenic and osteogenic lineage was investigated to confirm their stem cell potential.

3.3.1.1 ADSC specific morphology

To assess the fibroblast-like morphology of the ADSCs, cells isolated according the ADSC isolation protocol (see section 3.2.2.1) were cultured to passage 3 and analysed under the light microscope (Nikon Eclipse TS1000). They were compared with fibroblasts of similar morphology.

As shown in Figure 3.2 cells isolated from fatty tissue and expanded in tissue culture displayed the characteristic fibroblast morphology.
Figure 3.2: ADSC show a fibroblast like morphology. Representative pictures of ADSCs passage 3 and freshly isolated fibroblasts in low (10x) and high (20x) magnification. Both cell populations display a fibroblast-like cell shape. Scale bars represent 100 μm.
3.3.1.2 ADSC specific antigen profile using flow cytometry

ADSCs have a specific antigen profile; they are known to be CD90+, CD29+ and CD45- (Zuk et al., 2002, Tapp et al., 2009, Konno et al., 2013, Bourin et al., 2013). CD90 and CD29 are expressed in mesenchymal stem cells, including ADSCs (Bourin et al., 2013, Konno et al., 2013). ADSCs are negative for CD45 which distinguishes them from cells of the haematopoietic lineage (Bourin et al., 2013). Flow cytometry was performed to confirm that cells isolated from subcutaneous adipose tissue display this characteristic ADSC immunophenotype. Therefore, the surface antigen profile of isolated cells was analysed. In detail, flow cytometry was conducted on cells cultured until passage 3 for CD90, CD29 and CD45 antigens. For each antigen test 3.5 x 10^5 cells were used (described in section 3.2.5).

Figure 3.3A displays cells isolated from adipose tissue containing a CD29 and CD90 positive cell population. In total 97% of passage 3 cells tested (Figure 3.3C) expressed both CD90 and CD29 surface antigens. Thus, it confirms that cells isolated from adipose tissue express mesenchymal stem cell surface antigens. Furthermore, Figure 3.3B and C illustrate the lack of CD45 antigen in the cell population, which confirms that the cells are not originating from the haematopoietic lineage. This proves the antigen fingerprint of ADSCs which are reported to be CD90, CD29 positive and negative for CD45.

However, fibroblasts also express antigens that were investigated and are similar to ADSCs that are found in the SVF of the adipose tissue (Tapp et al., 2009). Throughout the isolation of ADSCs, fibroblast contamination is possible. Therefore, to ensure the work was undertaken with a single cell population of ADSCs and to clearly distinguish them from fibroblasts which show the same morphology and surface antigen expression as ADSCs, further differentiation studies were performed.
Figure 3.3: Isolated cells from adipose tissue express ADSCs antigen profile CD90+, CD29+ and CD45-. (A) The CD90 and CD29 expression of passage 3 cells isolated from adipose tissue as single staining in a representative histogram and together in a 2D dot plot including applied gates. The gates (red borders) were set in the emission border of unlabeled samples. Following the application on labeled cells, it visualised the emission of the antigen stained cells (quantitative number (red) in right hand corner of diagram) in single channels (histogram) and 2 channels (dot plot). (B) The staining for ADSCs negative surface antigen CD45 as histogram and 2D dot plot including applied gates, using the same gating strategy as in (A). (C) The quantitative expression of CD90, CD29 positive ADSCs marker and CD45 negative marker. Flow cytometry was carried out with passage 3 cells isolated from fatty tissue with n=3.
3.3.1.3 ADSC differentiation

To characterise the cells isolated from human adipose tissue, differentiation studies towards different mesodermal lineages were carried out. The differentiation study should also distinguish ADSCs from fibroblasts which have similar morphology (Figure 3.2) and similar appearance of surface antigens (Tapp et al., 2009). Both cell types were exposed to differentiation medium to assess their ability to differentiate towards the adipogenic, chondrogenic and osteogenic lineages. The cells were cultured in lineage specific differentiation medium until morphological changes in the ADSC culture were visible, followed by lineage specific staining (described in section 3.2.6 and 3.2.7). Adipose differentiation was stopped at 10 days, chondrogenic differentiation at 20 days and osteogenic differentiation at 30 days (Figure 3.4-3.6) for ADSCs as well as for fibroblasts.

ADSCs at passage 3 could be successfully differentiated towards the three mesodermal lineages (Figure 3.4-3.6). After 10 days the lipid droplets of maturing adipocytes were clearly visible with Oil-Red-O staining of triglyceride (Figure 3.4). Differentiation towards the chondrogenic lineage took 20 days and revealed a spindle-like cell morphology containing acidic polysaccharides, which are stained by alcian blue (Figure 3.5). Osteoblast-like cells appeared at approximately 30 days in the differentiation medium. The active alkaline phosphatase of these osteoblasts was stained and confirmed the differentiation of ADSCs towards this lineage (Figure 3.6). The control cells cultured in growth medium still displayed the fibroblastic morphology of ADSCs and fibroblasts. Furthermore, the control cells did not stain either for lipids, acidic polysaccharides nor alkaline phosphatase (Figure 3.4-3.6). This indicated the non-differentiation of cells cultured in growth medium.

Fibroblasts cultured in adipogenic media developed a rounder morphology when compared to positive control but did not stain positive for lipid droplet accumulation. The differentiation of fibroblasts in chondrogenic medium led to cluster forming cells without acidic polysaccharides. When cultured for 30 days in osteogenic medium, fibroblasts became confluent with similar morphology to the positive control. Also they did not accumulate alkaline phosphatase. The lack of
positive lineage specific staining confirms that fibroblast cannot differentiate along these mesodermal lineages. This proves the phenotype of cells isolated from fatty tissue as ADSCs, capable of differentiation, whereas fibroblasts did not display comparable properties, despite similarities in morphology and expression of surface antigens (Tapp et al., 2009).

Overall, it was demonstrated that the cells isolated from adipose tissue and used in this study express ADSC specific surface antigens and are able to differentiate towards different lineages (chondrogenic, osteogenic and adipogenic lineage). Furthermore, through the differentiation study fibroblasts and ADSCs could clearly be distinguished. Thus, possible culture contamination with fibroblasts can be excluded, which confirms the cells used in this study are ADSCs.
Figure 3.4: Differentiation towards adipogenic lineage shows lipid accumulation. Passage 3 ADSCs and fibroblasts were differentiated in differentiation media towards the adipogenic lineage for 10 days. The differentiation to maturing adipocytes is visible through lipid accumulating cells compared to cells grown in differentiation medium. Intracellular lipid was stained using Oil-red-O. Scale bars represent 100 µm. NON = growth medium, DIFF = differentiation medium.
Figure 3.5: Differentiation towards chondrogenic lineage and staining for acidic polysaccharides. Passage 3 ADSCs and fibroblasts were differentiated in differentiation media towards the chondrogenic lineage for 20 days. During chondrocyte differentiation intracellular acidic polysaccharides accumulated. Hence, the different cell lineages were stained with alcian blue for acidic polysaccharides with H + E counter stain. Scale bars represent 100 µm. NON = growth medium, DIFF = differentiation medium.
Figure 3.6: Differentiation towards osteogenic lineage and detection of alkaline phosphatase. Passage 3 ADSCs and fibroblasts were differentiated in differentiation media towards the osteogenic lineage for 30 days. Cells were stained for alkaline phosphatase which is present in differentiating osteoblasts. Scale bars represent 100 μm. NON = growth medium, DIFF = differentiation medium.
3.3.2 ADSC vs. the different scaffolds

3.3.2.1 Cell seeding of the different scaffolds

The purpose of this subsequent analysis was to evaluate the ability of the different scaffolds to retain the cells, which is an important factor in the tissue engineering process (Langer and Vacanti, 1993, Patrick, 2001, Choi et al., 2010a). Therefore, $3 \times 10^5$ ADSCs were seeded apically onto the scaffolds as specifically described in 3.2.9. The data gave insight into how the change in scaffold characteristics achieved through the modifications in the fabrication process (outlined in Chapter 2) affected the ability to retain cells. In addition, it should give information about the cell distribution throughout the scaffolds. To assess this, the distribution of cells throughout the scaffold and more precisely within either micropores or macropores was assessed (Figure 3.8-3.9).

To analyse the ability of the different scaffolds to support cell infiltration despite their high porosity they were seeded with cells ($3 \times 10^5$) (detailed in 3.2.8.1). The cells should infiltrate the scaffold and be found throughout the construct to ensure homogenous tissue regeneration (Martin et al., 2004).

Figure 3.7 represents the uptake of ADSCs in the biomaterials. Directly after seeding, most of the medium cell mix was taken up by the scaffolds. The pre-frozen scaffolds showed spontaneous absorbance of the cells. Specifically, as shown in Figure 3.7 after seeding the scaffolds, most of the liquid was absorbed by the biomaterials. This indicates uptake of the cell media mix into the scaffolds. In addition, it was observed that after overnight culture the freeze dried scaffolds were swollen and felt softer, more closely resembling soft tissue. Furthermore, the scaffolds illustrated a wetter morphology compared to the freshly seeded once, indicating re-hydration.
Figure 3.7: Uptake of ADSCs into the different scaffolds. ADSCs passage 3 were delivered in culture medium into the scaffolds to assess the ability of the scaffolds to retain cells. The scaffolds after seeding and overnight culture in growth media located in wells of a 12 well plate are shown. Scaffold II = -20°C pre-frozen scaffold, Scaffold III = -80°C pre-frozen scaffold, Scaffold IV =
3.3.2.2 Cell distribution throughout the different scaffolds

The cells seeded into a scaffold should ideally infiltrate the biomaterial and be distributed throughout it. This makes the process of newly forming tissue in and around the scaffold uniform and enhances matrix deposition (Martin et al., 2004). To measure cell infiltration, cells were cultured overnight in the scaffolds. The scaffolds were seeded with $3 \times 10^5$ ADSCs and sectioned from the middle extending towards the outer periphery, every 1000 µm. This yielded 4 different areas to assess. Cells were stained with DAPI to visualise cell nuclei in each layer (outlined in 3.2.8.1 and 3.2.9.2).

Cells were detected in each sectioned layer as represented in Figure 3.8. This confirms that the scaffolds are able to retain cells not just on the surface but throughout the entire construct.
Figure 3.8: Cell distribution throughout the scaffolds. The cell distribution throughout the scaffolds is presented. ADSCs were seeded into the scaffolds. (A) After overnight culture the scaffolds were frozen and sections were taken from the middle onwards every 1000 µm to obtain at least 4 areas (blue 1-4). (B) Sections of each area were stained with DAPI to assess the distribution of cells in the layer. Representative pictures are shown here. Scale bars represent 100 µm. green = gelatin scaffold, white = nucleus. Scaffold II = -20°C pre-frozen scaffold, Scaffold III = -80°C pre-frozen scaffold, Scaffold IV = freeze dried scaffold.
3.3.2.3 Cell distribution within micropore and macropore structure of the different scaffolds

The distribution of the cells within the micropores and macropores was investigated. The cells should ideally be found in both macropores and micropores to allow homogenous distribution and tissue regeneration (Martin et al., 2004). According to the pore size characterisation (outlined in Chapter 2) the cells should be able to infiltrate within the micropores and macropores of the different versions of the scaffold. Therefore, $3 \times 10^5$ ADSCs per scaffold were seeded and cultured for 10 days in the biomaterials as detailed in section 3.2.8. Sections from the centres of the scaffolds were immunostained with actin to visualise the cells (described in section 3.2.9.1). Random pictures were taken and the cells were allocated to micropore or macropore according the size differences of the pores (outlined in section 3.2.9.3). This delivers valuable information of the cell distribution in the microporous macroporous structure of the scaffolds.

Figure 3.9A illustrates the assessment of the cell distribution within the micropores and macropores. Through actin immunostaining the cells were visualised and able to be localised. A large amount of cells ($77\% \pm 23\%$) are observed in the macropores compared to the smaller amount of cells ($23\% \pm 23\%$) located in the micropores of scaffold II (Figure 3.9A, B). In contrast, in scaffold III 40\% ($\pm 26\%$) of the cells were localised in the micropores, but the majority is located in the macropores of the biomaterial ($60\% \pm 26\%$). Scaffold IV showed an even distribution of the cells within the microporous ($50\% \pm 10\%$) and macroporous structure ($50\% \pm 10\%$) (Figure 3.9A, B).

In conclusion, cells are located in the micropores as well as in the macropores of the different scaffolds. The main location of cells in scaffold II was the macropores. An almost 40\%: 60\% distribution of cells in micropores to macropores was found in scaffold III. An even cell distribution was supported by the design properties of scaffold IV.
Figure 3.9: Cell distribution in micropores and macropores of the different scaffolds. ADSCs passage 3 were delivered in the different scaffolds. After 10 days of culture 4 random pictures were taken of the microporous macroporous structure of the different biomaterials. Cell appearance either in micropores or macropores was noted and is represented in (A) with n = 30. (B) Representative pictures of micropores and macropores with cells are shown. Scale bars represent 50 µm. Scaffold II = -20°C pre-frozen scaffold, Scaffold III = -80°C pre-frozen scaffold, Scaffold IV = freeze dried scaffold. green = gelatin scaffold, blue = nucleus, red = actin
3.3.2.4 ADSC viability and proliferation studies

The next step was to analyse the suitability of the scaffold materials and the scaffolds for ADSCs culture. Therefore the viability of the cells when cultured on the different materials used to produce the scaffolds was tested. Furthermore, a viability study was conducted on ADSCs within the scaffolds for a time frame of 10 days (Figure 3.10). To complete the study, the proliferation ability of ADSCs within the scaffolds was investigated (Figure 3.11). The information about the viability and proliferation capacity of ADSCs on the scaffold materials and within the scaffolds is essential to ascertain the suitability of the biomaterials and different scaffolds to support cell growth and survival.

3.3.2.4.1 ADSCs viability on the scaffold materials

To assess the ADSC viability on the materials used for the production of the different scaffolds, ADSCs (1 x 10^4 cells/ml) were seeded on TCP coated with the materials used for the scaffold preparation (detailed in section 3.2.10.1.1). The cells were left overnight to attach. The following day the metabolic activity of the cells was detected as described in 3.2.10.1.2, to illustrate the immediate effect of the materials on ADSCs. Gelatin cross linked with glutaraldehyde has been shown by others to have a growth retarding effect on ADSCs which lasts one week (Korurer et al., 2014) but can be detected in culture as early as within one day. Therefore, this experiment was vital to check if the gelatin cross linked with glutaraldehyde used in this study would show a similar effect.

Viability was measured on plates coated with gelatin and gelatin cross linked with glutaraldehyde. There was no significant difference in cell number between the different materials (Figure 3.10A). Here, an average of 9,040 (± 4925.91), 8,000 (± 2831.18) and 10,000 (± 3222.33) cells were measured when seeded overnight on gelatin, cross linked gelatin and on plastic with same initial seeding density of 10,000 cells. The observed slight decrease in ADSC number on gelatin, cross linked gelatin and TCP can be traced back to the high biological variability of the primary cell source.
Consequently, the scaffold material gelatin cross linked with glutaraldehyde was therefore shown to support ADSC culture. No growth retarding effect on ADSCs was observed. Therefore, the scaffold materials are clearly suitable for the development of an ADSC culture system.

However, the cells are not cultured in a 2D environment in the scaffold, but instead in a 3D environment, where they are surrounded by the materials. Therefore, additional viability studies were undertaken with ADSCs cultured in the different scaffolds. Here, cell viability on the single materials and a cultivation time of 10 days was chosen to underpin cell viability within the scaffolds. To analyse the viability of the ADSCs when cultured in the different scaffold versions for a time frame of 10 days, live and dead assay was carried out. The live and dead assay was applied as the metabolic reagents used to assess cell viability in 2D studies are absorbed by the scaffolds, yielding false results. Scaffolds II-IV, which were shown to be most suitable for further investigations with ADSCs (outlined in Chapter 2), were seeded apically with $5 \times 10^5$ cells and cultured for 10 days. The application of the live/dead assay as described in 3.2.10.2 followed.

Figure 3.10B represents live/dead staining of the centre sections of the different scaffold versions. Despite the interference of the autofluorescence of the gelatin, it was possible to detect more living green than red fluorescent dead cells in all scaffolds and all areas assessed.

The viability study with materials and the different scaffold versions clearly showed that ADSCs can be cultured on the materials and within the scaffolds. Furthermore, cells are viable after 10 days of culture in the scaffolds.
Figure 3.10: Cell viability on scaffold materials and in the different scaffolds. (A) The viability of ADSCs cultured on the scaffold materials (gelatin, cross linked gelatin) overnight. The cell viability was assessed using the CCK-8 proliferation assay. (B) The cell number $x$ was calculated with the help of a standard curve, the formula was determined through the application of a trend line with a coefficient of regression $R^2 = 0.9979$. Error bars indicate standard deviation. $n = 3$. TCP served as control. (B) Live and dead assay was performed on ADSCs cultured for 10 days within the different scaffold versions. The green fluorescence displays living cells and red dead cells. Cells cultured on TCP either treated with ethanol or not served as negative or positive control. White arrows are pointing at dense areas of living cells. Scaffold II = -20°C pre-frozen scaffold, Scaffold III = -80°C pre-frozen scaffold, Scaffold IV = freeze dried scaffold. Scale bars represent 500 µm.

Material vs. cells

![Material vs. cells](image)

Standard curve CCK-8

![Standard curve CCK-8](image)

Scaffold II

![Scaffold II](image)

Scaffold III

![Scaffold III](image)

Scaffold IV

![Scaffold IV](image)

Control (+)

![Control (+)](image)

Control (-)

![Control (-)](image)
3.3.2.4.2 Cell proliferation within the different scaffolds

For successful tissue reconstruction, cells should be retained inside the scaffold and continue to proliferate to ensure long-term bulking of tissue (Patrick, 2001, Choi et al., 2010a). Therefore, the proliferation ability of ADSCs cultured in the different scaffold types was evaluated. Thus, $3 \times 10^5$ ADSCs per scaffold were seeded and cultured in the biomaterials. After 1 day and 10 days of culture the scaffolds were harvested. Sections from the centre of each scaffold were immunostained with DAPI and actin to localise the cells. The centre is the most difficult part to reach sufficient nutrient supply. If proliferation occurs there the scaffold design counteracts the necrosis seen in the application of large soft tissue fillers such as fat grafts (Dong et al., 2015). Further, it favours the application of the scaffolds in larger soft tissue defects. Four random pictures of the centre area of each scaffold type were taken.

The cell number of 1 day and 10 days in culture was evaluated and compared using Image J image analysis to quantify the number of cell nuclei (detailed in section 3.2.10.3). The time frame of 10 days was chosen to give cells enough time to proliferate. Day 1 is the day after seeding the scaffolds and day 10 the harvest day.

Despite non-significant differences in the proliferation rate of ADSCs within the scaffolds visible trends were observed (Figure 3.11). In detail, the average cell number after 10 days was noticeably doubled in all scaffolds (Figure 3.11A-D), representative images are shown in Figure 3.11D.

Overall, it was shown that cells cultured for 10 days display the ability to proliferate in the centre of the different scaffold version.
Figure 3.11: ADSC proliferation within the different scaffold versions. ADSCs passage 3 were cultured for 1 day and 10 days in the different scaffolds. (A-C) The cell count at day 1 and day 10 in the different scaffolds as quantified using Image J is represented. Error bars indicate standard deviation. Three scaffolds of each type were analysed through counting the nuclei of 4 random pictures taken from the central area of each scaffold tested. (D) Representative images of ADSCs cultured for 1 and 10 days in the different scaffolds. Yellow = auto fluorescence of gelatin scaffold, red = actin, white = nucleus. Scale bars represent 100 µm. Scaffold I = -20°C pre-frozen scaffold, Scaffold II = -80°C pre-frozen scaffold, Scaffold IV = freeze dried scaffold.
3.3.2.5 ADSC phenotype preservation within the different scaffolds

In the next step, the potential of the scaffolds to support the preservation of the ADSC phenotype was analysed by adipogenic, chondrogenic, osteogenic as well as stem cell marker gene expression. This gives an insight into the ability of the different scaffold environments to preserve ADSC stemness or to trigger adipogenic, chondrogenic or osteogenic differentiation of the ADSCs. The preservation of ADSCs stemness without further influencing the differentiation is advantageous. Thus, the scaffolds can be used for ADSC delivery and culture. Directed adipogenesis can be controlled through addition of differentiation factors. Therefore, ideally the scaffolds should not support spontaneous differentiation.

For this purpose, 3 x 10^5 ADSCs were seeded and cultured for 10 days in the different scaffolds. RNA was isolated and gene expression for the stem cell marker CD90, the pre-adipocyte gene PPARG and the adipocyte marker GLUT 4 was assessed. As controls, ADSCs cultured on TCP with and without adipogenic differentiation medium were utilised. GAPDH gene expression was used to normalize the Ct values. Gene expression was quantified as fold-change in expression compared to cells grown on TCP without differentiation (detailed in section 3.2.11).

No significant change in the CD90 expression between ADSCs cultured within the scaffold and on TCP was noted. Gene expression analysis revealed a CD90 expression of 0.4 - 0.6 fold (± 0.003, ± 0.28, ± 0.07) when ADSCs were cultured with growth media in the different scaffolds. Whereas, ADSCs cultured with differentiation medium on plastic (described in Figure 3.12 as DIFF) showed a non-significant increase of 1.3 (± 0.45) fold change in the expression of the stem cell marker (Figure 3.12A).

The expression of the pre-adipocyte gene PPARG was increased by 33 fold (± 8.03) in cells cultured with differentiation medium compared to ADSCs cultured on TCP. No significant change in expression was observed for cells cultured in the different scaffolds (Figure 3.12B).
A significant difference was observed however, in the GLUT 4 expression of cells cultured with differentiation medium, with a fold change increase of 96 (±4.41, p=0.02). Cells cultured in the different scaffold versions showed no significant change in GLUT 4 expression compared to cells cultured on TCP. ADSCs cultured in scaffold II displayed a slight decrease to 0.44 fold (± 0.29). A fold change of 0.35 (± 0.07) was observed for ADSCs cultured in scaffold III. When cultured in scaffold IV the ADSCs displayed 0.78 fold expression (± 0.30) compared to cells on TCP. Therefore, the GLUT 4 expression in cells in the scaffolds was not significantly different from cells grown on TCP (Figure 3.12C).

Additionally, to exclude the capacity of ADSCs to spontaneously differentiate towards other mesodermal lineages when cultured in the different scaffolds, gene expression analysis for chondrogenic and osteogenic genes were performed. Hence, the gene expression of the chondrogenic marker SOX 9 and the osteogenic marker RUNX 2 was assessed (Choi et al., 2012, Bourin et al., 2013, Açil et al., 2014, Roxburgh et al., 2015). No significant change in the expression of these genes was observed when ADSCs were cultured in the scaffolds compared to cells cultured on TCP (Figure 3.12D,E).

In summary, the different scaffold environments do not induce spontaneous differentiation of ADSCs towards the adipogenic, chondrogenic or osteogenic lineage in a time frame of 10 days. Further, the cells retain their stem cell phenotype. Consequently, the scaffolds support the preservation of ADSCs for at least 10 days.
Figure 3.12: Multilineage gene expression of adipose stem cells cultured in the different scaffolds. ADSCs passage 3 were cultured for 10 days in the different scaffolds. (A) The gene expression of the stem cell marker CD90 and (B, C) the adipogenic differentiation marker PPARG and GLUT 4 was investigated. (D, E) Gene expression for chondrogenic differentiation marker SOX 9 and osteogenic differentiation marker RUNX 2 is represented. The gene expression was assessed using qRT-PCR. GAPDH was utilised for normalisation. The fold change was calculated to ADSCs grown on TCP. ADSCs cultured in differentiation medium on plastic (DIFF) were used as positive control for adipogenic differentiation. Error bars represent standard deviation. All experiments were performed with n= 3.
3.3.3 Long term *in vitro* study

To investigate the degradation, hence the structural change of the scaffolds as well as the cellular behaviour within the gelatin sponges over time a preliminary long term *in vitro* study was conducted. This study was carried out on scaffold III and IV due to their favourable performance in the cell distribution study when compared to scaffold II (outlined in section 3.3.2.3). Here, the cells were shown to distribute throughout the microporous macroporous biomaterial structure of scaffold III and IV in almost even (40%: 60%) and even (50%: 50%) manner. This would ascertain homogenous tissue regeneration within *in vivo* setting (Martin *et al.*, 2004). Despite similar performance of the design feature of scaffold II in cell infiltration, viability, proliferation and differentiation the even distribution of the cells within the structure was not given (distribution of 23%: 77% within the microporous macroporous structure). Thus, even tissue regeneration in *in vivo* setting cannot be guaranteed. Therefore, scaffolds III and IV were seeded with $3 \times 10^5$ cells/scaffold and cultured in growth medium over a time frame of 56 (2 months) days as described in section 3.2.12.1. Subsequent histological analysis of the scaffold structure as well as ADSC cytoskeleton stain was performed to investigate the microporous and macroporous construct structure and ADSC morphology (specified in 3.2.12.2). The time frame of 2 month was chosen to investigate the structural and cellular remodelling of the scaffolds over a longer duration than so far tested 10 days. Further, Chang *et al.* showed slow gelatin/hyaluran scaffold degradation within 2 month *in vivo* studies in mouse and porcine models. The slow biomaterial degradation demonstrated positive effects on tissue integration as well as adipose tissue regeneration (Chang *et al.*, 2013). Thus, a slow degradation rate while cell remodelling of the lost tissue takes place is advantageous in adipose tissue engineering.

The porous scaffold structure was retained after 56 days of *in vitro* culture as presented in Figure 3.13. Small micropores ($< 160 \, \mu m$) within the gelatin walls as well as larger macropores ($\geq 1 \, mm$) were clearly recognisable when compared to scaffolds cultured for 1 day with ADSCs in growth medium. At the same time structures were revealed which were noticeably different as demonstrated in Figure 3.13. Micropores of scaffold III were more uniform and spherical when compared to scaffolds cultured for 1 day (Figure 3.13, 3.14). Furthermore, the microporous as
well as the macroporous structure of scaffold IV appears fibrous when related to constructs cultured for a shorter time (Figure 3.13, 3.14). ADSCs were present in high numbers in the centre of the scaffolds. Specifically, they were located within the biomaterial structures filling the large macropores and displaying a spherical morphology (Figure 3.13).

Thus, scaffold III and IV cultured over 56 days with cells, presented structurally stable parts as well as parts which are subjected to degradation and remodelling. ADSCs within both scaffolds filled the macropores and adopted a spherical shape. The fact that the cells filled the macropores within the centre of the sponges suggests that the constructs support not just cell infiltration but also cell proliferation as well as metabolite diffusion for the duration of at least 56 days. These preliminary results are promising due to ADSC populating and filling the porous structure. Thus, the scaffold design features for the support of ADSC survival, infiltration and viability are confirmed. Furthermore, the observation of the cells populating the porous structure, correlates with the investigated slow degradation rate of the biomaterials. The monitored desirable slow degradation is displayed in structural unstable and stable parts of the construct. Here, a slow degradation rate is favourable to provide enough time for the cellular regeneration of the lost tissue.
Figure 3.13: Microporous macroporous scaffold structure is retained after 56 days in vitro culture. The microporous macroporous structure of scaffold III and IV is represented at day 1 and after 56 days of in vitro ADSC culture in growth medium. Green = scaffold structure, Scale bars represent 200 µm.
Figure 3.14: Porous scaffold structure is remodelled by ADSCs. Represented are scaffold III and IV after 56 days in in vitro culture with ADSCs. Different magnifications (low (10x) and high (20x)) of cells within the microporous macroporous gelatin structure are displayed. ADSCs were stained for cytoskeleton protein actin and cell nucleus. Green = scaffold, red = actin, blue = DAPI. Scale bars represent 100 µm (10x) and 50 µm (20x).
3.4 Discussion

For a successful tissue engineering approach to a clinical problem, the scaffold as well as the cell source and its behaviour within the scaffold are key parameters. Therefore, the general requirements for the cell source for tissue engineering are:

1. The cell source should be compatible with the host.
2. Easy availability through minimally invasive procedures and culture in bulk are desirable.
3. The cells should be able to proliferate to restore tissue volume.
4. The cells should be able to provide or differentiate into tissue specific cells that resemble the native tissue.

(Gomillion and Burg, 2006, Wong et al., 2010, Bauer-Kreisel et al., 2010).

ADSCs are a promising and popular cell source in adipose tissue engineering. They fulfil the requirements of an ideal cell source for adipose tissue engineering. ADSCs are mesodermal stem cells found in abundance in the fatty tissue (Zuk et al., 2001), are available through minimally invasive methods and are able to be cultured and differentiated into lineages of mesodermal origin. In the context of tissue engineering, they should show viability and proliferation potential within the scaffold and patient surroundings (Gomillion and Burg, 2006). Furthermore, the cells should infiltrate and settle throughout the scaffold to provide even tissue distribution and enhance matrix deposition (Carrier et al., 1999, Hemmrich et al., 2005b, Martin et al., 2004, Hong et al., 2006, Thevenot et al., 2008). Another important feature for adipose tissue regeneration of bulk defects is the slow degradation of the scaffold while the donor and host cells reconstruct the lost tissue components (as described in 1.3.1.3.2). The aim of this chapter was to test the ability of the scaffolds described in Chapter 2 to retain ADSC and promote adipose tissue reconstruction. This cell based study will further help to select the most promising scaffold out of the tested ones for further ECM based studies. For this purpose, cell seeding, distribution, material-cell biocompatibility, proliferation, differentiation and biomaterial degradation were analysed. To achieve this, cells isolated from subcutaneous abdominal tissue were first characterised to confirm their ADSC phenotype. The cell seeding and
distribution throughout the scaffold was investigated next by histological analysis. The materials used in the scaffold manufacturing process were then tested for biocompatibility in combination with the cells by using viability assays and proliferation analysis on the scaffold materials in isolation and within the scaffolds. The potential to preserve the ADSC stem cell phenotype during scaffold culture was studied by gene expression analysis. Finally, the most promising scaffolds were selected and tested within a preliminary long term in vitro study on their degradation behaviour.

3.4.1 ADSC characterisation

ADSCs are fibroblast-like cells that express mesodermal stem cell marker such as CD90 and CD29 and are negative for haematopoietic lineage marker such as CD45. Due to their mesodermal origin they are able to differentiate towards the adipogenic, chondrogenic and osteogenic lineage (Zuk et al., 2001, Zuk et al., 2002, Bourin et al., 2013, Zuk, 2013).

Our study confirmed that cells isolated from abdominal tissue show fibroblast-like morphology and express CD90 and CD29 antigens. The cells are negative for CD45, which distinguishes them from the haematopoietic lineage. This phenotype is consistent with work shown by other groups (Zuk et al., 2002, Tapp et al., 2009, Konno et al., 2013).

ADSCs are known to differentiate towards the mesodermal lineages, which are adipogenic (fat tissue), osteogenic (bone) and chondrogenic (cartilage). Therefore, the differentiation towards these 3 lineages was tested and achieved through culture in osteogenic, chondrogenic and adipogenic differentiation media. The specific media contained established lineage specific differentiation factors (Zuk et al., 2001, Zuk et al., 2002, Bunnell et al., 2008). Adipogenic differentiation is known to be induced by isobutylmethylxanthine, dexamethasone and insulin (Pittenger et al., 1999, Zuk et al., 2001). Adipogenic culture media containing these components induced lipid droplet accumulation characteristic of adipocytes. The droplets were stained with Oil-Red-O and confirmed the differentiation to the adipogenic lineage. Chondrogenic differentiation was induced with TGF-β which supports the
expression of collagen II and cartilage specific glycoproteins (Hennig et al., 2007). The lineage specific staining was performed with alcian blue which stained chondrocyte associated sulphated proteoglycans in blue (Zuk et al., 2001, Bunnell et al., 2008). Osteogenic differentiation was induced with a commercially available medium probably containing ascorbic acid (Pittenger et al., 1999, Zuk et al., 2002). When ADSCs were cultured in this differentiation medium alkaline phosphatase activity was visible which indicates osteogenic differentiation.

Others used similar media for differentiation and displayed similar results regarding staining, morphology and differentiation time (Zuk et al., 2001, Zuk et al., 2002, Bunnell et al., 2008, Roxburgh et al., 2015). Due to the morphological and immunophenotype similarities to ADSCs, fibroblasts were also differentiated towards the same lineages (Tapp et al., 2009). Results revealed a clear difference in cell morphology and an inability to differentiate into the different lineages. Thus, despite having a similar morphology, it was possible to show that the cells used in this study were indeed ADSCs and not fibroblasts.

Overall, it was confirmed that the cells (ADSCs, cell culture passage 3) display stem cell characteristics typical for ADSCs as used widely in the adipose tissue engineering community (Zuk et al., 2001, Zuk et al., 2002, Bunnell et al., 2008, Tapp et al., 2009, Brayfield et al., 2010, Konno et al., 2013, Wang et al., 2013b, Phull et al., 2013, Açil et al., 2014, Cheung et al., 2014, Korurer et al., 2014) and that they differ from fibroblasts. This confirms that the cells isolated from human adipose tissue are a homogenous ADSC cell population. Therefore, the cells isolated from human adipose tissue could be used for further studies.

3.4.2 Cell seeding and distribution of ADSCs throughout the different scaffolds

The aim of this analysis was to assess the suitability of the scaffold design to support cell uptake, cell infiltration and distribution throughout the construct. If the scaffold design would not support cell uptake, tissue regeneration through cell proliferation and differentiation could not be achieved. Additionally, if cells could not infiltrate and distribute evenly within the scaffold, even tissue regeneration would not be guaranteed as observed by Yu et al. (Yu et al., 2013). Yu et al. manufactured porous
foams based on decellularised adipose matrix. Histological investigations revealed uneven cell distribution restricted to the scaffold surface. In subsequent \textit{in vivo} rat studies no expected increase in adipogenesis and vessel infiltration of the cell seeded compared to the unseeded constructs was observed (Yu \textit{et al.}, 2013). Consequently, the cell harbouring and distribution within the scaffold are important perquisites for construction a tissue engineered scaffold. If they are not fulfilled this means exclusion of the scaffolds from further studies. Thus, this analysis also assists the selection of the most suitable scaffold out of the three scaffolds obtained through different freezing temperatures for further in-depth analysis.

Therefore, ADSCs were seeded into the scaffolds to test the uptake of cells. It was shown that scaffolds II-IV absorb the cells spontaneously like a sponge. This is due to rehydration of the lyophilised sponges. This rapid sponge-like absorption was seen before in freeze dried biomaterials and is frequently applied in the tissue engineering community (Hemmrich \textit{et al.}, 2005b, Lai \textit{et al.}, 2013, Lloyd \textit{et al.}, 2014). Lai \textit{et al.} used lyophilisation to prepare gelatin disks for corneal reconstruction seeded with corneal epithelial cells (Lai \textit{et al.}, 2013). Lloyd \textit{et al.} applied freeze drying to load collagen–glycosaminoglycan scaffolds with fibroblasts and keratinocytes as skin replacement (Lloyd \textit{et al.}, 2014). Further, for adipose tissue reconstruction, the dehydrating process was used in commercial hyaluronan benzyl ester collagen sponges subsequently coated with hyaluronic acid and freeze dried. In this way scaffolds with improved absorption were created (Hemmrich \textit{et al.}, 2005b). In the present study, the spontaneous absorption of cells marks an important advancement of the original gelatin scaffold, which did not absorb the cell solution well (Phull \textit{et al.}, 2013).

However, for the utilisation of the scaffolds in soft tissue reconstruction in clinical application, ADSCs should infiltrate throughout the scaffold to promote tissue formation evenly throughout the construct (Martin \textit{et al.}, 2004). To assess if this was indeed the case, the scaffolds were sectioned in 4 areas from the centre to the periphery. In each area, nuclei were detected by nucleic acid stain with DAPI. This confirmed that the cells can be found throughout the scaffold structure. Noticeable was that cells in the macropores were predominantly found aligned on the pore wall.
but not filling the large spaces. The alignment of cells on large pore walls but not filling the centre was observed by Bellas et al. as well (Bellas et al., 2015). This group constructed porous silk sponges different concentrations with pore sizes ranging from 300 µm - 500 µm. After 90 days in vivo rat studies the porous foams were completely infiltrated by cells and tissue (Bellas et al., 2015). Thus, the decreased surface area for cell attachment provided through the large pores (Murphy and O’Brien, 2010, Bellas et al., 2015) might not negatively affect in vivo settings. However, the cell distribution throughout the scaffold provides an optimal starting point for even adipose tissue regeneration and confirms that seeding through surface application is suitable for the cell delivery into the sponges. Further quantification studies of the cell number detected in each area would provide additional information of the even cell distribution throughout the scaffold as done in the proliferation study, described in section 3.3.2.4.2 and discussed in paragraph 3.4.3.

The distribution of the cells in either micropores or macropores was investigated. The designed structure should accommodate cell proliferation and differentiation. Therefore, the microporous structure of the scaffolds was altered through the application of diverse freezing techniques (outlined in detail in Chapter 2, section 2.3.2). The larger macroporous structures were designed by particle leaching of alginate beads to gain space for cell proliferation and differentiation. The micropores should also locate cells to maintain an even cell distribution and allow cell-cell contact for matrix deposition and communication.

Scaffold II supported the location of the majority of cells in the macroporous structure. Scaffold III and IV illustrated a 40%: 60% and even cell distribution respectively in micropores and macropores. The observed distribution relates to the pore sizes characterised in this study (specified in Chapter 2). Scaffold II had a narrow size range from 3.55 µm to 30.96 µm located in thick gelatin walls separating the macropores. The pore size makes the cell infiltration possible. But the space theoretically only can harbour small numbers of ADSCs due to their average size of 22 µm in diameter (Ryu et al., 2013). Consequently, cells accumulated in the macropores in greater quantities than in the other two scaffolds. This heterogeneous internal cell distribution is a disadvantage in gaining an even tissue regeneration
through an even cell distribution. Hence, cells would accumulate in the macropores, start to proliferate and differentiate and therefore support tissue regeneration only from these locations. Other parts of the scaffold would be neglected by cellular infiltration which results in uneven tissue regeneration. This non-homogenous tissue regeneration may not restore the natural contour and might result in contour defects. This was seen with the application of larger fat grafts where the central cellular parts have been shown to die due to necrosis, therefore destroying the normal contour resulting in disfiguring irregularities or contour defects (Nishimura et al., 2000, Tremolada et al., 2010, Tabit et al., 2012). Furthermore, such small pores displaying a high risk of blockage through infiltrating host cells. This blockage can cause tissue death through the prevention of vessel infiltration and regeneration of the centre parts as observed with pores < 50 µm by Cao et al. (Cao et al., 2006). Consequently, scaffold II was shown not to be an ideal candidate for adipose tissue reconstruction.

Scaffolds III and IV were manufactured with a micropore size range up to 160 µm. This not only supports cell infiltration as shown and prevents pore blockage through host cell infiltration, it also provides space for a greater number of cells. The slight difference between scaffold III and IV in cell distribution can be explained due to a broad but more even pore size range of micropores in scaffold IV (outlined in Chapter 2, section 2.3.2.2). Thus, the broad micropore size range of those scaffolds is more suitable for a scaffold for adipose tissue regeneration.

The knowledge and insights gained from this study confirmed the ability of the frozen scaffolds to spontaneously absorb cells which can subsequently be found throughout the scaffold. This characteristic is advantageous and marks an important advancement in the development of a scaffold for adipose tissue reconstruction. Here, the cells are absorbed into and throughout the scaffold in one step. No additional step such as multiple seeding or waiting for cell attachment is necessary. Through one step the scaffold design is able to quickly absorb and retain cells that are viable and can proliferate (discussed in section 3.4.3). This would make it easy to handle the constructs during practical clinical application. Furthermore, through the freeze drying process the scaffolds can be stored at room temperature and do not
need specific storage requirements. This simplicity makes this scaffold design suitable for clinical applications.

Overall, this analysis provides valuable information about the scaffold materials and the ability of the cells to infiltrate the scaffolds and be distributed throughout. Cells could be detected in the microporous macroporous structure of the scaffolds. Scaffold II showed increased cell accumulation in macropores due to size restriction of the microporous structure. This may be disadvantageous in the process of even tissue regeneration and may also restrict fluid transport thought the bulk construct. It is therefore an exclusion criterion for further studies.

3.4.3 ADSC viability and proliferation studies

A suitable scaffold for tissue engineering should not be toxic to the cells, but support cell viability and cell proliferation (Bauer-Kreisel et al., 2010), to ensure that cells within the scaffold can replace the lost tissue. The biomaterial used for the scaffold in this study are from a natural source and were not expected to have any negative effect on the viability of the cells (Farris et al., 2010, Galateanu et al., 2012).

ADSCs seeded on tissue culture plates coated with the different scaffold materials were indeed viable after 24h. Furthermore, it was shown that ADSCs are viable and proliferate when seeded and cultured within the scaffolds. Consequently, these results confirmed that the scaffold materials are non-toxic for ADSCs and can be used to expand and deliver the cells. The use of gelatin in combination with ADSC has already been suggested as suitable for tissue engineering by others (Hong et al., 2005, Lin et al., 2008, Farris et al., 2010, Zhou et al., 2011, Lin et al., 2011, Phull et al., 2013, Korurer et al., 2014). However, gelatin is soluble in aqueous solutions with a melting point ≥40°C and shows poor mechanical properties (Singh et al., 2002, Farris et al., 2010). To produce a stable shape, to improve thermal and mechanical properties of the gelatin scaffold the widely applied cross linker glutaraldehyde was used. Despite the possible toxicity of glutaraldehyde, the gelatin cross linked with glutaraldehyde did not affect the proliferation of the cells when compared to TCP. The washing steps applied during scaffold fabrication are therefore suitable for the removal of residual cross linker (described in Chapter 2) as
also noted by others (Bigi et al., 2001, Chang et al., 2013, Imani et al., 2013). Furthermore, glutaraldehyde is used clinically in a range of applications (Jayakrishnan and Jameela, 1996, Chao and Torchiana, 2003) and is suggested to be safe when used in low concentrations (< 2%) (Bigi et al., 2001), as it was done in this study (0.35% final cross linker concentration in construct).

The live and dead assay performed on ADSCs cultured for 10 days within the scaffolds revealed visible higher values of living cells compared to dead cells. It has to be mentioned that the autofluorescence of the gelatin prevented the quantification of the exact cell numbers. However, the observation confirmed that cells in the centre of the scaffold are viable. The centre areas are the most difficult areas to be reached by perfusion and oxygen, and inadequate supplies of these have been shown to result in necrosis (Sutherland et al., 1986, Tabit et al., 2012). Consequently, the results imply that the scaffold design with different pore sizes allowed the survival of cells also in the central areas by supporting diffusion of nutrients and gases. This finding highly supports the suitability of the scaffolds in adipose tissue reconstruction.

Proliferation of ADSCs within the scaffolds was further analysed. All scaffolds visibly supported the proliferation of ADSCs. Proliferation in the central areas of each scaffold was investigated after seeding was performed via surface application to ascertain whether cells could infiltrate central areas and continue to proliferate. This data confirmed that the scaffolds were suitable for further cell culture studies as cells could easily infiltrate the central areas and continue to proliferate (Choi et al., 2010b, Chang et al., 2013, Cheung et al., 2014).

3.4.4 ADSC differentiation potential in 3D scaffold culture

The 3D scaffold environment can influence cell differentiation due to porosity, topography and cell-material interaction and cell-cell interaction. Cells can also be influenced to synthesise and secrete ECM components in a 3D environment. In addition, cell-ECM interactions have been shown to further influence cell differentiation (Meng et al., 2014). It is known that ADSCs can differentiate towards mesodermal linages such as the adipogenic, chondrogenic and osteogenic lineage.
(Zuk et al., 2001, Zuk, 2013). Therefore, to investigate the effect of the 3D scaffold environments on the spontaneous differentiation potential of ADSCs, gene expression analysis were performed. The gene expression of the stem cell antigen CD90, adipogenic, chondrogenic and osteogenic lineage specific genes was assessed. This analysis revealed that ADSCs cultured in the different scaffolds do not display significantly different expression of lineage specific markers. Instead, they showed similar expression as observed for ADSCs grown on TCP in proliferation medium, while the expression of the CD90 stem cell antigen was maintained and confirmed the present of stem cells in the scaffolds.

The results confirm what other differentiation studies using natural biomaterials have reported (Galateanu et al., 2012, Cheung et al., 2014). These studies show that ADSCs seeded within a biomaterial do not necessarily spontaneously differentiate towards the adipogenic lineage if not induced by differentiation medium. Cheung et al. prepared composite scaffolds based on natural materials, respectively methacrylated glycol chitosan and methacrylated chondroitin sulphate. ADSCs were delivered into the scaffold with decellularised adipose tissue and differentiation was subsequently induced. Adipogenic gene expression was observed in scaffolds cultured in differentiation medium but not in decellularised adipose tissue or scaffolds cultured in growth medium (Cheung et al., 2014). Therefore, the ADSCs were preserved and remained stem cells as in our gelatin sponges. This makes these scaffolds potential candidates for further studies.

3.4.5 Long term in vitro study

Despite the support of cell infiltration, viability and differentiation scaffolds for the reconstruction of larger contour defects should degrade slowly to allow the transferred stem cells and host cells to regenerate the lost tissue. In the case of fat graft transfer the cellular regeneration time was estimated to be 12 months (Yoshimura et al., 2011), therefore the degradation time of an ideal scaffold for adipose tissue regeneration should be at least within this time frame. However, due to time limitations the presented analysis was a preliminary degradation study. The investigation was conducted within 2 months with a cell seeded construct in growth medium to more closely mimic in vivo conditions. Furthermore, the study was
performed on scaffold III and IV due to their favourable features of cell infiltration, distribution, viability and conservation of the ADSC stem cell phenotype. Scaffold II was excluded due to disadvantages in cell accumulation within the macroporous of the construct structure, resulting from the size restriction of the microporous structure. This can jeopardise even tissue regeneration and also restrict fluid transport throughout the bulk construct (discussed in section 3.4.2).

Nonetheless, in this time frame, initial degradation was clearly visible due to change of the microporous macroporous structure of the scaffolds. The modulation of the porous structure of biomaterials when seeded with cells or in in vivo studies has been observed by others (Lin et al., 2011, Korurer et al., 2014, Bellas et al., 2015, Fan et al., 2015). Bellas et al. displayed that porous silk sponges seeded with ADSCs were completely remodelled after 90 days in in vivo rat studies (Bellas et al., 2015). Korurer et al. subjected hyaluronic acid (HA)/gelatin-fibrin scaffolds to in vitro studies with ADSCs for 21 days. They revealed changes in pore structure (larger pores) and metabolisation of the biomaterial while the overall porous structure was still maintained (Korurer et al., 2014). The structural change observed in the present study is probably related to the cell source used as noticed by Korurer et al. and based on enzymatic digestion (Korurer et al., 2014, Fan et al., 2015). Fan et al. tested in vitro the degradation of HA based scaffolds with PBS and enzymes (hyaluronidase and lysozyme). After 21 days scaffolds in PBS displayed larger pores whereas scaffolds cultured in the presence of enzymes showed a significant effect on the scaffold structure resulting in a larger pore diameter and a looser structure (Fan et al., 2015). In the present study scaffold IV revealed a visibly looser structure when compared to scaffold III indicating a greater degree of degradation. Both scaffolds displayed similar physical features but differences in pore size distribution resulting in different cellular distribution within the constructs as described in section 3.2.9 and discussed in paragraph 3.4.2. Scaffold IV supports even cell distribution resulting in higher cellular degradation visible in the overall increased fibrous scaffold structure when compared to scaffold III.

Furthermore, in this study ADSCs revealed a dominant spherical morphology as seen by others who cultured ADSCs in 3D environments longer than 10 days (Lin et
al., 2011, Fan et al., 2015). The cells were found in increased numbers and clustered in the macropores which clearly indicates cell proliferation. This also reveals that the scaffold design supported sufficient metabolite diffusion and waste removal. Thus, it confirms the interconnectivity of the porous structure of the gelatin sponges which allows metabolite diffusion. It further confirms the adequate size of the microporous structure for cell infiltration and migration. Additionally, the degradation of the scaffold structure through ADSCs indicates that ADSCs secrete enzymes, indicating the possibility that the cells also deposit ECM within the porous structure. ADSCs are known to secrete ECM degradation enzymes and ECM components similar to native adipose ECM which supports material degradation and cell differentiation (Vallée et al., 2009, Mariman and Wang, 2010, Wronska and Kmiec, 2012). Additional histological staining and protein analysis for adipose specific ECM components, adipogenic differentiation and ECM degradation enzymes such as laminin, lipid accumulation and MMPs would underpin this hypothesis.

This preliminary study is encouraging due to the visible support of cell proliferation and potential ECM secretion into the scaffolds when seeded with ADSCs. Furthermore, it strongly confirms the scaffold design features of a micorporous and macroporous structure for adipose tissue regeneration. Additionally, the scaffold structure is noticeably altered although the microporous macroporous features are still recognisable. Therefore, a slow biodegradation while the cellular component would replace the missing tissue maybe possible with the scaffolds presented here. Despite the promising results of this preliminary study, further experimental repeats and quantifications related to ECM secretion and differentiation are necessary. However, the preliminary results are promising regarding the degradation time of the scaffold and their design features. Therefore, the scaffolds display a platform for further advancement of the biomaterials. Here, the incorporation of further ECM signals would be possible and would provide an element of control of the ADSC differentiation towards the adipogenic lineage, therefore adipose tissue regeneration.

Nonetheless, throughout the whole study a high biological variability between samples was observed especially in the quantitative studies of cell viability, proliferation and gene expression. The high standard deviations made it difficult to
quantify significance in the data as observed in the proliferation and gene expression analysis. Such a biological variation is generally encountered in the application of primary cells such as ADSCs (Roxburgh et al., 2015). Here, the influence of donor age, gender, BMI, health, donor site and cell passage number is recognized to influence ADSC differentiation and proliferation potential (Sun et al., 2011, Sachs et al., 2012, Oñate et al., 2012, Handel et al., 2012, Russo et al., 2014). In the present study the consented tissue was donated from various patients with different genders and broad age range. To eliminate donor site and cell passage number differences, ADSCs were sourced exclusively from subcutaneous abdominal tissue and used in experiments only at passage 3. However, other factors such as donor variability, age and health were not able to be excluded due to patient confidentiality. The donor variability was shown to influence the ADSC adipogenic differentiation by Russo et al. (Russo et al., 2014). Further, the effect of donor age on proliferation and differentiation of ADSCs was studied by different groups (Zhu et al., 2009, de Girolamo et al., 2009, Choudhery et al., 2014). Choudhery et al. undertook a comprehensive study on the effect of age on ADSCs. They compared ADSCs isolated from young (< 30 years), adult (35-60 years) and aged (> 60 years) donors. Within this study they revealed that donor age has a negative impact on ADSC proliferation and chondrogenic and osteogenic differentiation (Choudhery et al., 2014). Patient health also significantly influences ADSC differentiation and proliferation capacity. Onate et al. revealed through comparative studies with obese and non-obese patients, the inverse influence of health on adipogenic differentiation and proliferation (Oñate et al., 2012). This may explain any variation seen in our proliferation and gene expression study.

Despite the biological variation it was possible to note trends in ADSC proliferation. Proliferation ability of ADSCs when cultured in the different scaffolds was assessed histologically and quantified through immuno staining and cell count. This analysis showed higher cell numbers, therefore cell proliferation within the scaffolds after 10 day culture.

In conclusion, this analysis outlines the potential of scaffold III and IV to support ADSC infiltration, proliferation and phenotype. The different scaffolds maintain ADSC stem cell phenotype over 10 days in culture and do not induce adipogenic,
chondrogenic or osteogenic differentiation. Furthermore, encouraging results within the long term preliminary in vitro study showed implication of the scaffold design for adipose tissue reconstruction. Here, favourable slow biomaterial degradation was observed while the cells remodelled the construct structure. Therefore, they can be utilised for further studies to advance the scaffold properties such as controlled differentiation of the ADSCs population within the biomaterial. Thus, they provide a platform for the integration of an adipose environment into the scaffold, which supports adipogenesis.

3.4.6 Conclusion

Characterisation of cells isolated from subcutaneous adipose tissue through immunophenotyping of ADSC specific antigens indicated that ADSCs were the main cell type obtained using our protocol. Further differentiation studies along the mesodermal lineages confirmed this phenotype and indicated that a homogenous cell population clearly distinct from fibroblasts was present in cultures.

The design feature of spontaneously cell absorption was crucial for the ADSC infiltration and distribution in and throughout the scaffolds. Here, scaffold II displayed main cell location within the macroporous structure resulting from the small size micropores compared to scaffold III and IV. The unfavourable cell distribution as observed in scaffold II lead to exclusion of the construct from further studies. The risk of micropore blockage through infiltrating host cells, possible restriction of metabolite flow throughout the constructs and uneven tissue regeneration would be too high.

ADSCs were viable and proliferated in the centre of the scaffolds. This marks an essential feature in the process of engineering a scaffold for subsequent clinical application.

The differentiation study revealed that the ADSC stem cell phenotype is preserved when cells are cultured for 10 days in the scaffolds.
Additional preliminary long term *in vitro* studies over a 2 month time course with the selected scaffolds III and IV displayed desirable slow biodegradation of the gelatin sponges while allowing the migration and proliferation of the cells throughout the constructs.

Concluding, scaffold III and IV displayed the characteristics required of suitable scaffolds for tissue reconstruction. Both scaffold designs allow cell uptake, infiltration, distribution and proliferation. Furthermore, they support the preservation of ADSC stem cell phenotype over 10 days in culture and do not induce spontaneous adipogenic, chondrogenic or osteogenic differentiation. The biomaterial design also showed implication to support a slow degradation rate while the cells filled and remodelled the construct structure. Therefore, they are suitable for ADSC culture and further studies with an artificial adipose environment.
Chapter 4: ECM based adipose stem cell niche

4.2 Introduction

ECM has been recognised for centuries but has had a number of different names during that time. Before the early 1800s it was known as the “fiber” observed in tissue. With advancing scientific techniques and methodologies, ECM components were discovered. Thus, in the early 1900s it was widely called ground substance (Grundsubstanz). Finally, in the 1930s, the cell surrounding matrix was named ECM. Since then the ECM has continued to be investigated and functions beyond its role in structural support have been revealed (Piez, 1997).

The native adipose ECM is known to play a part in stem cell proliferation, induction of morphological changes and differentiation (Mariman and Wang, 2010, Divoux and Clément, 2011, Watt and Huck, 2013). The cells sense changing ECM signals through adhesion proteins, mostly via a family of molecules known as integrins. Thus cells adhere to the surrounding ECM, sense mechanical cues and respond (for example change in morphology, proliferation and differentiation) to the changing surroundings. The communication is also reciprocal, where cells influence the composition of their acellular surroundings (Watt and Huck, 2013). The ECM consists of different components in varying compositions depending on the developmental stage of the adipose tissue (Bucky and Percec, 2008, Choi et al., 2010a, Mariman and Wang, 2010, Divoux and Clément, 2011, Watt and Huck, 2013). This is evident in the development of mature adipocytes, where a number of intensive changes in the ECM composition take place. During adipogenesis, the fibrillar ECM rich in collagen I and III changes to a laminar ECM rich in laminin and collagen IV (Kitagawa, 1988, Halberg et al., 2008, Mariman and Wang, 2010, Divoux and Clément, 2011, Mori et al., 2014). This change plays an essential role in the differentiation of
mesenchymal stem cells towards the adipogenic lineage (Mariman and Wang, 2010, Divoux and Clément, 2011). Furthermore, individual structural ECM components such as fibronectin and collagen have been shown to influence cell morphology, attachment and differentiation (Spiegelman and Ginty, 1983, Sánchez et al., 2000, Lygoe et al., 2007, Lam and Longaker, 2012). It has been shown that the blockage of fibronectin integrin receptor α5 through anti-integrin antibodies added to a fibroblast culture inhibits their differentiation to myofibroblasts when induced through TGF-β1 (Lygoe et al., 2007). Furthermore, Sanchez et al. tested collagen, fibronectin and a mixture composed of entactin, collagen IV and laminin in concentrations ranging from 0.1 µg/cm², 1 µg/cm², 2 µg/cm² up to 10 µg/cm² for the support of hepatocyte differentiation. They revealed that fibronectin (2 µg/cm²) supported hepatocyte differentiation (Sánchez et al., 2000). Furthermore, Spiegelman and Ginty reported the suppression of preadipocyte differentiation when cultured on 5 µg/ 35 mm and 30 µg/ 35 mm dishes coated with fibronectin (Spiegelman and Ginty, 1983). Li et al. and Lam and Longaker presented the enhanced attachment and proliferation of ADSC cultured on laminin, collagen and fibronectin surfaces when compared to uncoated TCP (Li et al., 2008, Lam and Longaker, 2012).

Thus, the ECM further plays an important role as a key component of the stem cell niche and therefore acts as a regulator of stem cell fate (Votteler et al., 2010). In addition, in adipose tissue reconstruction it has been revealed that the preservation of an ADSC population is important for graft survival, tissue regeneration and adipose tissue turn over (Yoshimura et al., 2008a, Yoshimura et al., 2008b, Tremolada et al., 2010). Tremolada et al. published that fat graft survival partially depends on the survival of cells found in the graft (Tremolada et al., 2010). ADSCs are more resistant to pressure and ischaemia than adipocytes and most likely have a higher survival rate during fat grafting (Bauer-Kreisel et al., 2010, Choi et al., 2010a, Larocca et al., 2013). This contributes to the graft survival of ADSC supplemented fat (Tremolada et al., 2010). Yoshimura et al. observed improvement of clinical graft volume with cell assisted lipotransfer using ADSCs (Yoshimura et al., 2008a). These studies support the importance of the availability of a high number of ADSCs. Therefore, the preservation of ADSC stemness is important but also the support of adipogenesis. Here, natural
biomaterials, which support adipogenesis, mimicking the native adipose surrounding have been shown to promote adipose tissue reconstruction in \textit{in vivo} studies (Hong \textit{et al.}, 2006, Wang \textit{et al.}, 2013b, Poon \textit{et al.}, 2013). Consequently, the significance of the ECM as an adipogenic supportive surrounding as well as the preservation of ADSC stemness in the promoting of adipose tissue formation is shown. Therefore, the manufacture of a stem cell niche that provides signals for the time-related influence of cell behaviour, where the ADSC stemness is maintained and over time induces differentiation towards the adipogenic lineage based on natural adipose ECM is desirable in the preparation of a scaffold for adipose tissue reconstruction.

4.2.1 Aim of the chapter

In the present study the aim was to produce a stem cell niche within scaffolds III and IV which displayed promising physical and biological profile for adipose tissue reconstruction (see in Chapters 2 and 3). Scaffolds III and IV were found to support cell infiltration and distribution throughout the constructs and their pores. Furthermore, ADSCs cultured in the scaffolds were viable, proliferative and their stemness was preserved (outlined in Chapter 3). The construct design showed further implications for favourable slow degradation time while cells remodelled the porous structure (see Chapter 3). The microporous macroporous structure was also demonstrated to be tuneable by using alginate beads as macropore templates and the application of different freezing temperatures to vary the micropore size range (specified in Chapter 2). These features left scaffold III and IV with adjustable cell supportive structures and make them potential candidates for ADSC culture and further studies in adipose tissue regeneration.

An additional feature of the artificial stem cell niche should be to further act as an ADSC delivery matrix to fill the macropores of the gelatin sponges with cells. Thus, the scaffold acts as both a structural and shaping unit whereas the delivery matrix operates as the ADSC stem cell niche and supports adipogenesis. Further, the retainment of stemness should allow the ADSCs in \textit{in vivo} settings to proliferate, differentiate and support vascularisation. The stem cell population would contribute to adipose tissue regeneration and integration (Yoshimura \textit{et al.},}
2008a). The artificial adipose stem cell environment would, in addition, support adipose tissue formation.

Therefore, structural ECM components of the native adipose tissue were analysed for possible characteristics that allow the support of adipogenic differentiation and the maintenance of stemness on ADSCs in a 2D study. From the 2D study the most promising components elucidated were chosen and a delivery gel was cast (aim 2 described in 1.4.1). ADSCs were enclosed in the mix and delivered into the scaffolds. The newly created composite scaffold was analysed for distribution in the macropores and adipogenic differentiation was investigated by histological methods and qRT-PCR (aim 3 described in 1.4.1).
4.2 Materials and Methods

4.2.1 Materials

The plastic and consumables were purchased from Greiner Bio-One Ltd., UK unless stated otherwise.

4.2.2 ADSC isolation and culture

The stem cell isolation and culture was performed as described in Chapter 3, section 3.2.2.1. All studies were carried out using ADSCs that had reached passage 3. The harvest and cell count was performed as previously discussed (Chapter 3, section 3.2.2.2 and 3.2.2.3).

4.2.3 2D study of the influence of ECM components on ADSC morphology

In the 2D study ADSCs were grown for 3 days on glass coverslips coated with a range of ECM components. Afterwards the cells were directly and indirectly immunostained for actin and vinculin to help identify morphological differences.

4.2.3.1 Coating of glass coverslips with extracellular matrix components

ECM components were chosen according to the current concepts within the literature reporting ECM components of the adipose tissue (Choi et al., 2010a, Mariman and Wang, 2010, Divoux and Clément, 2011, Watt and Huck, 2013). Thus, human collagen I, III, IV, V, VI, laminin and fibronectin (BD Bioscience, UK) were selected. Glass coverslips (12 mm x 12 mm) sitting in a 24 well plate were coated with 250 µl of varying dilutions of the chosen components according the manufacturer’s instructions (BD Bioscience, UK) and the literature (Spiegelman and Ginty, 1983, Houdijk et al., 1985, Sánchez et al., 2000). Specifically, collagen I, III, IV, V, VI and laminin were diluted to a 10, 100, 200 µg/ml solution to create a surface coating of 1, 10, 20 µg/cm². Collagen I was diluted in 2 mM HCl (Sigma Aldrich, UK). Collagen III-VI were diluted in 10 mM Acetic acid (Sigma Aldrich, UK) whilst serum free culture medium was used to dilute laminin. Fibronectin is known to affect cell morphology and attachment when used at decreased concentrations (Spiegelman and Ginty, 1983). Therefore,
a concentration of 5, 30, 50 µg/ml was applied to provide a surface coating of 0.5, 3 and 5 µg/cm². The diluent was HBSS.

4.2.3.2 Seeding of coated glass coverslips

The stem cells were diluted with stromal medium to a stock solution of 50,000 cells/ml. From the stock solution 100 µl (5000 cells) was added to each well containing coated glass coverslips (detailed in section 4.2.3.1) and 1 ml of stromal medium. Non-coated glass coverslips were seeded with ADSCs and either cultured in stromal medium or adipogenic differentiation medium (control) (detailed in Chapter 3, section 3.2.6.1). To allow for morphological changes to happen the seeded coverslips were incubated for 3 days at 37°C, 5% CO₂. The culture medium was changed daily.

4.2.3.3 Morphology analysis

To illustrate and characterise the morphology of ADSCs grown on different ECM components immunostaining of the cytoskeleton and focal adhesion protein vinculin was carried out at day 3 of culture. Therefore, ActinRed™ 555 ReadyProbes® Reagent (Life Technologies Ltd., UK) Anti-Vinculin, hVin-1 staining (Life Technologies Ltd., UK) were applied.

4.2.3.3.1 Immunofluorescence staining

The immunofluorescent method is used to detect specific target antigens in cells or tissues. The method is based on fluorescent labelled antibodies that specifically bind the antigen of interest (Odell and Cook, 2013). The direct staining of the cytoskeleton is described in Chapter 3 section 3.2.9.1. The indirect method is based on a primary antibody that is specific to the antigen of the molecule of interest and a secondary antibody. The secondary antibody is specific to the primary antibody and fluorescently or colourimetrically labelled (Kroese, 2001). After application of both antibodies the labelled molecules were observed using fluorescent microscopy. The methodology is described below.
After media removal and rinsing in PBS the cells were fixed with 3% \( \text{v/v} \) PFA (Sigma Aldrich, UK) in PBS for 15 minutes. The fixation was stopped by rinsing the cell seeded coverslips in perm/quench solution (P/Q) (50 mM NH\(_4\)Cl, 0.2% \( \text{w/v} \) saponin (Sigma Aldrich, UK)), in PBS. After permeabilisation of the samples with P/Q for 15 minutes, coverslips were blocked in PBS, gelatin, azide, saponin solution (PGAS) (0.2% \( \text{w/v} \) fish skin gelatin (Sigma Aldrich, UK), 0.02% \( \text{w/v} \) saponin (Sigma Aldrich, UK), 0.02% \( \text{w/v} \) azide (Sigma Aldrich, UK), in PBS) for at least 5 minutes at room temperature. Incubation with the primary antibody (Table 4.1) followed for 1 hour at room temperature in a moist chamber. Samples were washed three times in PGAS. The secondary antibody (Table 4.1) was added and incubated in the dark in a humidified chamber for 30 minutes at room temperature. At this point the ActinRed TM 555 ReadyProbes\textsuperscript{®} Reagent was applied. The unbound antibodies were removed through washing with PGAS, PBS and water. The coverslips were mounted with ProLong\textsuperscript{®} Gold Antifade Mountant with DAPI (Life Technologies Ltd., UK) onto a glass slide. After overnight drying in the dark, samples were analysed using an Axio Scope A1 fluorescent microscope (Carl Zeiss Ltd., UK).

**Table 4.1: Immunofluorescence antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company (catalogue number)</th>
<th>Dilution (concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinculin (Anti-hVin-1)</td>
<td>Abcam®, UK (ab11194)</td>
<td>1:100 (100 µg/ml)</td>
</tr>
<tr>
<td>Anti-mouse dylight\textsuperscript{TM}</td>
<td>Jackson ImmunoResearch Europe (315-486-047)</td>
<td>1:100 (15 µg/ml)</td>
</tr>
</tbody>
</table>

**4.2.3.4 Aspect ratio**

Cells cultured for 3 days on different ECM components were stained according to the methods outlined in section 4.2.3.3.1. Fluorescence images were taken using the Axio Scope A1 fluorescent microscope (Carl Zeiss Ltd., UK). Four images across the section were taken at a magnification of 10x from each experimental condition. All cells in the field of view were analysed. In confluent areas, clearly defined cells were quantified. The aspect ratio of the cells was measured and calculated as described by others (Young et al., 2013). Specifically, 2 perpendicular lines were drawn across the nucleus of the cell. The length of the
longer line was then divided by the length of the shorter one. Thus, the ratio describes how round a cell is with perfectly round cells having a ratio equal to one.

**4.2.4 3D study of the selected ECM components in the scaffolds**

In the 3D study, ADSCs were cultured in the presence of the different scaffolds for 10 days. The ADSCs were delivered into the scaffolds with a collagen laminin hydrogel.

**4.2.4.1 Casting of collagen laminin gel**

To resemble the surface concentration used in the planar 2D studies as gel concentration, the concentration had to be converted towards a concentration applicable for 3D environments. Therefore, the method used for the transfer of a 2D concentration to a 3D gel, including the dimension of height is displayed in Figure 4.1 and described below.

In the 2D study 24 well plates (Greiner Ltd., UK) measuring 16.5 mm in height and 15.7 mm in diameter (d\textsubscript{2D}) (radius (r\textsubscript{2D}) 7.85 mm) were coated with a volume (V\textsubscript{2D}) of 250 µl ECM component. Thus, applying the formula \( V\textsubscript{2D} = r\textsubscript{2D}^2 \times h\textsubscript{2D} \times \Pi \) (h\textsubscript{2D} = \( V\textsubscript{2D} / (r\textsubscript{2D}^2 \times \Pi) \)) reveals the coating height of the surface was 1.3 mm. To resemble the same concentration within a 3D environment containing a volume (V\textsubscript{3D}) of 500 µl (determined through the liquid uptake study in Chapter 2, section 2.3.2.3.1), firstly the height of the gel to cast in 24 well plates (r\textsubscript{3D} = 7.85 mm) was calculated. Therefore, the formula \( h\textsubscript{3D} = V\textsubscript{3D} / (r\textsubscript{3D}^2 \times \Pi\textsubscript{3D}) \) was applied as well, giving a theoretical height (h\textsubscript{3D}) of 2.6 mm for a gel that would be cast out of 500 µl fluid. Next, the applied concentration/height of the 2D (c\textsubscript{2D}/h\textsubscript{2D}) studies was set in ratio with the used concentration x/per height of the 3D mould (x/h\textsubscript{3D}), producing the formula \( c\textsubscript{2D} / 1.3 \text{ mm} = x / 2.6 \text{ mm} \), where \( x = c\textsubscript{2D} / 1.3 \text{ mm} \times 2.6 \text{ mm} \). Finally, using this calculation dictated that a surface coating of 200 µg/ml and 100 µg/ml would be a 400 µg/ml and 200 µg/ml concentration of the 3D gel. From these concentrations a collagen laminin gel was produced as followed.
Chapter 4: ECM based adipose stem cell niche

Figure 4.1: Conversion of 2D concentration into 3D gel concentration. Shown are the calculations used to determine the concentration of the gel cast, resembling the surface concentration used in the 2D study. Therefore, the height of the 2D coating was determined using known radius ($r_{2D}$) and volume ($V_{2D}$) of the mould and coating solution. The height ($h_{3D}$) of the 3D gel was calculated the same way. The known height and concentration of the 2D approach was set in ratio with the concentration of the gel ($x$) and the determined height of the gel, leaving the formula $C_{2D}/h_{2D} = x/h_{3D}$. Thus, a 2D concentration of 200 µg/ml would be a 3D concentration of 400 µg/ml.

\[
\begin{align*}
V_{2D} &= 250 \mu l \\
d_{2D} &= 15.7 \text{ mm} \\
r_{2D} &= 7.85 \text{ mm} \\
h_{2D} &= h_{2D} \text{ mm}
\end{align*}
\]

\[
\begin{align*}
V_{2D} &= r_{2D}^2 \times h_{2D} \times x \left[\mu l\right] \\
250 \mu l &= (7.85 \text{ mm})^2 \times h \times x \left[\mu l\right] \\
h_{2D} &= 1.3 \text{ mm}
\end{align*}
\]

\[
\begin{align*}
V &= r^2 \times h \times x \left[\mu l\right] \\
500 \mu l &= (7.85 \text{ mm})^2 \times h \times x \left[\mu l\right] \\
h_{3D} &= 2.6 \text{ mm}
\end{align*}
\]

Example:

\[
\begin{align*}
c_{2D} &= 200 \mu g/ml \\
c_{3D} &= x
\end{align*}
\]

\[
200 \mu g/ml/1.3 \text{ mm} = x/2.6 \text{ mm} \\
x &= 400 \mu g/ml
\]

The collagen gel was prepared according to published methodologies (Ngo et al., 2006) on ice with chilled materials and reagents (Figure 4.2). Therefore, collagen solution 3 mg/ml (Stemcell Technologies, France) was diluted with medium to a 400 µg/ml concentration. To incorporate laminin, 200 µl of 1 mg/ml stock solution (Sigma Aldrich, UK) was added. The mixture was gelled by adjusting the pH with 6 µl 1 M sodium hydroxide (NaOH).

Figure 4.2: Casting of collagen laminin hydrogel. Collagen (Col) with a concentration of 400 µg/ml was mixed with laminin (Lam) 200 µg/ml. Compared to the culture medium the hydrogel is cloudy and viscous. Images were taken with a Canon camera.
4.2.4.2 ADSC delivery with collagen laminin hydrogel into the scaffold

The scaffolds were seeded with $1 \times 10^6$ cells. ADSCs were diluted to $1 \times 10^6$ cells/ml. After the dilution 1 ml of $1 \times 10^6$ cells was transferred into a 15 ml Falcon. The cell suspension was centrifuged at 1500 rpm for 4 minutes. The medium was aspirated. A 400 μg/ml collagen gel containing 200 μg/ml laminin was cast around the cells as described in 4.2.4.1. The gel mixing was carried out on ice. Therefore, the cell pellet was mixed with 67 μl of 3 mg/ml chilled collagen I solution (Stemcell Technologies SARL, France), 200 μl of 1 mg/ml chilled laminin (Sigma Aldrich, UK), 227 μl chilled stromal medium and 6 μl 1 M NaOH (Sigma Aldrich, UK). The soluble mixture containing cells was pipetted onto the scaffolds and incubated for 30 minutes at 37°C, 5% CO$_2$. After the incubation and setting of the gel, the scaffold was transferred into spinner bottles either containing stromal medium or adipogenic differentiation medium. The spinner bottles containing the seeded scaffolds were incubated for 10 days at 37°C, 5% CO$_2$ with continues rotation at 15 rpm. The medium was changed every 2-3 days.

4.2.4.3 Scaffold culture

ADSCs were cultured with the scaffold as described in Chapter 3, section 3.2.8.1.

4.2.4.4 Histological analysis

For the histological analysis of the seeded scaffolds, both direct and indirect immunohistochemistry methods were used. The detailed method is described below.

The scaffolds containing cells were harvested at day 10 of culture, fixed, frozen and 30 μm sections cut as described in Chapter 3, section 3.2.9.1. Immunohistochemistry staining of the actin cytoskeleton and glucose transporter 4 (GLUT 4) staining was carried out. The former histological staining technique is described in Chapter 3 section 3.2.9.1. The latter histological staining method is outlined below.
The sections were allowed to dry for 1 hour at room temperature and then fixed. The fixation of the scaffold occurred with a 50:50 Methanol: Acetone (*Sigma Aldrich, UK*) solution for 20 minutes at room temperature. After 3 x 3 minutes washes with PBS, the sections were blocked in horse serum (*Vector Laboratories Ltd., UK*). The excess serum was blotted off after 30 minutes of incubation time at room temperature. The incubation in primary antibody (GLUT 4) (Table 4.2) followed over night at 4°C. To remove non-specific binding the sections were washed in PBS for 5 minutes. The application of the secondary antibody anti-rabbit (*Vector Laboratories Ltd., UK*) (Table 4.2) was followed with an incubation time of 30 minutes at room temperature. Non-specific binding of antibodies was removed through washing the sections twice in PBS for 3 minutes, followed by washing in sterile water for 3 minutes to remove residual salts left by PBS. The dry blotted sections were mounted in ProLong® Gold Antifade Mountant with DAPI (*Life Technologies Ltd.; UK*). The stained slides were left overnight to dry and viewed with Axio Scope A1 Fluorescence microscope (*Carl Zeiss Ltd., UK*).

### Table 4.2: Antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Dilution (concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit Glucose transporter 4 (GLUT 4)</td>
<td><em>Bio-Rad AbD Serotec® Ltd., UK (4670-1704)</em></td>
<td>1:100 (50 µg/ml)</td>
</tr>
<tr>
<td>Dylight Goat Anti-rabbit IgG</td>
<td><em>Vector Laboratories Ltd., UK (DI-1549)</em></td>
<td>1:300 (50 µg/ml)</td>
</tr>
</tbody>
</table>

#### 4.2.4.5 Quantitative real time PCR

The gene expression was analysed using the qRT-PCR. The expression of the stem cell gene *CD90*, the preadipocyte gene *PPARG* and the adipocyte expression gene *GLUT 4* were carried out. The detailed methodology is based on RNA isolation, cDNA synthesis and qPCR reaction as described in Chapter 3 section 3.2.11. However, to remove the cells from the collagen laminin hydrogel, the RNA extraction had to be adapted. The modified method is described below.
4.2.4.5.1 RNA extraction from collagen laminin hydrogel

After 10 days of cell-scaffold culture the scaffolds were transferred into a 12 well plate. The scaffolds were cut in half. One half was used for histological analysis. The other half was utilised for RNA isolation. Therefore, the latter scaffold half was washed in PBS after media removal. One ml of DB (0.075% w/v collagenase type 1 (Sigma Aldrich; UK), 2% Pen/Strep, in HBSS) was added to the scaffold to extract the cells out of the collagen laminin environment. The digestion was stopped by adding 1 ml of medium after 10 minutes. The DB medium mix was transferred to a 15 ml Falcon and centrifuged at 2000 rpm for 5 minutes. The obtained cell pellet was used for RNA extraction applying the RNA isolation Kit - RNeasy Mini Kit (Qiagen; UK) (described in Chapter 3 section 3.2.11.1).

4.2.5 Statistical analysis

All experiments were performed in biological as well as in technical triplicates. The data are represented as mean ± standard derivation. Statistical significance was assessed using Microsoft Excel 2007 (Microsoft, UK). The 2 tailed unpaired students T-test was applied to determine statistical significance for the aspect ratio data. The one sample t-test was employed to determine statistical significance for the gene expression data using ADSCs cultured on TCP as a control. Significance was given for p ≤ 0.05. The aspect ratio data were plotted employing Microsoft Excel 2007. The qRT-PCR data were plotted using SigmaPlot 10 (Systat, USA).
Chapter 4: ECM based adipose stem cell niche

4.3 Results

The aim of this study was to create an adipose tissue environment that acts as a stem cell niche as described in Chapter 1 section 1.4.1. This artificial environment should closely mimic the native adipose surrounding, function as a delivery system for ADSCs into the scaffold and support the time-related differentiation of ADSC towards the adipogenic lineage. Thus, providing a heterogeneous cell population containing stem cells and ADSCs differentiating towards the adipogenic lineage. Here, it was shown that it is important to retain ADSCs which support adipose tissue survival as well as vessel infiltration. Both are important factors for survival of larger constructs. The time-related support of adipogenesis is essential to ascertain the differentiation of the stem cells towards the adipogenic lineage, therefore the regeneration of adipose tissue. In this way a microenvironment is created which promotes ADSC differentiation in vitro but also in a form that could be used in vivo. Furthermore, the presence of stem cells would support in vivo proliferation, differentiation and regeneration.

Firstly, a 2D study was carried out where morphological changes within the differentiating ADSCs were characterised as indicators of adipogenesis and stemness. Therefore, different ECM components known to be present in the native adipose tissue were chosen according to the literature (Spiegelman and Ginty, 1983, Li et al., 2008, Mariman and Wang, 2010, Divoux and Clément, 2011, Lam and Longaker, 2012). The chosen ECM components (collagen I, III, IV, V, VI, laminin and fibronectin) and their effect on the morphology of ADSCs were investigated. The morphological changes were compared to the changes observed when ADSCs are cultured in growth and adipogenic differentiation medium.

From this study the most promising components to create a 3D environment with potential to support the time-related adipogenesis of the stem cells were selected. The selected components were used to cast a gel that can support cell growth and functions as delivery matrix for cells. This optimised delivery matrix was applied to deliver cells on the scaffolds and maintain in culture for 10 days. As such, the potential of the delivery matrix to deliver cells into the scaffold was investigated,
as well as characterising its ability to support time-related adipogenesis of ADSCs.

4.3.1 2D study of the influence of ECM components on ADSC morphology

4.3.1.1 Morphological changes of differentiating ADSCs

The differentiation from stem cells to mature adipocytes is characterised through morphological changes from fibroblast-like to a spherical phenotype. This change occurs early in the adipogenic differentiation phase and is followed by adipogenic gene expression (Gregoire et al., 1998, Rosen and MacDougald, 2006, Lowe et al., 2011, Rosen et al., 2014). To determine an analysis method which illustrated this early morphological change for subsequent application on different ECM coated surfaces, adipogenic differentiation was induced in ADSCs. Therefore, ADSCs were subjected to adipogenic differentiation medium for 3 days, followed by immunochemical staining of the actin cytoskeleton and vinculin adhesion protein. A time frame of 3 days was chosen to exclude spontaneous differentiation due to cell-cell contact inhibition of confluent cells (Gregoire et al., 1998, Roxburgh et al., 2015). Specifically, $5 \times 10^3$ ADSCs were cultured either in growth medium or in adipogenic differentiation medium (described as controls in section 4.2.3.2). The applied media were shown in our previous investigations (described in Chapter 3, section 3.3.1 and 3.3.2.5) to preserve ADSC stemness or induce adipogenic differentiation. After 3 days the cells were stained for actin and vinculin and the aspect ratio was calculated (outlined in section 4.2.3.3 and 4.2.3.4), to quantify cell rounding.

Figure 4.3A shows that after 3 days of culture in adipogenic differentiation medium a morphological transition of the cells took place. When cultured in adipogenic differentiation medium, cells displayed a spherical appearance when compared to the fibroblast-like morphology of cells subjected to growth medium. Lamellipodia were clearly visible for cells grown in growth medium compared to cells in differentiation media. This contributes to the fibroblast-like phenotype of the ADSCs. Additionally, the adhesion protein vinculin was detected throughout the cells cultured in growth medium. In differentiating cells, the vinculin staining was localised in the cytoplasm close to the cell nucleus. The measured aspect ratio
quantified these visual changes (Figure 4.3B). The cells cultured in growth medium showed an average aspect ratio of 3.8. The differentiating cells displayed a significant 2 fold decrease to 1.8 (p= 0.01). This difference in aspect ratio confirms the rounder form of differentiating ADSCs compared to the longer and fibroblast-like phenotype of ADSCs.

In summary, ADSCs were confirmed to display morphological changes when differentiating. These changes were clearly visible after 3 days of culture in the presence of known differentiation factors, as demonstrated by immunocytochemical staining and quantification of the aspect ratio. The morphological differences between ADSCs and differentiating ADSCs can be applied to determine surfaces that promote adipogenesis or retain the fibroblast like morphology of ADSCs. Thus, components which induce a spherical or elongated phenotype of ADSCs can be identified.
Figure 4.3: Morphological changes of differentiating ADSCs. (A) ADSCs were cultured for 3 days in either growth (NON) or adipogenic differentiation (DIFF) medium on glass coverslips. For morphological analysis immunocytochemistry staining for the actin cytoskeleton and adhesion protein vinculin was performed. The change from fibroblast-like phenotype of ADSCs into more spherical differentiating cells was observed. (B) The aspect ratio confirms that the differentiating cells are significantly rounder compared to the cells in growth medium. The experiment was carried out as biological triplicates. Error bars indicate standard deviation. Significance is given for \( *p\leq 0.05 \). Scale bars represent 100 µm. red= actin; green= vinculin; blue= nucleus.
4.3.1.2 Screening of different extracellular matrix components

The hypothesis of this investigation is that the ECM components influence the morphology of ADSCs as shown in the study of Young et al. and described elsewhere in the literature (Mariman and Wang, 2010, Divoux and Clément, 2011, Young et al., 2013). These morphological changes are thought to be indications of adipogenesis promoted by ECM components, where a rounder morphology indicates maturing adipocytes (Lowe et al., 2011) as illustrated in 4.3.1.1. Furthermore, this study helped the selection of suitable ECM components in an appropriate concentration, which support the time-related adipogenesis of ADSCs. Additionally, it provided information about the behaviour of ADSCs on different surfaces.

To analyse the effect of ECM components on the morphology of ADSCs, potential candidates which are present in the adipose ECM were chosen according to the literature (Spiegelman and Ginty, 1983, Li et al., 2008, Mariman and Wang, 2010, Divoux and Clément, 2011, Lam and Longaker, 2012). Glass coverslips were coated with the selected components (collagen I, III, IV; V, VI, laminin and fibronectin) in different concentrations (Spiegelman and Ginty, 1983, Houdijk et al., 1985, Sánchez et al., 2000), seeded with $5 \times 10^3$ cells per coverslip and cultured (detailed in section 4.2.3). After 3 days of cell culture, immunocytochemical staining was carried out to visualise the actin cytoskeleton and the adhesion protein vinculin (outlined in section 4.2.3.3.1). In addition, to quantify the visualised morphological changes the aspect ratio of the cells grown under each condition was investigated (described in section 4.2.3.4).

It was demonstrated that the surfaces coated with different ECM components in varying concentrations influenced the shape of the ADSCs compared to ADSCs grown on glass coverslips (Figure 4.4, 4.5).

ADSCs grown on collagen I (Figure 4.4B) displayed a spherical morphology when grown on a concentration of 1 and 20 $\mu$g/cm$^2$ compared to ADSCs grown on uncoated glass coverslips (control) (Figure 4.4A). The aspect ratio allowed quantification of the rounder cell shape (Figure 4.5). Significantly smaller and
rounder cells were cultured on collagen I at 1 and 20 µg/cm² when compared to cells grown on glass. Cells cultured on coverslips had an average aspect ratio of 3.8. In contrast, cells grown on collagen I (1 µg/cm² and 20 µg/cm²) had observed aspect ratios of 2.6 (p= 0.01) and 1.9 (p= 0.0009), respectively. The latter is similar to the aspect ratio of differentiating cells (1.8 (Figure 4.5)). This suggests initiation of adipogenesis of ADSCs (as shown by the cells becoming more spherical in shape). The concentration of 10 µg/cm² influenced ADSCs to develop a flat spread morphology and aspect ratio similar to the cells grown on coverslips. Consequently, the adipogenic differentiation is possibly not influenced.

Collagen III (Figure 4.4B) was revealed to influence ADSC morphology at the lowest and highest concentrations investigated (1 µg/cm² and 20 µg/cm²). Cells were smaller which was suggestive of the initiation of adipogenesis and of the development of a soft surface. The aspect ratio for both conditions displayed a significant decrease compared to the control with 2.6 (p= 0.02, p= 0.005) (Figure 4.5). At a concentration of 10 µg/cm² the cell morphology and aspect ratio were similar to that observed with control cells (Figure 4.4, 4.5). This indicated no morphological effects of collagen III on the growth of ADSCs but that they were able to maintain a certain fibroblast like morphology.

The lower concentration of collagen IV (Figure 4.4B) (1 µg/cm²) resulted in ADSCs developing a visibly more rounded morphology. However, the corresponding aspect ratio is not significantly different when compared to the average ratio of the control cells (Figure 4.5). The concentrations of 10 µg/cm² and 20 µg/cm² also did not produce any obvious morphological changes. Furthermore, the aspect ratio of 3.5 confirms the preservation of the fibroblast-like morphology.

Cells grown on collagen V did not reveal any visual morphological difference when compared to ADSCs cultured on glass coverslips in growth media. The aspect ratio ranged from 2.8 to 3.0 confirmed the fibroblast-like morphology (Figure 4.4, 4.5).
ADSCs grown on collagen VI developed a visible rounder morphology in all of the concentrations investigated. The aspect ratio of cells cultured on a concentration of 1 µg/cm² of 1.8 (p= 0.09) was similar to differentiating ADSCs. The aspect ratio of 2.4 at the collagen VI concentration of 10 µg/cm² changed significantly (p= 0.04) when compared to cells grown on glass coverslips in growth media, indicating rounder cell morphology. Nevertheless, the error bars associated with the aspect ratio quantification indicated broad biological variability of the cell shape (Figure 4.4, 4.5).

Laminin treatment resulted in a visually increased confluence of ADSCs at a concentration of 10 µg/cm² corresponding with a fibroblast-like cell morphology (Figure 4.4B). Thus, this suggests the preservation of ADSC phenotype. The morphology differed significantly for the laminin concentration of 1 µg/cm² (p= 0.05) and 20 µg/cm² (p= 0.013). Thus, the cell aspect ratio of 2.5 indicated a smaller phenotype (Figure 4.4, 4.5).

Cells cultured on fibronectin showed morphological changes in all 3 tested concentrations (0.5 µg/cm², 3 µg/cm² and 5 µg/cm²) (Figure 4.4C). At 0.5 µm/cm² surface coating concentration ADSCs revealed a significantly (p= 0.04) rounded form whereas at 3 µm/cm² and 5 µg/cm² the cells appear to string together to form a clustered structure (Figure 4.4, 4.5). This reveals the influence of fibronectin on the cytoskeleton and differentiation of ADSCs, most likely suppression of adipogenesis due to the non-rounding of the cells.

Overall, morphological changes could be observed which indicated that the different ECM components influenced the morphology of ADSCs either towards a spherical shape or towards them maintaining the ADSCs fibroblast-like form. These morphological changes also confirmed the successful coating of the wells with ECM components. ADSCs cultured on collagen I with a concentration of 20 µg/cm² coated surfaces revealed the most significant change of the cell morphology. Here, a change from fibroblast-like to spherical shape resembling a similar aspect ratio to that of differentiating ADSCs was shown. The low cost and existing data (von Heimburg et al., 2001, Handel et al., 2012, Werner et al., 2014) relating to collagen I make it an ideal ECM component for further investigation.
Figure 4.4: Morphological changes of ADSCs grown on different ECM components. ADSCs at passage 3 were cultured in differentiation media and on different ECM components of varying concentrations. After 3 days immunocytochemical staining was carried out to investigate the morphology. (A) Shows the morphological change from fibroblast-like ADSCs to a rounder cell shape when cultured in adipogenic differentiation medium. (B) Col (collagen) I, III-VI and laminin were investigated with concentrations of 1, 10 and 20 µg/cm² for influence on ADSC morphology. The morphology was compared to control (A) ADSCs grown on uncoated glass coverslips. (B) The lowest tested concentration for the components showed smaller and rounder cells for Col I, III, VI and laminin (Lam). The medium concentration tested, influenced ADSCs to become more rounded when grown on Col VI. The highest applied concentration forced the ADSCs to become visibly smaller and rounder on Col I, III and laminin. (C) ADSCs were also cultured on fibronectin with concentrations of 0.5, 3 and 5 µg/cm². The lowest concentration forced the cells into a smaller spherical shape. On 3 and 5 µg/cm² fibronectin the cells grew into a tube-like structure. All conditions were tested on 3 biological replicates. The images are shown, display representative areas that reflect the observations across the replicates. Scale bars represent 100 µm. red= actin; green= vinculin; blue= nucleus. NON= cells cultured in growth medium, DIFF= cells cultured in adipogenic differentiation medium.
Figure 4.5: Significant changes of the aspect ratio. The aspect ratio was calculated from ADSCs grown in differentiation medium (DIFF) and on different ECM components in three concentrations. The aspect ratio of cells was taken from 4 areas of immunocytochemical images at magnification 10x. The ratio of each condition was compared to the ratio of stem cells grown in growth medium on uncoated glass cover slips (glass). A significantly rounded phenotype was visible for cells grown on – Col I (1 µg/ cm²; 20 µg/ cm²), Col III (1 µg/ cm²; 20µg/ cm²), Col VI (1 µg/ cm²; 10 µg/ cm²), Lam (1 µg/ cm²; 20 µg/ cm²) and Fib (0.5 µg/ cm²). All experiments were performed with 3 biological replicates. Error bars indicate standard deviation. Significance was given for *p≤ 0.05. Col= collagen, Lam = laminin, Fib= fibronectin.
4.3.2 3D study of the selected ECM components in the scaffolds

4.3.2.1 ADSCs delivery with collagen laminin hydrogel

Collagen I was selected as the basis for casting a gel to create an artificial environment which mimics the adipose tissue microenvironmental niche, containing ADSCs and maturing adipocytes. At the same time this environment should act as delivery matrix for cells to penetrate the scaffold and increase the macropore surface area for cell infiltration. Therefore, collagen I was chosen according to its performance in the 2D study (outlined in section 4.3.1). Collagen I significantly changed the morphology of ADSCs when cultured on a concentration of 20 µg/cm². At this concentration, it induced the ADSCs to adopt a spherical shape similar to ADSCs cultured in adipogenic differentiation medium. The round cell shape is indicative of the cells undergoing adipogenic differentiation into mature adipocytes (Bucky and Percec, 2008). As a second matrix component, laminin was selected. Laminin is known to be present in the surrounding matrix during the process of adipogenesis and induces cell proliferation (Li et al., 2008, Mariman and Wang, 2010). In our 2D study (described in section 4.3.1) it was shown that laminin at a concentration of 10 µg/cm² induced visibly a high cell confluence whereby cells adopted a fibroblast-like shape, similar to non-differentiating ADSCs. Thus, compared to the other components tested that conserved a fibroblast-like phenotype (collagen I (10 µg/cm²), collagen III (10 µg/cm²), collagen IV, collagen V, collagen VI (20 µg/cm²), fibronectin (3 µg/cm²), (5 µg/cm²)) an additional property of the component was indicated, increased cell confluence. Therefore, the cell shape as well as the visible higher cell confluence suggesting maintenance of the ADSC stem cell phenotype.

In an artificial matrix consistent of laminin and collagen, laminin would provide the signal for ADSCs to retain stem cells whereby collagen I would give indications for the adipogenic differentiation of ADSCs. Thus, firstly a stem cell population is maintained over time differentiation would be induced through perhaps remodelling or degradation of factors that induce stemness. Therefore, a time-related differentiation of ADSCs towards the adipogenic lineage through the selected ECM components could be given. Hence, displaying a cell population
where not all cells are differentiating at the same time. This would gain the utilisation of the favourable features from ADSCs and maturing adipocytes for adipose tissue regeneration. Further, it is known that collagen and laminin can form a gel (Guarnieri et al., 2007, Swindle-Reilly et al., 2012). Here, laminin aggregates in fibrils adjacent to the collagen fibres (Guarnieri et al., 2007).

Following, the components were combined within the gel to prepare an ADSC stem cell niche (as described in section 4.2.4.1 and represented in Figure 4.2) to support time-related adipogenesis of the ADSC population. Figure 4.2 shows that a gel which is more viscous as stromal medium was cast. The additional clouded appearance of the gel indicated fibrillogenesis of the collagen fibres, therefore successful gelling of the components. To assess the cell distribution, morphology and the adipogenic potential of the artificial adipose environment histological analysis and qRT-PCR were performed. Specifically, 1 x 10^6 ADSCs were enclosed into the delivery matrix and seeded into the scaffolds (outlined in section 4.2.4.2). After 10 days of culture in either differentiation or growth medium the scaffolds were harvested for immunohistochemistry of the actin cytoskeleton and GLUT 4 protein (detailed in section 4.2.4.3 and 4.2.4.4). Thus, the study should visualise the effect of the delivery matrix on ADSC adipogenic differentiation potency. Furthermore, gene expression analysis for CD90, PPARG and GLUT 4 was performed to investigate the adipogenic potential of the artificial matrix on the cells (specified in section 4.2.4.5).

An even distribution of ADSCs in the macropores of scaffold III was observed as represented in Figure 4.6A. The cells were found within the artificial matrix in the centre of the pores opposed the pore walls of the scaffold cultured in growth medium as well as in differentiation medium. The cells grown in growth medium displayed a fibroblast-like shape compared to the spherical shape of the cells cultured in differentiation medium (Figure 4.6B). When staining for GLUT 4, cells cultured in differentiation medium revealed expression of the adipocyte specific protein (Figure 4.6C).

The delivery of ADSCs in the collagen laminin matrix combined with the culture in growth medium showed an even cell distribution in the macropores of scaffold
IV. For the scaffold culture in differentiation medium cell accumulation in the periphery of the macropores was observed (Figure 4.7A). The cells did not differ in phenotype in the different media (Figure 4.7B). However, expression of GLUT 4 protein was observed when cells are cultured in differentiation medium (Figure 4.7C).

The gene expression analysis showed that the cell seeded scaffolds cultured in differentiation medium expressed significantly less of the stem cell gene CD90 (Figure 4.8A) compared to the control. Hereby, a fold change of 0.27 (± 0.13, p= 0.0046) and 0.21 (± 0.11, p= 0.0076) was detected in scaffold III and IV when containing ADSCs in the collagen laminin environment. Similar results were observed when ADSCs were differentiated on TCP, where a significant fold change of 0.53 (± 0.14, p= 0.0325) was detected. A slight decrease with a fold change of 0.58 (± 0.4) and 0.8 (± 0.55) was displayed by ADSCs delivered with the artificial adipose environment and grown respectively in scaffolds III and IV in growth medium.

The expression of PPARG, the pre-adipocyte expressing gene of ADSCs seeded in the scaffolds with the delivery matrix and cultured in normal growth or differentiation medium in scaffold III and IV was significantly different when compared to ADSCs cultured on TCP in growth medium (Figure 4.8B). A fold change increase of 2.8 (± 0.12, p = 0.006) and of 6.4 (± 1.7, p = 0.03) was recorded for cells seeded with the delivery matrix in scaffold III and IV in growth medium. A non-significant fold change was recorded when ADSCs were cultured in scaffold III and IV under influence of differentiation factors with a fold change of 9.01 (± 8.18) and of 34.81 (±17.4). Cells cultured on TCP in differentiation medium also showed a non-significant fold change of 6.2 (± 4.9) when compared to cells grown on TCP in growth medium.

The GLUT 4 gene expression was non-significantly different for cells seeded with the adipose environment and cultured in scaffold III and IV in growth medium (Figure 4.8C). A fold change of 1.46 (± 0.13) and a fold change of 0.54 (± 0.4) for scaffold III and IV was respectively identified. A significant fold change increase was noted for cells cultured in the delivery matrix in scaffold III and IV in
differentiation medium. Here, a fold change increase of 12.34 (± 2.6, p = 0.017) and of 41.78 (± 17.24, p = 0.05) respectively was detected when compared to ADSCs seeded on TCP cultured in growth medium. Furthermore, a non-significant fold change of 15.44 (± 17.55) was noted for cells subjected to differentiation factors and cultured on TCP compared to control sample in growth medium. The non-significant expression of cells cultured in differentiation medium on TCP is most likely due to the high biological variability shown by the error bars (Figure 4.8C).

In conclusion, the delivery matrix was shown to enhance the cell distribution in the macropores of the scaffolds. Additionally, it preserved the expression of the stem cell marker CD90 and supported the expression of the pre-adipocyte gene $PPARG$ in the cells delivered to the scaffolds and cultured in growth media. Therefore, indicating the presence of stem cells and ADSCs that are differentiating towards the adipogenic lineage, hence a heterogeneous cell population. Furthermore, visible but not significant increased expression of $GLUT 4$ was observed when cultured in differentiation medium, indicating adipogenic differentiation. This suggests that the artificial adipose environment assists adipogenesis when subjected to adipogenic inducing factors and on its own supports the maintenance of ADSCs and their differentiation towards the adipogenic lineage, thus also confirming the selection of the gels containing ECM components.
Figure 4.6: ADSCs delivered with the collagen laminin hydrogel are evenly distributed in the macropores of the -80°C pre-frozen scaffold III structure and show a rounder morphology. ADSCs were delivered into scaffold III with a collagen and laminin gel and cultured for 10 days in growth medium (NON) or differentiation medium (DIFF). (A) The distribution of ADSC in the macropores of the scaffold is represented. Scale bar represents 200 µm. (B) The ADSCs are able to differentiate which is illustrated through the morphological change of the actin cytoskeleton. The cells became a rounder phenotype. (C) They also express GLUT 4. Scale bars represent 50 µm. Yellow + green = auto-fluorescent scaffold; red = actin/ GLUT 4; blue = nucleus.
Figure 4.7: ADSCs delivered with the collagen laminin hydrogel are distributed in the macroporous freeze dried scaffold structure, are smaller and able to express Glut 4. ADSCs were delivered into scaffold IV with a collagen and laminin gel and cultured for 10 days in growth medium (NON) or differentiation medium (DIFF). (A) The distribution of ADSC in the macropores of the scaffold is shown. Scale bar represents 200 µm. (B) The ADSCs are able to differentiate which is illustrated through the morphological change of the actin cytoskeleton. (C) The differentiation is confirmed through the expression of GLUT 4. Scale bars represent 50 µm. Yellow + green = autofluorescent scaffold; red= actin/ GLUT 4; blue= nucleus.
Figure 4.8: ADSCs delivered with a collagen laminin hydrogel show expression of stem cell and adipogenic differentiation genes. ADSCs were delivered with a collagen laminin gel into the scaffolds and cultured for 10 days either in growth medium or adipogenic differentiation medium. As control ADSCs were grown on TCP and cultured in differentiation medium (DIFF). The fold change was calculated against cells grown on TCP in growth medium. The expression of CD90, PPARG and GLUT 4 was analysed. (A) The cells show expression of CD90 in all tested conditions with significant differences for cells cultured in differentiation medium in the scaffolds. (B) PPARG expression displays a significant increased expression for cells in the scaffolds cultured in DIFF. (C) A significant increase in GLUT 4 expression is revealed for ADSCs cultured in scaffold III and in differentiation medium. Significance is given for *p≤ 0.05. Error bars indicate standard deviation. The analysis was performed in technical and biological replicates (n= 3). III= -80°C pre-frozen scaffold, IV= freeze dried scaffold. DIFF= culture
4.4 Discussion

Approaches for developing a scaffold for adipose tissue regeneration have often focussed on scaffold based strategies, neglecting the interplay between the cells and the native adipose environment (Cheung et al., 2014). This environment is dominated by the ECM which consists of a variety of proteins such as growth factors, collagens, elastins, laminin and fibronectin (Mariman and Wang, 2010, Divoux and Clément, 2011, Watt and Huck, 2013). This highly active milieu is secreted by tissue specific cells and characterised through the reciprocal relation between ECM and cells where each is influenced by the other (Mariman and Wang, 2010). ECM, as part of the stem cell niche, plays an important role in cell differentiation (Kawaguchi et al., 1998). In the process of adipogenesis the composition of ECM dramatically changes from a fibrillar ECM with a high collagen level to a laminar ECM rich in laminin (Kawaguchi et al., 1998, Halberg et al., 2008, Mariman and Wang, 2010, Divoux and Clément, 2011, Mori et al., 2014). In vitro studies with individual ECM components revealed their potential for improved cell attachment, proliferation and suppression or support of differentiation (Spiegelman and Ginty, 1983, Li et al., 2008, Sánchez et al., 2000, Lam and Longaker, 2012).

The support of adipogenic differentiation was revealed to be especially important for adipose tissue regeneration. Lequeux et al. constructed large collagen sheets combined with ADSCs for adipose tissue reconstruction. In in vivo porcine model studies the group failed to reconstruct the hypodermis due to unsatisfying support of adipogenic differentiation (Lequeux et al., 2012). This confirms the support of adipogenesis as an essential feature for the regeneration of the lost adipose tissue. On the other hand the preservation of ADSCs is important for clinical application. ADSCs have been shown to support adipose tissue formation, tissue integration as well as angiogenesis (Yoshimura et al., 2008a, Yoshimura et al., 2008b, Tremolada et al., 2010). Thus, the present study followed the approach of the manufacture of an extracellular environment that supports time-related adipogenesis of ADSCs. Here, the time-related differentiation of ADSCs would retain a heterogeneous cell population, consisting of ADSCs and differentiating stem cells towards the adipogenic lineage. In this way both desirable features of
ADSCs and adipogenesis can be applied in the process of adipose tissue regeneration.

At the same time this milieu should be used as an ADSCs delivery matrix to transport ADSCs into the gelatin scaffolds and support the cell population resident in the macropores. In this way the surface area of the macropores is increased, from cell localisation onto the construct walls towards additional cell accumulation within the large pores. Thus, an increased surface for cell attachment and proliferation would be provided (Murphy and O'Brien, 2010).

Therefore, the aim of this study was to advance and improve scaffold III and IV to create a composite scaffold containing an artificial adipose stem cell niche which, as outlined earlier supports time-related ADSC adipogenesis. Scaffold III and IV were chosen due to their favourable structure that assists ADSC infiltration, distribution and proliferation. Furthermore, the scaffolds supported the preservation of ADSC stemness and showed implication of a slow degradation rate while cellular remodelling of the constructs occurred. To develop the artificial stem cell niche extracellular matrix components were tested for their ability to induce adipogenesis of ADSC in a 2D morphological study. Promising components were chosen to create a gel suitable for ADSC delivery and support of time-related adipogenesis. After applying the gel into the scaffolds the resultant adipogenic induction was investigated by qRT-PCR.

4.4.1 2D study of the influence of ECM components on ADSC morphology

4.4.1.1 Morphological changes of differentiating ADSCs

To study the influence of different ECM components on the differentiation potential of ADSCs towards the adipogenic lineage, morphological changes in cell appearance were investigated. It is well known that morphological changes take place during the early development of lipid laden mature adipocytes. Adipogenesis can be divided into 2 phases; the commitment and the terminal differentiation phases. During the commitment phase, the mesenchymal stem cell commits to the adipogenic lineage. This process cannot be identified visually but the terminal differentiation phase can. An early sign of the second phase is the
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morphological change from fibroblast-like cells to more rounded cells. This is followed by lineage specific gene expression (Gregoire et al., 1998, Rosen and MacDougald, 2006, Lowe et al., 2011, Rosen et al., 2014). In the present study these features were applied to identify differentiating ADSCs.

To prove this early morphological change in shape, adipogenesis was induced in ADSCs grown on TCP using differentiation medium. This medium contained adipogenic lineage inducing molecules such as isobutylmethylxanthine and dexamethasone (Pittenger et al., 1999, Zuk et al., 2001). After three days of culture, morphological changes were observed and the cells displayed significantly different aspect ratios when compared to cells cultured in growth medium. This aspect ratio confirmed spherical cell shape of ADSCs cultured in differentiation medium when compared to the higher aspect ratio of the fibroblast like ADSCs. As confirmed by others the in vitro cell rounding of adipogenic committed cells was observed and significantly rounder cells were identified and quantified (Gregoire et al., 1998, Rosen and MacDougald, 2006, Young et al., 2013, Chang et al., 2013, Rosen et al., 2014).

For the present study it was demonstrated that ADSCs committed to the adipogenic lineage can be analysed using immunochemical techniques that detect actin and vinculin combined with aspect ratio quantification in the time frame of three days of culture. Therefore, this method can be used to identify surfaces that support cell rounding or cell elongation. Furthermore, successful application of a similar methodology was already performed by the group around Young et.al. (Young et al., 2013). They applied the aspect ratio measurement to quantify cell rounding on bright field images of ADSCs grown on surfaces of varying stiffnesses. Those surfaces contained decellularised extracellular matrix. These authors showed the smaller the aspect ratio, the rounder the cell, and the more it is differentiated towards the adipogenic lineage and a mature adipocyte phenotype (Young et al., 2013).
4.4.1.2 Screening of different ECM components for the induction of a spherical cell shape on ADSCs

Adipose ECM is described to contain a broad range of molecules from growth factors to adhesion proteins (laminin, fibronectin) and collagens. Collagens make up a considerable amount of the non-cell mass of the adipose tissue. They are particularly responsible for the structural characteristics of the ECM (Mariman and Wang, 2010, Divoux and Clément, 2011). Structural characteristics such as stiffness influence stem cell fate transmitted through adhesion of cell-ECM, for example differentiation (Huebsch et al., 2010, Trappmann et al., 2012, Watt and Huck, 2013). Thus, collagen, laminin and fibronectin are known to play a role in the process of adipogenesis. Furthermore, collagen synthesis is a prerequisite for adipogenesis where the fibrillar adipose ECM changes to a laminin rich ECM (Ibrahim et al., 1992, Mariman and Wang, 2010). As a consequence, collagen I and III content decreases and an increase of collagen IV, V, VI content was observed (Gregoire et al., 1998, Mariman and Wang, 2010, Divoux and Clément, 2011). The laminin content was found to increase during maturing of adipocytes and laminin itself is an essential component together with collagen IV of its surrounding basal lamina (Durbeej, 2010, Chiu et al., 2011). The fibronectin concentration was found to decrease during adipogenesis and strongly influence the cytoskeleton (Taleb et al., 2006, Divoux and Clément, 2011). These important interactions of specific ECM components in adipogenesis make them crucial candidates in the development of an artificial adipose stem cell niche.

This study was designed to select appropriate ECM components in an appropriate concentration which support time-related adipogenesis of ADSCs. Thus, coverslips were coated in different concentrations of collagen I, III, IV, V, VI, laminin and fibronectin. Three different concentrations were selected for each component guided by the results observed within the manufacturer’s suggestions and the literature (Spiegelman and Ginty, 1983, Houdijk et al., 1985, Sánchez et al., 2000). After 3 days of culture in growth medium, the morphology was analysed using immunocytochemistry staining and quantitative aspect ratio calculation. Adipogenesis related morphological changes are early observed events in the process of maturing adipocytes as described by Gregoire (Gregoire...
and seen in our study (outlined in section 4.3.1.1 and discussed in section 4.4.1.1). Therefore a time frame of three days was selected to exclude spontaneous adipogenesis resulting from growth arrest due to cell-cell contact of confluent cells (Gregoire et al., 1998, Roxburgh et al., 2015).

Overall, it was observed that the ECM components significantly influenced the morphology of ADSCs. A rounder cell shape was quantified for ADSCs grown on collagen I, collagen III, collagen IV, laminin and fibronectin.

In the case of collagen I and III a morphologically different spherical cell shape was observed at the lowest and highest concentrations tested. These fibrillar molecules are mainly found in the reticular fibre network of the stromal adipose ECM. During adipogenesis it was determined that collagen I and III decrease while adipocytes are maturing (Nakajima et al., 2002, Mariman and Wang, 2010, Divoux and Clément, 2011, Chiu et al., 2011). The study of Molina et al. demonstrated that at day 3 of induced preadipocyte differentiation a higher level of collagen I and III are present when compared to the induction day with subsequent decrease (Molina et al., 2009). However, the effect of cell rounding at a low and high collagen I and III concentration was seen before on fibroblasts cultured on different concentration of collagen I from 0.00075 mg/ml to 0.8 mg/ml (Gaudet et al., 2003). Gaudet et al. related fibroblast rounding at low and high concentration to the receptor saturation model which is based on receptor (integrin) availability of surface and cell (Figure 4.9). At low collagen I concentration (<0.025 mg/ml) fewer integrin binding sites are presented to the cell. Therefore, less focal adhesion points are formed, resulting in cell rounding. These focal adhesions points are under the influence of a strong tensional force. At an intermediate collagen I concentration fibroblast spreading was observed. Here, the receptors are saturated, resulting in cell spreading. At higher collagen I concentration (>0.025 mg/ml) a high number of integrin adhesion points are presented to the cell, which also expresses more integrin receptors. This results in a dense interaction of cells with the collagen fibres, visible through the rounded cell shape and the focal adhesion is subject to a lower force (Gaudet et al., 2003). In the present study similar morphological observations were made for collagen I and III and therefore, can be related to the receptor saturation model.
Collagen VI is a bead forming collagen and known to increase during adipogenesis reciprocal to the appearance of collagen I and III (Kadler et al., 2007, Mariman and Wang, 2010, Divoux and Clément, 2011). In detail, during adipogenesis collagen VI expression initially increases followed by a decline and finally reaching a plateau of expression which is higher than on the day of adipogenesis induction (Nakajima et al., 2002, Molina et al., 2009). However, our study displayed that at a concentration of 1 µg/cm² and 10 µg/cm² ADSCs were significantly rounder than observed with the highest condition tested. This indicates these specific concentrations have an influence on cell morphology with potential influence on related adipogenesis. The variance between different donors was higher than seen in collagen I. Collagen VI is an important structural protein for restricting the size of mature adipocytes. In fact, obese collagen VI deficient mice display increased adipocyte expansion compared to wild type mice (Khan et al., 2009). In our study, a longer cultivation time followed by subsequent gene expression analysis would likely have revealed more detail of its function in adipogenesis.

Laminin and fibronectin were observed to have a rounding effect on ADSC morphology. ADSCs were rounder on a low fibronectin concentration (0.5 µg/cm²). The more spherical morphology was also displayed on ADSCs grown on low and high laminin concentration. This again can be explained by the receptor saturation model (Figure 4.9). Similar cell morphology was observed by Lam and Longaker (Lam and Longaker, 2012). They investigated the morphology, attachment, proliferation and preservation of stemness of stem cells such as ADSCs on different coated surfaces. Laminin and fibronectin surfaces were revealed to influence a round morphology of ADSCs and preserved the expression of stem cell specific genes (Lam and Longaker, 2012). However, in the present study a fibroblast-like form as well as a higher confluent of cells was observed at a laminin concentration of 10 µg/cm², suggesting the preservation of ADSCs as seen in the study of Lam and Longaker (Lam and Longaker, 2012). In this current study additional gene expression investigations for adipogenic markers would provide more evidence of possible differentiation or non-differentiation.
However, in the current study ADSCs cultured on 3 and 5 \( \mu \text{g/cm}^2 \) (30 \( \mu \text{g/ml} \), 50 \( \mu \text{g/ml} \)) fibronectin concentration revealed visually different cell morphology when compared to the other conditions. ADSCs formed a tubular structure. Similar structural formations were observed by Spiegelman and Ginty (Spiegelman and Ginty, 1983). This group undertook studies on pre-adipocyte differentiation on mouse pre-adipocytes grown on different fibronectin concentrations (5 \( \mu \text{g/35 mm (in diameter)} \), 30 \( \mu \text{g/35 mm (in diameter)} \)). They concluded that fibronectin plays a role in suppression of differentiation through influencing the actin cytoskeleton (Spiegelman and Ginty, 1983). However, it has to be mentioned that some research shows that fibronectin does not inhibit differentiation. More recently, Hemmrich et al. cultured human preadipocytes on 200 \( \mu \text{g/ml} \) fibronectin. Preadipocytes displayed enhanced adipogenesis after induction with differentiation medium (Hemmrich et al., 2005a). Smas and Sul and Gregoire et al. also reviewed the controversial effect of fibronectin on inhibition and supporting adipogenesis. They concluded that when cells are allowed to become rounded the inhibitory effect of fibronectin is overcome as seen in the study of Hemmrich et al, where cell rounding was induced through differentiation media (Gregoire et al., 1998, Smas and Sul, 1995, Hemmrich et al., 2005a). Thus, in the present study ADSC morphology was influenced by fibronectin. Here, a widespread and spindle-like cell morphology with cells arranging into a tube formation was observed. This characteristic morphology, as

![Figure 4.9: Receptor saturation Model.](image-url)
shown in the work of Spiegelman and Ginty (Spiegelman and Ginty, 1983) is reminiscent of the phenotype seen when ADSCs are differentiating into endothelial cells (Oñate et al., 2012). Therefore, these fibronectin conditions were excluded from further studies. Nevertheless, important knowledge about the influence of fibronectin on ADSCs was gained. Further studies such as gene expression analysis for endothelial cell marker CD31, long term culture as well as blockage of fibronectin receptor during differentiation would produce additional valuable information about the influence of fibronectin on ADSC differentiation towards the adipogenic lineage and other mesodermal cell lineages such as endothelial cells (Lygoe et al., 2007, Aubin et al., 2015).

In summary, the screening of diverse ECM components for their influence on ADSC morphological change as an indicator for adipogenesis revealed possible candidate components that influence adipogenic differentiation of the stem cell. Collagen I, III, VI, laminin and fibronectin were shown to support a spherical ADSC phenotype. Furthermore, the influence of fibronectin on the cytoskeleton and possible differentiation towards other lineages was indicated. Therefore, this study delivers a framework for possible ECM components which influence ADSC morphology and induce differentiation. However, to support potential effects on adipogenesis further studies are required. Adipogenic gene expression as well as longer culture time with subsequent adipogenic lineage specific staining should be conducted. This would reveal insights in adipogenic induction potential of the ECM candidates on ADSCs. Further, combinations of the possible candidates such as collagen I and III on adipogenesis of ADSCs would provide additional information. Proliferation and attachment studies would add valuable information in the support of the ECM components of ADSC culture. Besides, the range of concentrations of ECM molecules seemed to affect the morphology of ADSCs differently. This underpins the importance of determining the right concentration of the ECM components as shown by others (Spiegelman and Ginty, 1983).

Nonetheless, studies undertaken revealed among other observations, that collagen I supports cell rounding. Collagen I belongs to the family of collagens (Kadler et al., 2007). Collagens are natural, biocompatible, cost saving biomaterials used for
adipose tissue regeneration as well as having been shown to support adipogenic differentiation (von Heimburg et al., 2001, Hiraoka et al., 2006, Mojallal et al., 2008, Kimura et al., 2010, Lin et al., 2011, Girandon et al., 2011, Casadei et al., 2012, Lequeux et al., 2012, Freric et al., 2012, Jabaji et al., 2013, Werner et al., 2014, Acil et al., 2014). Furthermore, collagen I is a major structural protein of the adipose ECM. Collagen I at a concentration of 20 µg/cm² can form a gel (400 µl/ml as described in section 4.2.4.1) and therefore presents a possible candidate for the creation of an artificial stem cell niche. Additionally, laminin at a concentration of 10 µg/cm² increased cell confluence visually and a fibroblast-like cell morphology was maintained. According to other studies laminin is involved in enhancing attachment and proliferation (Placzek et al., 2009, Lam and Longaker, 2012). In addition, laminin is known as the major component of the basement membrane (Durbeej, 2010, Chiu et al., 2011). Laminin also plays a role in maintenance and remodelling of adipose tissue (Choi et al., 2010a). Thus, laminin presents a further candidate for the development of an adipose stem cell niche, which would assists time-related adipogenesis. Thus, providing a cell population in different stages of adipogenesis, from stem cells to differentiating ADSCs.

These initial selection studies were performed in 2D settings. The 2D studies help to understand how the cells respond to the introduced stimuli (Ravi et al., 2015), specifically of the tested ECM candidates. Here, the 2D investigations were applied for the selection of individual ECM components out of multiple candidates for the creation of an artificial adipose ECM matrix. Two dimensional analysis are further cost and time saving when compared to 3D investigations, where the scaffold materials, increase of ECM constituents and cell number would be needed. Consequently, the cost and the duration of the experiments would also expand. Thus, 2D approaches were a cost saving and time efficient approach for the selection of promising ECM candidates for further 3D investigations.

Nevertheless, the interaction of cells in 2D and 3D is different with 3D environments mimicking the native environment more closely (Meng et al., 2014). In 3D environments cell adhesion to their surroundings is not planar as in 2D settings, a third dimension is added and based on different integrin formation
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(Huebsch et al., 2010). Additionally, the forces influencing cell spreading are different. The diffusion of soluble factors like nutrients is also organised differently. In 2D conditions the nutrients are applied apically whereas in 3D environments the nutrients diffuse the construct all around (Baker and Chen, 2012). Susante et al. showed that chondrocytes cultured in collagen I gels regain a fibroblast like morphology and dedifferentiated (Susante et al. 1995). Lin et al. displayed that culturing ADSCs on microcarriers based on collagen I and IV revealed increased differentiation in 3D compared to 2D culture (Lin et al. 2014). Therefore, further studies were conducted with collagen I and laminin in a 3D environment.

4.4.2 3D study of ECM components in the scaffolds

To advance the study a gel was cast consisting of collagen I (400 µg/ml, resembling 20 µg/ cm² of the 2D studies, described in section 4.2.4.1) and laminin (200 µg/ml resembling 10 µg/ cm² of the 2D studies, described in section 4.2.4.1). Collagen I was chosen because of its performance in the 2D study where it induced cell rounding, thus indicating adipogenesis. Furthermore, it is a major structural component of the native adipose ECM (Mariman and Wang, 2010, Divoux and Clément, 2011). The fibrillar collagen is also differently expressed during adipogenesis (Nakajima et al., 2002, Mori et al., 2014). Laminin was chosen due to its visually increasing effect of ADSCs proliferation and attachment, indicating maintenance of ADSCs stemness. The protein was also selected because of its role in adipogenesis and as basement membrane protein (Mariman and Wang, 2010, Divoux and Clément, 2011, Lam and Longaker, 2012, Mori et al., 2014). Hence, collagen I and laminin were utilised for the creation of an artificial stem cell niche featuring time-related support of adipogenesis. Here, the stem cell population would be maintained through laminin while over time adipogenic differentiation would be induced. Specifically, through the presence of collagen I and the remodelling or degradation of the stemness inducing constituent.

To analyse the effect of the incorporation and delivery of ADSCs within this native environment a gel was cast and ADSCs were delivered into the two
scaffold types and cultured for 10 days. The scaffolds were maintained either in growth or differentiation medium to evaluate the potency of adipogenic differentiation. Afterwards histological staining of actin and GLUT4 as well as adipogenic gene expression analysis were performed. This gave information about cell distribution within the macropores and GLUT4 expression as well as adipogenic supportive effects of the collagen laminin hydrogel within the scaffolds.

The histological analysis for actin cytoskeleton showed that in scaffold III after 10 days in culture in growth medium ADSCs clustered and presented fibroblast-like elongated cell morphology. As expected a more spherical cell phenotype was observed when cultured in differentiation medium, indicating adipogenesis (Young et al., 2013). In scaffold IV a fibroblast-like morphology of ADSCs was shown when either cultured in growth or differentiation medium. The similar cell morphology of differentiation and non-differentiating cells in a 3D environment was observed by others (Huebsch et al., 2010). Huebsch et al. investigated the differentiation ability of MSCs in alginate hydrogels differing in stiffness. They noted MSC differentiation on soft surfaces towards the adipogenic lineage and on stiffer surfaces towards the osteogenic lineage. Thereby the cell morphology did not change. These observations were traced back to different integrin binding formation in 3D environment compared to 2D environment (Huebsch et al., 2010). Therefore, the similar cell morphology observed in our study may be an artefact of different integrin binding in 3D and therefore not necessarily evidence of non-differentiation or differentiation.

Further, the cells were distributed in macropores except in scaffold IV cultured in differentiation medium where an alignment along the macropore wall was observed. The successful cell distribution with a delivery matrix was confirmed by our own group and others (Handel et al., 2012, Phull et al., 2013, Cheung et al., 2014). To reach an even cell distribution within the macropores, they have to be filled with the delivery matrix. However, the location of the ADSCs at the rim of the macropores in scaffold IV indicates little infiltration of the collagen laminin mixture into the pores. For further studies the applied delivery matrix volume should be increased to ascertain optimal filling of the porous structure.
Nevertheless, this delivery matrix provided sufficient environmental cues to support adipogenesis induced through differentiation factors and shown through GLUT4 staining. The process of adipogenesis was further demonstrated by the results of the gene expression analysis. The gene expression analysis revealed a visible decrease in *CD90* expression and a visible increase of *PPARG* and *GLUT4* expression of ADSCs delivered with the collagen and laminin gel into the scaffolds.

Furthermore, the cells in scaffold III and IV cultured in growth medium showed an increase in the expression *PPARG* and no difference in the GLUT 4 expression. This explains the lack of detection of differentiation marker protein GLUT4 in the histological analysis (shown in Chapter 4, Figure 4.7, 4.8). These observations were not statistically significant which might be due to the high biological variability. The inductive effect on adipogenesis through the collagen laminin hydrogel as observed through the upward trend in the expression of *PPARG* when cultured in growth medium, manufactures a closer cell-cell contact and therefore initiates spontaneous differentiation. Additionally, it prevents cell wash out which occurs through cultivation and replacing of media (Flynn et al., 2008). Furthermore, in the present study the influence of the collagen laminin gel on cell morphology through stiffness cannot be excluded (Huebsch et al., 2010).

Throughout the whole study a high biological variability was observed as discussed previously (Chapter 3, outlined in section 3.3.2 and 3.4.4). This variability is due to use of primary human cells. Here, age, gender, BMI and health affect the differentiation potential of ADSCs (Sun et al., 2011, Sachs et al., 2012, Oñate et al., 2012, Russo et al., 2014). Due to the high biological variability no significance was determined within the gene expression study but trends were visible. Specifically, the support of adipogenesis through the collagen laminin gel was evident when induced with differentiation factors. Furthermore, non-significant but visible adipogenic inductive effects of the collagen laminin hydrogel were demonstrated when cells were delivered into the scaffold and cultured in the growth medium. Therefore, indicating the presence of ADSCs and ADSCs differentiating towards the adipogenic lineage, thus the time related adipogenic differentiation of the stem cells. To date, biomaterials that have been
shown to induce adipogenesis revealed integration in the host tissue as well as increased adipose tissue formation (Uriel et al., 2008, Wang et al., 2013a, Young et al., 2014). Uriel et al. derived gels from adipose tissue which showed adipogenic induction of ADSCs after 1 week in vitro. In comparative in vivo studies in a rodent model the gel alone displayed an increased adipose tissue formation compared to matrigel (Uriel et al., 2008). Further, Young et al. observed that decellularised adipose tissue seeded with ADSCs showed integration with the host tissue, increased adipose tissue formation as well as the induction of vessel formation (Young et al., 2014). Consequently, the observed effect of adipogenic induction supports the use of the scaffolds in adipose tissue reconstruction.

Overall, this study illustrated the development of an artificial adipose stem cell environment from the selection of appropriate ECM components to the subsequent development of a gel that was applied to deliver ADSCs into the scaffold. This allowed the investigation of the adipogenic differentiation potential of the resulting composite scaffold. It was demonstrated that the adipose stem cell environment can be used as delivery matrix and supports the distribution of the cells within the large macropores of the scaffolds, thus increasing the surface area for cell attachment and proliferation.

Furthermore, this delivery matrix integrated into the scaffolds supported adipogenic differentiation when induced with differentiation factors. Additionally, the manufactured composite scaffolds also supported spontaneous adipogenic differentiation. Such spontaneous adipogenic induction has already been shown by others to be advantageous when applied in vivo for tissue integration as well as adipose tissue formation (Uriel et al., 2008, Wang et al., 2013a, Young et al., 2014).

To date, composite scaffolds based on natural scaffold materials in adipose tissue reconstruction display good tissue integration in in vivo settings (Cheung et al., 2014). Cheung et al. manufactured chitosan based scaffolds and delivered ADSCs into the scaffold with decellularised adipose matrix. An in vivo rodent model showed implant integration, adipogenesis and fat formation. However, a fast
degradation of 12 weeks was revealed (Cheung et al., 2014). Further the application of decellularised adipose matrix is hampered due to the discrepancy of donor volume to gained matrix volume (Sano et al., 2014). Flynn et al. presented a composite scaffold based on hyaluronic acid and ADSCs delivery with decellularised placental matrix. This combination revealed increased adipogenesis when induced through differentiation factors but did not show any induction of adipogenesis (Flynn et al., 2008). Handel et al. applied a commercial polypropylene sponge and delivered ADSCs into it with a 0.5% collagen hydrogel. In in vitro studies they observed also adipogenic supportive effects of the matrix but not induction (Handel et al., 2012). Phull et al. combined the microporous macroporous scaffold with a fibrin hydrogel mimicking a stimulating extracellular environment. The fibrin delivery matrix distributed cells throughout the porous structure, filled the scaffolds macropores with cells and adipogenic differentiation was observed when induced with adipogenic differentiation cocktail (Phull et al., 2013). However, fibrin does not mimic the native adipose environment consisting of a multitude of structural proteins such as collagen. Therefore, it was necessary to construct an environment closer to the native adipose acellular component (Frantz et al., 2010).

The composite gelatin sponges developed here, therefore, represents a unique combination of an artificial adipose stem cell environment that induces and supports adipogenesis. Furthermore, this artificial environment is not reliant on tissue donation as with decellularized ECM. In addition, after 10 days in culture the structure of the scaffolds is fully retained indicating longer degradation time, which is desirable for adipose tissue reconstruction (Yoshimura et al., 2011, Bellas et al., 2013b). The artificial adipose stem cell environment also improves cell distribution within the scaffold and resembles more closely the native adipose environment (Mariman and Wang, 2010, Divoux and Clément, 2011). The combination within a scaffold structure which is designed to support cell infiltration and porosity supporting vascularisation is advantageous. Thus, the outer structural component the scaffold can be altered in porosity and patients demanding shape. Here, both scaffold types integrating the collagen laminin hydrogel, displayed similar features in cell infiltration, distribution and support of adipogenesis. Therefore, future in vivo studies for their suitability for adipose
tissue reconstruction and promotion/support of adipogenesis should be undertaken on both scaffolds.

### 4.4.3 Conclusion

In this study ECM components were revealed to influence ADSCs morphology in 2D studies dependent upon the concentration of the component. Morphologically, a spherical cell shape was indicative of adipogenic differentiation and elongated cell morphology was related to preservation of stemness.

From the 2D study, a combination of ECM components (collagen I and laminin) was chosen to support time-related adipogenesis of ADSCs in \textit{in vitro} studies. It was displayed that ADSCs can be delivered within the artificial stem cell niche into the selected scaffolds III and IV and that this supported cell population within the porous gelatin sponge structure.

Subsequent differentiation studies revealed that the newly developed scaffolds composed of the gelatin sponge with internal artificial ADSC stem cell niche support adipogenesis as well as the preservation of ADSC stemness, thereby closely resembling the native adipose environment. Further, the composite scaffold displayed adipogenic inductive features that are favourable in \textit{in vivo} settings for adipose tissue formation and tissue integration.

In conclusion, novel composite scaffolds were designed based on an outer scaffold structure and inner artificial stem cell niche. The outer structural component, the scaffold, is tuneable in shape, porosity and pore size and allows efficient uptake and distribution of cells and ECM throughout its porous environment. The internal stem cell niche was designed to assist cell distribution within the macroporous scaffold structure and support adipogenesis and the retainment of ADSC stemness. Hence, this study leaves two potential scaffolds which have been evaluated \textit{in vitro} to advance to further \textit{in vivo} studies for adipose tissue reconstruction.
Chapter 5: General discussion

Adipose tissue is the largest connective tissue in the human body and widely known for its role in energy storage (Smahel, 1986, Patrick, 2001, Sethi and Vidal-Puig, 2007). It also operates as an endocrine organ including functions such as thermogenesis, reproduction and other metabolic activities. Additionally, the fatty tissue provides mobility and cushioning of the bordering tissue layers due to its location and texture properties (Sethi and Vidal-Puig, 2007, Bucky and Percec, 2008, Choi et al., 2010a, Frerich et al., 2012, Cinti, 2012). This tissue is rich in adipocytes and further contributes to our body’s individual shape (Bucky and Percec, 2008, Choi et al., 2010a, Frerich et al., 2012). Consequently, if the adipose tissue is lost due to age, disease, traumatic accidents, full thickness burns or congenital malformation this can lead to severe and deforming contour defects. Affected patients suffer not just physically but also emotionally due to the deformation (Patrick, 2001). The emotional impact of the disfigurement are often more disturbing for the patient than the defect itself (Partridge, 2015).

Current strategies to restore the body contour such as fat grafting, cell enriched lipotransfers and the use of synthetic filler materials are unpredictable and often result in unsatisfying outcomes (Brayfield et al., 2010). Fat grafts are subjected to a high resorption rate and due to necrosis of the central areas of the graft are not applicable in cases of major defects such as those resulting from traumatic accidents (Tremolada et al., 2010, Tabit et al., 2012). The application of cell enriched lipotransfers is also only applied to the reconstruction of smaller defects. Synthetic materials are often recognised by the body as foreign and can cause toxic and allogenic effects, migrate or be absorbed (Tremolada et al., 2010, Tabit et al., 2012, Philips et al., 2012). Consequently, described methods often require multiple applications of donor fat or synthetic filler materials and do not restore the natural feel and desired aesthetic appearance of the tissue that has been lost (Tabit et al.,
2012). Furthermore, the requirements for restoring contour defects, displayed in the surgical procedures carried out to reconstruct body deformities increases yearly (Surgeons, 2015). Accordingly, the limitation in current treatment methods paired with the increasing need to restore the body contour raises the demand for adipose tissue fillers. The tissue engineering discipline provides a rational approach for the development of replacement constructs for this lost tissue (Langer and Vacanti, 1993).

The tissue engineering strategy incorporates the fabrication of a scaffold structure to provide shape and a support complex or scaffold for cells to attach, proliferate and differentiate, which contributes to tissue formation while the scaffold slowly degrades. Natural biomaterials display a multitude of bioactive features when compared to synthetic materials (Placzek et al., 2009). Among natural biomaterials, gelatin has been shown to present favourable characteristics for the application in adipose tissue reconstruction. It is biocompatible, has a natural feel, is non-toxic and forms gels that are shapeable and adaptable to defects of different forms and dimensions (Lin et al., 2008, Bigi et al., 2001, Yao et al., 2012, Grover et al., 2012, Kirsebom et al., 2013, Chang et al., 2013). Furthermore, it has already been applied in vitro and in vivo as small scaffolds for adipose tissue reconstruction and shown to support cell attachment, proliferation, migration and adipogenesis (Lin et al., 2008, Korurer et al., 2014). Like other natural biomaterials the collagen derivative displays a rapid degradation rate and poor mechanical properties which can be improved through chemical cross linking with glutaraldehyde (Bigi et al., 2001, Farris et al., 2010).

Tissue engineering also integrates a cell source, which is able to proliferate and differentiate into the lost cells of the tissue being bioengineered. For adipose tissue reconstruction a remarkable cell source are the tissues own stem cells- ADSCs. ADSCs are an abundant stem cell source, which can be harvested using minimal invasive methods from liposuction or abdominoplasties (Konno et al., 2013, Zuk, 2013). These cells are predisposed to differentiate into adipocytes but are also capable of differentiating in other mesodermal cell lines such as chondrogenic or
osteogenic cells (Zuk et al., 2001, Brayfield et al., 2010). Hence, ADSCs represent an ideal cell source for adipose tissue reconstruction.

Besides the cell source, a further aspect of the tissue engineering discipline is the delivery and incorporation of appropriate extra cellular signals that support the cells and scaffold in tissue remodelling and regeneration (Bauer-Kreisel et al., 2010). Current adipose tissue engineered substitutes based on synthetic or natural biomaterials are restricted either in material properties which do not mimic the natural tissue in feel, its complex acellular surrounding and/or degrade too rapidly (Mauney et al., 2007, Wang et al., 2013a, Bellas et al., 2015). Further, products that displayed tissue integration, adipogenesis and angiogenesis in vivo are limited to areas of a few centimetres/microlitres (Kimura et al., 2010, Moioli et al., 2010, Young et al., 2014). They have been shown to cover large and thin dimensions, but are clearly unable to reconstruct bulk soft tissue defects such as would be required after for example breast tumour removal (Lequeux et al., 2012).

This project set out to construct a bulk scaffold suitable for adipose tissue reconstruction of larger dimensions. Therefore, the scaffold, the cell source and the extracellular signal aspects of the tissue engineering discipline were considered and combined to develop and create novel constructs that would be ready to advance towards in vivo studies for adipose tissue regeneration.

Findings and observations from this project have contributed to the following conclusions:

1. Through the application of a particulate leaching method, combined with the utilisation of ice as porogen, the construction of a novel range of bulk microporous macroporous scaffolds advanced in microporosity and macroporosity could be fulfilled.

2. The incorporation of the advanced microporous macroporous structure within the gelatin scaffolds combined with freeze drying, can support cell infiltration, distribution and viability as well as the preservation of ADSC stemness.
3. Individual ECM components can be combined to an artificial ADSC stem cell niche that can be incorporated within the scaffolds, which aids the delivery of cells throughout the porous structure and supports adipogenesis.

4. The combination of an outer tuneable scaffold structure with physical and biological profile suitable for adipose tissue reconstruction and internal artificial ADSC stem cell niche yields a novel range of scaffolds with potential for adipose tissue reconstruction.

The study conclusions outlined above fulfil the project goals described under the study aims (outlined in section 1.4.1). The outcome resulted from experimental work, which was carried out and divided into the three main aspects of the tissue engineering strategy: 1. scaffold, 2. cell source, 3. extracellular signals resulting in study conclusions as described in subsequent section.

5.1. Scaffold preparation and characterisation

In this study scaffolds based on gelatin with small diameter (< 160 µm) and larger diameter pores (≥ 1 mm) were constructed through the application of a recently developed solvent casting and particulate leaching technique based on alginate beads and a solvent gelatin matrix (Phull et al., 2013). This method was further developed and adapted to create a reproducible, controlled and time efficient scaffold preparation method applicable for bulk constructs. Specifically, the reagents used for the alginate bead preparation and dissolution were fine-tuned to obtain uniform spherical and homogenous cross linked alginate beads for the use as macropore templates. The leaching technique also required further adjustments due to its uncontrolled and time consuming nature. As a result of this, a technique to control the alginate dissolution from the bulk scaffolds was developed by direct injection of the dissolution solution (trisodium citrate) into the constructs. To date, methods that used the reversible gelation of alginate beads to create large pores within scaffolds applied the dissolution solution via diffusion within thin matrixes such as 15 µm (Delaney et al., 2010) or 5 mm in depth (Tomei et al., 2009). Calcagnile et al. recently addressed this problem as well and used heat to shrink the enclosed alginate beads with subsequent residual removal through water flow out of larger scaffolds.
(> 1cm) (Calcagnile et al., 2014). Hence, the advanced leaching methodology out of scaffolds with large dimensions presented here demonstrates an improved, efficient, timesaving and controlled approach for the removal of alginate beads from larger constructs. Further, through histology it was shown that macropores (≥ 1 mm) can be produced in the bulk gelatin sponges with the improved alginate bead leaching method.

In the next step the control over the pores within the gelatin structure, termed micropores was gained. This mechanism provides a tool to construct different micropore sizes and therefore, varying range of micropores can be selected according to the demand of the cell source. It was decided to apply ice as porogen with subsequent freeze drying to construct a porous structure. The use of ice crystals in the construction of pores displays a generic and bio-clean method without the application of additives or organic solvents (Kang et al., 1999). Furthermore, this process can be combined with the particulate leaching technique, consequently the macroporous structure is retained. Hereby, the rule was followed that the lower the freezing temperatures the smaller the formation of ice crystals hence the smaller the pores that formed (Mao et al., 2003). The subsequent freeze drying step sublimes the ice out of the pores without thawing, leaving pores behind producing a porous structure (Kang et al., 1999, Nazarov et al., 2004).

The initial temperature that the scaffold was subjected to was room temperature without freezing or freeze drying. This resulted in the production of a biomaterial similar to the scaffold constructed by Phull et al. (Phull et al., 2013) and called scaffold type I in the present study. Furthermore, a range of freezing temperatures were applied to yield derivatives of scaffold type I. This was achieved using temperatures at - 20°C (scaffold type II), at - 80 °C with subsequent freeze drying (scaffold type III) and just by freeze drying with a condenser temperature of - 55°C (scaffold type IV). Through this application 4 different scaffolds (I-IV) were developed differing in the range of micropore sizes produced. The resultant biomaterials were characterised macroscopically for their construct size and histologically for macropore and micropore size. Through swelling, the liquid uptake of the constructs was investigated. Furthermore, the porosity and interconnectivity
were analysed with the liquid replacement method and the investigation of the time needed for liquid to pass through the scaffolds. This permitted the selection of those scaffolds, which were physically suitable for cell based studies. Macroscopic measurements confirmed that scaffolds were produced with the pre-freezing and freeze drying method (Phull et al., 2013) that can be ranked in the range of bulk constructs for adipose tissue reconstruction (average dimensions of 1 cm in diameter and 1 cm in height) (Hemmrich et al., 2008, Kimura et al., 2010, Lequeux et al., 2012, Chang et al., 2013, Debels et al., 2015a). These scaffolds also could provide a platform for scaling up due to the shapeable gelatin matrix and the adjustment of the amount of alginate beads and reagents used to the desired dimensions.

Furthermore, histological investigations revealed that the application of alginate beads can be utilised as templates for the manufacture of large pores, termed macropores. It has to be mentioned that using histological assessment of sectioned scaffold showed a range of macropore sizes, which is likely an artefact of the sectioning process and resulted in microscopic analysis revealing different pore diameters. Nevertheless, the leaching process still succeeded in the manufacture of large pores in all scaffold types ranging up to 3.09 mm in diameter which provides theoretically enough space for maturing adipocytes that can reach a size up to 190 µm in diameter (Skurk et al., 2007).

It was further shown through histological analysis that different micropore size ranges could be constructed depending on the freezing temperature as shown by others (Mao et al., 2003, Imani et al., 2013) with pore size diameters ranging from 3.55 µm up to 159.18 µm. However, the lowest applied freezing temperature did not produce the smallest micropore size as seen by others (Mao et al., 2003, Imani et al., 2013). This may be due to merging of the small pores into larger pores as observed in scaffold III frozen at -80°C (detailed in Chapter 2, section 2.3.2.2.2). Scaffold IV, frozen under freeze dryer conditions displayed the most stable distribution of micropores probably due to immediate sublimation of water resulting from the freeze drying process. Furthermore, throughout the scaffolds outer larger pores with inner smaller pores were observed as a result of freezing from the outside towards the inside and predominantly merging of the peripheral pores into larger formations.
This created a wide range of micropore size. However, this should not be a limitation as seen by others that applied successfully constructs \textit{in vitro} and \textit{in vivo} studies with larger outer and smaller inner pores (Bellas et al., 2015, Korurer et al., 2014). Thus, the constructed inner micropores were wide enough to potentially contribute to cell and nutrients infiltration as well as waste removal. All presented scaffolds displayed a soft natural feel and were further physically characterised for their suitability in cell based studies. Therefore, the liquid up take, porosity and interconnectivity were chosen to investigate through analysis of the swelling behaviour, the liquid replacement method and ability to support liquid flow throughout the materials. The liquid up take and interconnectivity are essential for cell infiltration, metabolite exchange during cell culture and therefore for the survival of the cells used (Fan \textit{et al.}, 2015). High porosity is necessary for increased space for cell infiltration, attachment, proliferation and differentiation. This characterisation revealed that scaffold II-IV showed increased liquid uptake and porosity compared to scaffold I. Consequently, scaffold I was excluded from further studies due to the demand of high porosity for scaffolds in cell based studies and \textit{in vivo} adipose tissue reconstruction (Zhong \textit{et al.}, 2012, Korurer \textit{et al.}, 2014).

However, at the end of the scaffold preparation and characterisation study (Chapter 2), 3 scaffold types differing in micropore size but similar in macroporous structure were characterised and ready to be moved towards the cell based investigation. Those scaffolds displayed suitable porosity, liquid uptake and different ranges of micropore sizes when compared to the scaffold prepared similar to the recent construct of Phull \textit{et al.} (Phull \textit{et al.}, 2013). As such, the selected scaffolds were manufactured through an improved particulate leaching method and the use of ice as porogen into a novel range of advanced constructs for adipose tissue engineering.

\subsection*{5.2. Scaffold design and its interaction with ADSCs}

In the next step, the selected scaffolds were characterised further. In this cell based study the scaffolds design should be tested for their suitability to be seeded and cultured with ADSCs, therefore the scaffolds potential to be applied in \textit{in vivo} settings. Furthermore, it should distinguish the scaffold types that can be moved
towards the subsequent tissue engineering component, which is the incorporation of extracellular signals for the support of adipogenesis.

In the present study, cells isolated from subcutaneous adipose tissue were confirmed to be ADSCs through immunophenotype and differentiation studies as by others (Zuk et al., 2001, Zuk et al., 2002, Bunnell et al., 2008). The selected scaffolds (II-IV) were tested for their suitability to support ADSC seeding, infiltration, distribution, viability and preservation of the stem cell phenotype. Additionally, in a preliminary cell based in vitro analysis over 2 months the biomaterial degradation behaviour was investigated. This evaluated if the scaffold design supported cell infiltration, cell distribution throughout the construct and promotes homogenous tissue regeneration (Carrier et al., 1999, Weinand et al., 2009). Furthermore, it was important to confirm that the natural biomaterial used was biocompatible with the stem cell source and does not support differentiation towards other lineages such as chondrogenic or osteogenic lineage (Zuk et al., 2001). The information of the degradation behaviour should provide implications if the scaffold design supports a desirable slow degradation rate while cells remodel the construct structure (Yoshimura et al., 2011).

The constructed scaffolds displayed a sponge-like absorbance of cells that had been suspended in normal growth media, additionally demonstrating a favourable feature of freeze dried materials (Hemmrich et al., 2005b, Lai et al., 2013, Lloyd et al., 2014). This feature assisted the distribution of the cells throughout the constructs and was revealed by histological investigation of serial sections through the scaffold. Furthermore, histological analysis displayed differing cell distribution within micropores and macropores of the scaffolds. Scaffold II supported cell infiltration mainly within the macropores whereas scaffold III and IV were shown to harbour cells within both structures. Therefore, scaffold II was excluded from further studies due to heterogeneous cell distribution within the porous structure resulting from the small range of micropore sizes. The design of scaffold II would potentially jeopardise even tissue reconstruction and display a high risk of pore blockage through cells (Cao et al., 2006). Scaffold III and IV were ascribed as favourable candidates to take forward for further studies to produce homogenous tissue regeneration. Viability studies with live/dead and metabolic assays confirmed cell
viability on the natural scaffold materials as well as within the scaffolds after 10 days. The support of cell viability was seen by others that utilised scaffolds based on gelatin (Yao et al., 2012, Kirsebom et al., 2013, Chang et al., 2013). The preservation of ADSC stemness during culture within the scaffolds was confirmed through gene expression (stem cell marker CD90, adipogenic genes PPARG, GLUT 4, chondrogenic gene SOX 9 and osteogenic gene RUNX 2) analysis.

In a subsequent cell based degradation study the structural stability of the scaffolds was tested over 2 months. Such a study mimics the in vivo conditions more closely than in vitro enzymatic or PBS based degradation analysis (Grover et al., 2012, Bellas et al., 2015). Degradation of the scaffolds was revealed through histological staining. Hence, changes in the microporous macroporous structure of the scaffolds were observed indicating degradation as shown in other studies (Korurer et al., 2014, Bellas et al., 2015). Scaffold IV displayed an increased degradation that was visible by virtue of the looser structure of the gelatin sponge. This loosening most likely resulted from the more even cell distribution within the porous structure when compared to scaffold III. ADSCs increasingly cellularised the porous structure within the centre of the scaffolds, revealing a spherical shape and indicating matrix deposition. The latter should be investigated in future studies. The slow degradation rate observed with the scaffolds is a desirable feature for adipose tissue reconstruction. It allows the cells to proliferate and differentiate to reconstruct the lost tissue while the biomaterial slowly degrades (Yoshimura et al., 2011). Due to time limitations and the length of the study only preliminary, but promising, data were revealed. Further repeats, quantifications and specific extracellular staining should be carried out in future studies.

Nevertheless, the results gained through the cell based study made the use of the scaffolds viable for ADSC stem cell culture and also the incorporation of adipogenic supportive ECM factors.

By the end of the biological characterisation study (Chapter 3) scaffold III and IV were selected because of their favourable cell distribution within micropores and macropores, which would support even adipose tissue reconstruction. Furthermore,
both versions of the scaffold displayed an ability to support ADSC stem cell preservation, therefore can be utilised within ADSC stem cell culture. In addition, scaffold III and IV show a favourable slow degradation rate which would allow cellular regeneration of the lost tissue while the construct slowly reduces in size. Furthermore, the scaffolds can be moved forward to advance their properties through the incorporation of extracellular signalling. The signalling was achieved by the design of an extracellular matrix environment, which delivered ADSCs into the scaffolds, harbours ADSCs and supports their differentiation towards the adipogenic lineage. This differentiation is essential for the support of adipose tissue reconstruction.

In conclusion, the first aim of the project was met. The goal included the development of an improved scaffold based on alginate bead leaching out of a solvent gelatin matrix. Additionally, this construct was intended to display features suitable to support and promote adipose tissue regeneration (as described in Chapter 1, section 1.4.1). Thus, the preparation and characterisation studies revealed a novel range of adipose tissue engineered microporous macroporous scaffolds, which displayed a physical and biological profile suitable for the utilisation in adipose tissue reconstruction.

5.3. ECM based adipose stem cell niche

To further develop the scaffolds towards an advanced construct suitable for in vivo studies the third component of the tissue engineering strategy was approached: the integration of extracellular factors that support adipose tissue reconstruction. Thus, the creation of an artificial stem cell niche. This stem cell niche should be used to deliver ADSCs within the microporous macroporous structure to assist the distribution of cells within the large macropores. At the same time it would eliminate the decreased surface area for cell attachment typical for large pores (Murphy and O'Brien, 2010). The decreased surface area used by cells was observed by the phenomenon of cells aligning on the macroporous wall in the present study (described in chapter 3, section 3.3.2) and by others (Bellas et al., 2015). Additionally, this niche should also support the preservation of ADSC stemness as
well as their time-related differentiation towards the adipogenic lineage, gaining a heterogenous cell population. Here, both the support of stemness and differentiation are desirable features for the design of adipose tissue constructs. ADSCs were shown to have higher resistance in ischaemic conditions and contribute to blood vessel infiltration resulting in increased fat graft survival (Kilroy et al., 2007, Yoshimura et al., 2008a). Furthermore, assisting adipogenesis supports the regeneration of new adipose tissue (Hong et al., 2005, Wang et al., 2013b).

In 2D in vitro studies structural components (collagen I, III, IV, V, VI, laminin and fibrinogen) which are known to be present in the adipose extracellular matrix and recognised to play a role in adipogenic differentiation were tested for their support of adipogenesis (Mariman and Wang, 2010, Divoux and Clément, 2011). During adipogenesis a change from a collagen rich to a laminin rich environment takes place, whereby collagen is essential for the terminal differentiation process (Ibrahimi et al., 1992). The investigation of individual components was chosen over the application of growth factors or decellularised adipose tissue to gain a mechanism of control, quality and quantity of the constituents used. To date, the application of growth factors is in need of the fine tuning of the growth factor doses delivered and release mechanism (Lu et al., 2014). The application of decellularised adipose tissue is restricted to the availability of donor tissue and decellularisation methods to gain high volume of non-cellular mass (Sano et al., 2014). Furthermore, altering methods were shown to influence the protein content of this biomaterial (Poon et al., 2013). Therefore, the analysis of selected ECM components on ADSCs behaviour delivers a mechanism of control over the quality and quantity of the ECM constituents used for the manufacture of an artificial stem cell niche, influencing ADSC differentiation.

In the present study, to investigate the support of adipogenic differentiation of ADSCs through chosen components, their influence on ADSC morphology was investigated. Morphological changes are early signs of the terminal differentiation phase of adipogenesis. Here, the stem cell is already committed to the adipogenic lineage and further matures to lipid laden adipocytes (Bucky and Percec, 2008). Thereby, their morphology changes from fibroblast-like to spherical cells followed
by lineage specific gene expression (Gregoire et al., 1998, Rosen and MacDougald, 2006, Lowe et al., 2011, Rosen et al., 2014).

Initially, it was confirmed that the aspect ratio of immunocytochemical stained differentiating ADSCs showed significant changes of cell morphology compared to non-differentiating cells. It was proven that a change from elongated fibroblast-like undifferentiated to spherical differentiating cells took place as confirmed by others (Chang et al., 2013, Young et al., 2013). This analysis tool was subsequently employed to assess cells cultured on surfaces coated with varying concentrations of chosen ECM components. This investigation of the induction of morphological change on ADSCs by such a variety of ECM components with different concentrations is so far unique. It revealed that tested components influence significantly the morphology of ADSCs. Specifically, collagen I, III and laminin showed significant spherical ADSCs on tested highest and lowest concentration confirming the receptor saturation model which is based on surface and cell adhesion proteins (Gaudet et al., 2003). Additionally, fibronectin at intermediate (3 µg/ml) and high (5 µg/ml) concentration influenced a tube like accumulation of ADSCs indicating suppression of adipogenic differentiation as observed with preadipocytes by the group around Spiegelman and Ginty (Spiegelman and Ginty, 1983). Therefore, this study adds further to the knowledge of ADSC cell biology and behaviour when grown on individual ECM components. It also opens areas for further in depth studies with the native adipose extracellular mass. Importantly, this investigation revealed laminin and collagen I as possible candidates to assist cell proliferation and to support spherical cell morphology, therefore indicating the support of adipogenesis. Due to a lack of available time no further evaluations could be performed such as long term culture with subsequent gene expression analysis. Additionally, cells behave differently in 2D and 3D environments (van Susante et al., 1995, Huebsch et al., 2010, Lin et al., 2014). Different integrin binding of cells to 2D and 3D environments promote different cellular behaviour such as differentiation (Huebsch et al., 2010). In a 3D environment varying spreading forces are present when compared to 2D planar surroundings (van Susante et al., 1995, Lin et al., 2014). The nutrient diffusion is also differently organised (Baker and Chen,
2012). Consequently, this study was moved further towards the 3D approach within the scaffolds.

In the next step, a gel was cast containing collagen I and laminin combined with ADSCs. The mixture was seeded apically onto the selected scaffolds III and IV. Through histological sectioning and cytoskeleton staining the gel proved to be usable as a delivery matrix for ADSCs into the scaffolds. Accordingly, the gel dispersed the ADSCs within the macroporous structure of the constructs as observed by others (Flynn et al., 2008, Handel et al., 2012, Phull et al., 2013, Cheung et al., 2014). Furthermore, histological and gene expression analysis confirmed the support of adipogenesis of the artificial ADSC stem cell niche within the scaffold when subjected to adipogenic inducing factors. The composite scaffold also revealed the preservation of ADSC stemness (expression of CD90 stem cell marker) and adipogenic inductive effects as determined by the expression of the early adipogenic differentiation marker PPARG when cultured in growth medium. Therefore, the presence of a heterogenous cell population. Furthermore, adipogenic inductive materials were shown to enhance adipose tissue formation in vivo, therefore illustrating a favourable feature for scaffolds in adipose tissue reconstruction (Wang et al., 2013a, Werner et al., 2014, Young et al., 2014).

However, it was not possible to determine the origin of the adipogenic inductive effect due to time limitations. The induction through the scaffolds can be excluded due to differentiation studies carried out in chapter 3, section 3.3.2.5. However, in this study an increased cell number was applied. Therefore, the induction of adipogenesis resulting from contact inhibition of the ADSCs cannot be excluded. Contact inhibition of ADSCs is known to trigger spontaneous adipogenesis (Chang et al., 2013, Roxburgh et al., 2015). Although the control cells cultured in growth medium on TCP did not show increased expression of the adipogenic marker when compared to cells cultured within the scaffolds. Therefore, the inductive adipogenic effect most likely results from the laminin and collagen used in the gel. The gel might additionally provide a soft, more conducive environment. Soft surfaces are known to induce adipogenesis of ADSCs (Young et al., 2013). It is likely that future studies using different cell numbers within the collagen laminin gel followed by
subsequent gene expression analysis would provide additional insights regarding the induction of adipogenesis.

In conclusion, the second project aim was fulfilled that intended to create of an artificial ADSC stem cell niche. This niche was expected to allow for the cell delivery into the porous scaffold structure as well as supporting a heterogeneous cell population consisting of ADSCs and stem cells differentiating towards the adipogenic lineage (as outlined in Chapter 1, section 1.4.1). The further advanced developed scaffolds, containing an artificial ADSC stem cell niche, support ADSC differentiation towards the adipogenic lineage as well as ADSC stemness. Furthermore, the inductive adipogenic features of the composite scaffold strongly imply the support of adipogenesis.

This confirms the third project aim, to combine the chosen scaffolds with the artificial ADSC stem cell niche to obtain a construct with corresponding physical and biological features for adipose tissue reconstruction. Thus, it was displayed that optimised final constructs are composite scaffolds with a biological and physical profiles suitable for adipose tissue reconstruction as summarised in Figure 5.1. Further, the constructs described here can be moved forward towards in vivo investigations for adipose tissue regeneration.
Chapter 5: General discussion

**Gelatin**

**Literature:** mechanical support, shape is adjustable, natural feel, bioactive, biocompatible, clinically applied, utilised in *in vitro* and *in vivo* investigations for adipose tissue reconstruction

**Support:** viability as well as preservation of the stem cell phenotype of ADSCs within the constructs, displays a slow degradation rate while cells proliferating

**Investigated:** through metabolic, Live/Dead assay, histological investigations, gene expression analysis and long term study with subsequent histological analysis

**Macropore**

**Average size:** 1.7 mm, 1.61 mm in scaffold III and IV (pre-freezing - 80 °C with subsequent freeze drying or freeze drying -55 °C)

**Support:** of cell infiltration (ADSCS, 22 μm), proliferation and differentiation (mature adipocytes, 190 μm) and metabolite diffusion

**Investigated:** through serial sections, immunohistological staining, cell count and gene expression analysis, cell viability

**Micropore**

**Different size distribution:** in scaffold III, IV gained through diverse freezing regimes (pre-freezing - 80 °C with subsequent freeze drying or freeze drying -55 °C), Pore diameter up to 160 μm

**Support:** of cell infiltration (ADSCs, 22 μm), interconnectivity, metabolite diffusion

**Investigated:** through serial sections combined with histological investigations, liquid diffusion, liquid replacement method, viability of cells

**Liquid up take**

**Support:** Cell seeding via sponge-like absorbance achieved by re-hydration of the constructs via apical seeding with cell-media mix

**Investigated:** through liquid up take as well as histological serial sections

**Porosity and interconnectivity**

**Porosity:** up to 65% of the scaffold volume as well as interconnectivity gained through the microporous macroporous biomaterial structure designed via particulate leaching, different freezing regimes and subsequent freeze drying

**Support:** of cell seeding, infiltration, proliferation, differentiation and metabolite diffusion

**Investigated:** through the liquid replacement method and ability of liquid to flow through the constructs, histological investigations, cell count, gene expression as well as viability of cells

**Artificial extracellular ADSC stem cell niche**

**Gel:** combination of collagen type I and laminin

**Support:** of cell delivery in the construct, increasing the surface area for cell distribution within the macroporous structures, ADSC differentiation towards adipogenic lineage, preservation of ADSC stem cell character

**Investigated:** through histological and gene expression analysis

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**Figure 5.1:** Overview of the investigated features of the scaffolds designed to support adipose tissue reconstruction. The designed scaffolds are made of gelatin which was confirmed to support cell viability, preserved the ADSCs stem cell character as well as showed a promising slow degradation rate while the cells were proliferating. The constructs contain macropores as well as micropores. These structures were presented to support cell infiltration, proliferation, differentiation as well as provide the scaffold with interconnectivity and porosity and assist metabolite diffusion. The biomaterials also showed cellular up take through sponge-like absorbance of cell-media mix, which was investigated through the liquid up take. The fashioned artificial extracellular ADSC stem cell niche was proven to act as delivery matrix of the cells in the scaffolds, where it assisted their distribution throughout the porous environment. Further, the artificial matrix also supports ADSC differentiation towards lipid laden adipocytes as well as preserving the ADSC stem cell character. Thus, the gelatin sponges are able to be moved forward in the process of developing an engineered product for adipose tissue reconstruction.
5.4. From bench to bed side-revealing further study hurdles

The presented study aiming to develop a scaffold for adipose tissue reconstruction by applying the tissue engineering strategy was limited to in vitro analysis. Despite this restriction the investigations show promising implications as well as potential limitations for the final application of the constructs within clinical settings. Thus, possible hypothetical applications revealing further study hurdles of the gelatin sponges are discussed in the following paragraphs.

5.4.1. Physical implication for the utilisation of the scaffolds in clinical settings

For translation into clinical settings the improved scaffold preparations as well as the used biomaterials display advantageous features. Specifically, the gelatin sponges would represent a favourable biomaterial. Gelatin is a low cost material (Bigi et al., 2001), therefore the sponges can be prepared within a moderate budget (Place et al., 2009). The further developed scaffold preparation integrates a subsequent freeze drying step. This provides a porous biomaterial structure (Kang et al., 1999, Nazarov et al., 2004) and equips the construct with the potential for long term aseptic storage at room temperature till further use (Bellas et al., 2013b). This potential still needs to be investigated through weight loss, gelatin and cross linker release over time (Bigi et al., 2001, Grover et al., 2012). At the same time the resultant sponge-like absorbance of the cellular component within the dehydrated materials displays a simple way of cell seeding (described in Chapter 3, section 3.2.9). After the rehydration of the biomaterials through the seeding of cells suspended in culture medium, they showed the ability to be compressed and returned after release to their originating hydrated structure. This feature would support the application of the sponge to the defect side through a minimal skin incision, although the compression has to be further investigated through compression tests (Young et al., 2013).

Additionally, the improved scaffold preparation advanced to a more rapid construct manufacture, displayed in the optimised alginate leaching method (outlined in Chapter 2, section 2.3.1). Thus, the scaffold can be prepared within a short time and would minimise patients waiting duration.
However, the method was manually driven and in the case of high throughput preparation of the constructs this would be limited to the availability of human resources. Here, the manufacture of the biomaterials through rapid prototyping, also referred to as 3D manufacture would display an economically priced and highly reproducible alternative strategy. This approach is based on the production of a digital 3D format, resembling the sliced scaffold structure. This digital design is subsequently printed through layer by layer assembly to produce a porous architecture (Derby, 2012). A commonly used 3D printing method is inkjet printing where the biomaterial is applied in a similar fashion to normal printing with ink (Derby, 2012, Pati et al., 2014, Pati et al., 2015). Within this process the 3D architecture can be controlled (Wang et al., 2006, Derby, 2012, Miller et al., 2012). This strategy is already investigated for adipose tissue reconstruction (Pati et al., 2015). Pati et al. prepared porous scaffolds based on decellularised adipose matrix with a polycaprolactone framework using 3D printing. The hybrid constructs (1 cm diameter, 0.5 cm height) seeded with ADSCs showed adipogenesis, increased vascularisation and tissue integration in an in vivo mouse study for 12 weeks when compared to injectable decellularised adipose tissue (Pati et al., 2015). Furthermore, gelatin was already used within the layer by layer approach (Wang et al., 2006). Wong et al. used gelatin cross linked with glutaraldehyde to print a porous scaffold for liver regeneration. They showed hepatocyte survival and proliferation in a 2 month in vitro study (Wang et al., 2006). Therefore, a digital 3D design could be produced of the average dimensions of the presented microporous macroporous scaffold structure. These porous design features (detailed in Chapter 2, section 2.3.2) displayed successful results for ADSC viability and proliferation of up to 2 month in vitro culture (described in Chapter 3, section 3.3.2 and 3.3.3). Here, gelatin could be dispensed layer by layer into a standardised conformation. Thus, the developed construct displays potential for high throughput manufacture.

To further gain an element of control, therefore for scaling up the scaffold preparation the use of advanced bioreactors could be considered. Bioreactors also mimic more closely in vivo conditions when compared to traditional 2D culture. Here, 2D cultures are operator dependent, thus they are difficult to reproduce and underlying a high risk of contamination. Additionally, the concentration and
diffusion of nutrients are outside the range of physiological conditions (Gugerell et al., 2015). Bioreactors are devices that allow the culture of 3D scaffolds under controlled environmental and operating conditions such as pH, time, temperature and metabolite diffusion. Accordingly, bioreactors provide an environment which mimics more closely in vivo conditions and at the same time adds an element of control and standardisation to in vitro culture conditions (Martin et al., 2004). Therefore, bioreactors demonstrate a tool which is optimal for scaling up the scaffold manufacture and culture. Today there are diverse bioreactors in use from simple spinner bottles and rotation wall vessels to perfusion bioreactors and devices that apply controlled mechanical forces onto the culture (Martin et al., 2004, Bellas et al., 2013a, Lin et al., 2014, Gugerell et al., 2015).

In the present study spinner bottles (spinner flask bioreactors) were used (detailed in Chapter 3, section 3.2.8), which enhance the distribution of cells within a scaffold and assist the continuous flow of metabolites throughout the construct (Bellas et al., 2013a). Next to the flow rate various other variables define the in vivo environment such as temperature, pH and perfusion rate. More advanced bioreactors can be programmed to control these factors such as perfusion bioreactors (Martin et al., 2004, Gugerell et al., 2015). These bioreactor types can be programmed to balance the metabolite diffusion throughout the construct, the shear stress impacting the porous scaffold structure as it would be in vivo settings and the accommodation of the synthesis of ECM within the 3D environment (Martin et al., 2004). Here, Gugerell et al. applied perfusion bioreactors for the culture of ADSCs within UV cross linked methacrylated gelatin (0.5 cm thick) to mimic more closely in vivo conditions. They showed higher cell viability within the bulk gelatin hydrogels under perfusion culture when compared to static culture conditions (Gugerell et al., 2015). Thus, the utilisation of more advanced bioreactors in future studies, especially for larger scaffolds should be considered.

Nonetheless, the gel properties of the scaffold material itself are also advantageous. In the present study, the scaffolds were able to adapt the size and shape of the chosen mould (detailed in Chapter 2, section 2.3.2). The construct could therefore be individually shaped depending on patients demand. The defect dimensions could be
transferred to the construct through the application of an appropriate mould. The scaffold could then be moulded in a controlled time frame. Therefore, studies for scaling up the scaffold size should be conducted whereby proportional adjustments of cross linker concentration as well as gelatin concentration should be undertaken to ascertain scaffold design properties, shape stability and desired stiffness.

The possible increase of the scaffold design size for application in large recipient beds is dependent on diverse factors such as material limitations including mechanical features, swelling rate, porosity and degradation properties of the construct (Bigi et al., 2001, Chang et al., 2013, Imani et al., 2013). When a construct is scaled up in size it is important to keep these design features constant across the diverse dimensions. This would verify the related physiological design features such as stiffness, porosity, cell uptake, cell distribution, cell viability and biomaterial degradation time. Imani et al. investigated the influence of different gelatin concentrations, glutaraldehyde concentrations and freezing temperatures on gelatin construct properties. They showed the dependence of pore size and distribution on the freezing temperature with lower freezing temperatures gaining smaller pores. They further demonstrated the linear effect of freezing temperature and gelatin concentration on porosity. Here, lower freezing temperatures and decreased gelatin concentration gained higher porosity. Additionally, they demonstrated the relation between cross linker concentration and construct degradation rate, with rising glutaraldehyde concentration prolonging the biomaterial degradation time.

Furthermore, Imani et al. revealed the reverse effect of gelatin concentration and cross linker concentration on the construct water uptake. The group also showed the link between gelatin concentration and construct stiffness whereby increased gelatin concentration increased the material stiffness (Imani et al., 2013). Bigi et al. demonstrated further the linear relationship between cross linker concentration and stiffness (Bigi et al., 2001). Accordingly, the regents used as well as the freezing protocol of the biomaterials should remain constant while increasing the size. Here, the size limit of the constructs design has to be determined at which the physical scaffold properties remain stable while keeping the preparation protocol constant.
Another factor which is essential to consider when increasing the design size would be the biological size limitation. As shown for large fat grafts, sufficient oxygen supply is crucial for the survival of cells as well as grafted tissue (Dong et al., 2015, Doi et al., 2015). Here, ADSCs can remain viable for up to 3 days under hypoxic conditions (Yoshimura et al., 2011). Consequently, the successful scaffold size increase is not just dependent on the physical but also on the biological volume restriction. Therefore, the increased biomaterial size should be investigated in in vitro and in vivo setting for cell viability.

A further aspect that has to be accounted for when increasing the scaffold dimensions is the clinical application. Here, the construct size would be restricted to dimensions that are comfortable to handle during surgery. Therefore, constructs which are large but prone to break due to increased volume would be disadvantageous. Furthermore, the physical space in which to transplant the construct differs from patient to patient not just in dimension but also in the density of vascular supply and tissue compliance (Rubin, 2014, Khouri et al., 2014a).

Thus, a large construct could exceed the recipient vasculature, as experienced by application of too larger amounts of fat grafts (overgrafting) (Rubin, 2014, Khouri et al., 2014a). In addition, different recipient sites have different compliances. Subcutaneous tissue shows to be most compliant and scarred and irradiated tissue (characterised through fibrosis) (Haubner et al., 2012, Walmsley et al., 2015) the least, restricting the volume that can be accommodated within the recipient bed (Khouri et al., 2014a). Consequently, the stretching and expanding of scar tissue through meshing, improved mega volume graft (in the range of 250 ml) survival (Khouri et al., 2014b). Furthermore, Khouri et al. summarized their experience gained through 1000 performed mega volume autologous fat transfers. They showed that after the application of a certain graft volume within a recipient bed the compliance of the tissue decreases, which increases the interstitial fluid pressure resulting in the decrease of the capillary circulation. This emanates in the inhibition of the oxygen supply which restricts vessel formation and negatively effects the graft survival. They further reveal the improved fat graft survival after the application of a vacuum based brava external breast expander which increases the physical traction
on the breast tissue (Uda et al., 2014), resulting in the increase of the blood supply as well as tissue compliance when applied before breast augmentation (Khouri et al., 2014a).

Therefore, the enlargement of the scaffold dimensions is not just dedicated by the physical and biological limitations of the construct design but also by the individual clinical settings. Accordingly, those thresholds need to be investigated in future studies.

In present study, our gelatin sponges measured a size of 1 cm in diameter and 1 cm in depth (described in Chapter 2, section 2.3.2.1), which ranks them into the range of bulk scaffolds for adipose tissue reconstruction (Hemmrich et al., 2008, Kimura et al., 2010, Lequeux et al., 2012, Chang et al., 2013, Debels et al., 2015a). The sponges were also stable enough to be handled throughout the whole study without material waste through construct breakage. It was further shown that cells are viable and proliferate for at least 2 months in in vitro cell culture (outlined in Chapter 3, section 3.3.2.4 and 3.3.3). This demonstrates that the applied construct size can be investigated for the application in in vivo studies. However, the scaling up of the scaffold dimensions needs to be further investigated in future studies. In the case of larger wounds exceeding the scaffold dimensions, multiple smaller sized constructs could be packed within the recipient bed. This would increase the potential of earlier vascular supply for the cells within the constructs as well as the biomaterial integration within the patients tissue, as observed with the application of fragmented instead of integral fat grafts (Dong et al., 2015).

However, not just scaffold dimensions have to be considered within the clinical application of our gelatin sponges, also the different toughness of diverse patient sites needs to be accounted for. This would ascertain the reconstruction of the natural feel of the lost tissue and contribute to the physical as well as emotional wellbeing of the patient. The stiffness of adipose tissue is location as well as function dependent (Gefen et al., 2001, Samani and Plewes, 2004, Alkhouli et al., 2013). The gelatin concentration, cross linking concentration and the pore size are known variables that influence biomaterial stiffness (Bigi et al., 2001, Grover et al., 2012, Imani et al.,
These parameters should be investigated in further studies. Thus, the scaffold possibly provides a platform for the alteration of the construct dimensions and stiffness depending on the patients’ demand.

Furthermore, with the current study it was confirmed that the scaffold design of interconnected micropores and macropores supports cell infiltration, viability and proliferation (described in Chapter 3, section 3.3.2). Therefore, confirming the constructs support of adequate nutrient diffusion and waste removal. Thereby, the sponges were applied as hydrogels, mimicking the native ECM characteristics like mechanical properties, adhesion sites, swelling ration as well as ECM proteins (collagen) (Henderson et al., 2015). Here, other preparation methods of the microporous scaffold structure could be investigated such as cyrogellation to advance the manufacture. Cryogels are gaining increasingly popularity in the tissue engineering community due to their simple and inexpensive preparation. Further, they show a highly interconnected porous structure with pores ranging up to 200 µm. The mechanical properties also can be modulated through freezing temperature, cross linking and polymer concentration (Singh et al., 2010, Dainiak et al., 2010, Bhat and Kumar, 2012, Henderson et al., 2015). Cryogels are prepared through gelling of natural or synthetic polymers under sub-zero temperatures. Here, ice crystals are forming out of the polymer solvent. The ice crystals dispense the polymers to dense areas, later the constructs walls, while the crystals grow till they connect which each other (provision of high interconnectivity). Upon thawing the ice crystals melt and leave a porous network behind (Henderson et al., 2015). Thus, cryogels have been widely used in the tissue engineering community to investigate dermal substitutes, constructs for muscle, cardiac tissue, bone regeneration and adipose tissue reconstruction (Singh et al., 2010, Dainiak et al., 2010, Bhat and Kumar, 2012, Chang et al., 2013, Henderson et al., 2015). Accordingly, this method could be applied to manufacture the microporous structure within presented gelatin sponges.

Additionally, alternative materials for the preparation of the macropores could be analysed. Here, Miller et al. used carbohydrate glass a mixture of glucose, sucrose and dextran as sacrificial element to prepare a vasculature network within 3D
constructs. The carbohydrate glass could be removed by emerging the scaffold in water (Miller et al., 2012). This natural glass could display an alternative material used for the preparation of macropores within our gelatin sponge.

Accordingly, the investigation of different materials and preparation techniques could display an alternative approach for the time efficient preparation of the scaffolds and equip the presented sponges with additional advanced properties such as even pore size distribution and advanced porosity and interconnectivity. These properties could support the utilisation of the constructs for different anatomical sites.

5.4.2. Biological implications for the utilisation of the scaffold in clinical settings

Consequently, to the physical implication for the use of the scaffolds in clinical settings, the materials could be manufactured depending on the patient needs and seeded with ADSCs. The cells could be either autologous or allogenic source depending on the patients capacity to donate tissue. This provides advantageous, minimally invasive use of patients’ material. The application of ADSCs was shown to support angiogenesis and adipogenesis (Doi et al., 2015). The seeded materials consisting of ADSCs and artificial stem cell niche should be cultured for a sufficient time within growth and adipogenic differentiation media to reach cell attachment, preventing cell loss due to wash out, sufficient cell proliferation and starting adipogenesis to stimulate tissue regeneration (Doi et al., 2015). Doi et al. investigated the origin of adipose tissue remodelling through fat grafts in green fluorescent mice. They revealed that ADSCs delivered within the fat grafts are predominantly responsible for adipocyte differentiation. Additionally, they speculated that the differentiation of ADSCs in the graft towards the adipogenic lineage is caused by neighbouring apoptotic adipocytes (Doi et al., 2015).

Accordingly, the delivery of both ADSCs as well as adipocytes should be considered. ADSCs and adipogenesis were shown to be present through gene expression as well as histological investigations within our reported composite scaffold (detailed in Chapter 4, section 4.3.2). These in vitro results provide evidence
that the constructs have the potential to support adipose tissue formation in vivo. However, the cell seeding number as well as culture time would depend on defect and scaffold dimensions and needs to be investigated as well as in vitro structure stability. The current study revealed promising evidence of structure stability within 10 days and suggested beginning construct degradation within 2 months of in vitro culture (described in Chapter 3, section 3.3.3 and Chapter 4 section 4.3.2.1).

After sufficient culture time the scaffolds could be surgical implanted by incision subcutaneously to bulk the defect without the need of multiple interventions. Importantly, to be safely applied within organisms the constructs should be biocompatible. Hence, gelatin was shown to be biocompatible in in vivo studies (Chang et al., 2013). Chang et al. applied hyaluronic acid-gelatin scaffolds in in vivo mouse and porcine model for a period of 2 months. Throughout the study the animals were healthy and showed no sign of inflammation, necrosis or skin infection. Further, an encapsulation of the biomaterial within a collagen capsule, separating the material from the native tissue as seen with synthetic biomaterials (Cao et al., 2006, Ratner, 2015) was not observed. On the contrary, the construct integrated within the native tissue, contained mature adipocytes and supported angiogenesis (Chang et al., 2013). Additionally, clinical and pharmaceutical materials based on gelatin such as wound dressings and drugs are FDA approved and already in use (Bigi et al., 2001). This would also enhance the chance of future regulatory application of our constructed gelatin sponge (Pashuck and Stevens, 2012).

Furthermore, the cross linker glutaraldehyde is in clinical use as bioprothesis heart valves (Jayakrishnan and Jameela, 1996, Chao and Torchiana, 2003). The integrated artificial environment within the scaffold is made of collagen which is extensively applied as skin substitutes in clinic and with laminin part of the native adipose environment (Halim et al., 2010, Mariman and Wang, 2010). Additionally, the scaffold based on natural materials supported cell viability in our in vitro analysis which were confirmed through metabolic assays, live and dead staining as well as proliferation investigation in present study (detailed in Chapter 3, section 3.3.2.4).
The utilisation of ADSCs is also already clinically applied in the form of cell enriched lipotransfers and displays improved outcomes observed in decreased graft resorption resulting in minimised volume loss within small tissue defects (Yoshimura et al., 2008a, Yoshimura et al., 2008b). Furthermore, ADSCs do not express HLA II antigen which makes allogenic transplantation possible (Zuk, 2013). The non-toxicity of the biomaterials confirmed in our in vitro studies, the known use of the scaffold materials and ADSCs in in vivo as well as in clinical application display supporting reasons for the possible application of presented composite scaffolds in clinical settings. However, illuminating in vivo studies as well as pre-clinical trials are essential to verify clinical application (Pashuck and Stevens, 2012).

Nevertheless, the implantation of natural biomaterial within a healthy tissue environment is known to result in macrophage infiltration. It is observed throughout the first weeks post-transplantation. Those macrophages cause the immune response due to the present of the material followed by material integration within the surrounding tissue, tissue remodelling and regeneration with a decrease in inflammation which is characteristic of the tissue repair phase (Bellas et al., 2013b, Bellas et al., 2015). Pro-inflammatory macrophages are observed throughout the tissue repair phase whereby in the later stages of healing predominantly macrophages secreting angiogenic factors, specific for tissue remodelling are present (Schultz et al., 2011, Bellas et al., 2015). Here, Hong et al. applied commercial gelatin sponges for 2 weeks in a mouse model and noted a fibrous capsule surrounding the construct, indicating tissue repair (Hong et al., 2006). Consequently, scaffolds based on natural biomaterials offer a favourable format for the application as an adipose tissue substitute.

The remodelling and regeneration process is supported by transplanted cells as well as by host cells. Here, Doi et al. and Dong et.al. showed with a chimeric green fluorescent protein (GFP) mouse model system and fat exchange with wild type mouse that graft (Dong et al., 2015) as well as host ADSCs contribute to adipogenesis (predominantly graft ADSCs) and vessel ingrowth. The observed vasculogenesis resulted through graft ADSCs as well as host bone marrow derived endothelial cells (Doi et al., 2015). In this way the tissue remodelling could take
place, whereby the infiltrating cells also include macrophages and other cells such as endothelial cells. Thus, the scaffold should provide an interconnected pore system with pore sizes large enough to accompany cell infiltration preventing pore blockage resulting in tissue death (Cao et al., 2006).

In the current study, the scaffolds presented pore interconnectivity and sufficient pore diameter for cell infiltration confirmed through fluid flow studies as well as histological investigations (described in Chapter 2, section 2.3.2.2 and Chapter 3, section 3.3.2). Through the use of ice as porogen the microporosity was altered. This provides a platform for further adjustment if necessity is revealed caused by pore blockage in in vivo studies. Furthermore, the construction of macropores also delivers sufficient space for maturing adipocytes which can reach a size up to 190 µm (Skurk et al., 2007) and was shown to be restricted with pore diameters smaller than 40 µm (von Heimburg et al., 2001). The infiltrating as well as scaffold cells are able to secrete ECM degradation enzymes such as MMPs and would contribute to the degradation of the scaffold while the cellular component replaces the lost tissue. These process would probably start from the outside to the inside due to higher cellular density of the surrounding tissue (Bellas et al., 2013b). A preliminary long term in vitro degradation study showed that the scaffolds slowly degraded while the cellular components filled the scaffold structure (Chapter 3, section 3.3.3). However, in vivo studies would reveal insights into the degradation behaviour and support of tissue remodelling of constructed scaffolds.

5.4.3. Vascularisation remains a key challenge within bulk tissue engineered constructs

Crucially, for a biomaterial to be integrated within the native tissue as well as support tissue regeneration through the combined cell source, vascularisation is essential. The vascular system supplies nutrients, oxygen and provides a path of waste removal for the inserted construct containing cells. The vascular support is essential for larger scaffolds due to restriction of the diffusion limit of oxygen to 100 µm - 200 µm (Novosel et al., 2011). Furthermore, the lack of sufficient vascularisation is a major cause of large fat graft failure due to ischaemic conditions within the central parts (Dong et al., 2015). The hypoxic environment in central
areas of bulk fat grafts causes death of adipocytes after 24 hours; after 3 days apoptosis of ADSCs follows (Yoshimura et al., 2011), preventing tissue regeneration.

In the tissue engineering community sufficient vascularisation of bulk engineered constructs still presents a key challenge (Novosel et al., 2011, Dew et al., 2015). Different strategies are followed from the incorporation of angiogenic factors (Kimura et al., 2010, Lu et al., 2014, Ting et al., 2014) to in vitro co-culture with endothelial cells (Bellas et al., 2013a) and scaffold design features such as channel or pore incorporation (Stosich et al., 2009, Druecke et al., 2004).

Here, our scaffolds displayed a high porosity which is favourable and would not just support cell infiltration, attachment, proliferation but also nutrients and waste diffusion (Fan et al., 2015). Furthermore, our composite scaffolds were characterised through large macropores up to 3.09 mm. Large pores were presented to increase vessel infiltration within the first days of implantation (Druecke et al., 2004). Therefore, the gelatin sponges display promise to support vessel infiltration. Additionally, in the presented long term in vitro study of 2 months, within central parts of the large construct the cells were still alive and cellularised the large porous structure (detailed in Chapter 3, section 3.3.3). This suggests the potential for cell survival in in vivo studies. The use of ADSCs would also assist vessel formation through secretion of angiogenic factors (Doi et al., 2015). Furthermore, the artificial ADSC stem cell niche consists of laminin and collagen I. Laminin is known to increase endothelial cell proliferation (Schultz et al., 2011), thus the scaffold integrated laminin could contribute to host endothelial cell proliferation and therefore vessel infiltration. Endothelial cells are major components within the endothelia coated tubular network of the vasculature (Novosel et al., 2011, Dew et al., 2015). Accordingly, the artificial ADSC stem cell niche could have a supportive influence on endothelial cell proliferation and therefore vessel infiltration. These promising features have to be evaluated through future in vivo studies.
5.4.4. Application of the scaffold in clinical challenging environments

In clinical settings the application of the biomaterial within healthy tissue is not guaranteed. Worldwide a constant increase of overweight (body-mass index (BMI) 25-29) and obesity (BMI ≥ 30) is recorded. For the British population it is estimated that every seventh out of ten citizen will be overweight or obese by 2020 (Wang et al., 2011). Overweight and obese adipose tissue is characterised through an altered tissue surrounding (Divoux and Clément, 2011). Therefore, an additional challenge presents material biocompatibility and integrations within challenging environments (Ratner, 2015, Oliva et al., 2015). Oliva et al. revealed that biomaterial (dentrimer/dextran based) adhesion in colitis (inflamed tissue) rabbit model differs from healthy tissue. In the inflamed tissue model biomaterial adhesion was decreased when compared to healthy tissue. This observation resulted from different ECM composition within the environments.

In obesity, obese tissue results from high levels of excess energy causing adipocyte hypertrophy by too little energy expenditure. It is characterised through the changed secretory function of macrophages and hypertrophic adipocytes which is displayed in constant low grade inflammation and hypoxic state of the fatty tissue. This can further cause insulin resistance and type 2 diabetes (Stumvoll et al., 2005, Hajer et al., 2008, Lee et al., 2010, Cinti, 2012). Additionally, macrophages and adipocytes are known to secrete matrix degradation enzymes such as MMPs (MMP 1, MMP 4, MMP 9, MMP 10, MMP 14) which is accompanied by intensive remodelling of the surrounding ECM (Mariman and Wang, 2010, Divoux and Clément, 2011, Wronska and Kmiec, 2012).

The changing ECM of obese adipose tissue characterised through an increased collagen content (Divoux and Clément, 2011) and hypoxic state of the fat could have influence on material integration and adipose tissue reconstruction effects of the biomaterial as well as the cell source. The material could fail to integrate. The hypoxic environment could cause the apoptosis of cells, resulting in unsuccessful vessel infiltration and adipose tissue regeneration.
Consequently, the scaffold interaction with altered tissue environments needs to be considered. Possible strategies such as the alteration of the integrated adipose stem cell niche within our sponge currently consisting of collagen I and laminin, to resemble more closely the obese surrounding could be followed. This could improve the integration of the construct within the tissue (Oliva et al., 2015). Here, a different ratio of collagen to laminin could be investigated to mimic the collagen rich environment of the obese surroundings. Other combinations could be tested as well. For example, the application of fibronectin. Current studies provided implications for possible support of ADSCs differentiation towards endothelial cells through low concentrations of fibronectin (outlined in Chapter 4, section 4.3.1.2). This would support the vessel infiltration within the construct out of the hypoxic environment. Furthermore, the presented gelatin sponge could be modified with additional cell adhesion sites such as the RGD (arginine, glycine, aspartic acid) peptide (Halberstadt et al., 2002). Modified alginate with fibronectin RGD attachment sites showed vascular ingrowth, decreased inflammation as well as improved tissue integration when compared to alginate without the adhesion motive within a sheep in vivo model (Halberstadt et al., 2002). Furthermore, incorporation of growth factors such as FGF, VEGF or PDGF within the gelatin sponge through swelling or integration of for example, heparin for binding the hormones within the construct (Kimura et al., 2010, Lu et al., 2014, Ting et al., 2014) could also support vessel ingrowth and angiogenesis. The improved vascularisation within the biomaterial could counteract the hypoxic environment found in obese patients.

The scaffolds developed in this project could not only be applied as a cellularised clinical device; they also could be used without cells for smaller soft tissue defects where host cell infiltration would contribute to tissue regeneration as seen with other investigated constructs (Lu et al., 2014, Debels et al., 2015a). Presently, fragmented fat grafts have displayed increased vessel formation as well as decreased necrosis of central areas when compared to integral grafts (Dong et al., 2015). Thus, the application of more than one ADSC seeded construct within larger defects could be also possible.
5.4.5. Hypothetical clinical utilisation of the composite scaffold

The potential subsequent studies described here would further characterise and evaluate the use of the constructed microporous macroporous scaffold for adipose tissue reconstruction. With the knowledge gained from present and future studies the scaffold could become a possible candidate for adipose tissue reconstruction. Here, it could be utilised for adipose tissue loss, after the wound healed and left an obvious contour defect. The wound should be healed to ascertain a non-inflamed wound bed which could influence the scaffold based tissue regeneration (Oliva et al., 2015). The treatment of the disfigurement could proceed with the patient’s consent for the medical intervention. At the first day of treatment the defect would be assessed possible using 3D laser scan (Howes et al., 2014). Howes et al. used this technique to measure the volume of breasts before and after reconstruction surgery (Howes et al., 2014). In case of the contour defect it could be applied to estimate macroscopically the dimensions of the replacement material and potential cell number used. At this point the decision can be made if the scaffolds are seeded with cells or applied without cells as small sized filler material supporting host cell infiltration and tissue regeneration.

For larger defects the patient would donate lipoaspirate (Yoshimura et al., 2008a, Yoshimura et al., 2008b) which would be used to isolate, expand and characterise ADSCs for later combination within the gelatin sponge. The donation of lipoaspirate displays a standard procedure and is considered as minimally invasive method (Zuk, 2013). Following lipoaspirate donation, the patient could be discharged while awaiting further invention. During the time of ADSCs expanding to the desired cell number (1-3 weeks) and characterisation, the scaffold could be moulded to the acquired dimensions. The preparation of the scaffold would include the production of the mould and the use of FDA approved materials within a clean room. This would ascertain the utilisation of sterile materials and minimise possible undesired wound infections. After cell expansion, thus after 1-3 weeks, the cells could be seeded onto the scaffold with the application of the artificial stem cell niche as delivery vehicle.
After 10 days culture in growth media, according to our studies the cells should be viable and starting to differentiate along the adipogenic lineage. At this stage they contain a population of ADSCs which should be able to contribute to vessel infiltration and adipogenesis, therefore adipose tissue regeneration. They also contain developing adipocytes which contribute to the tissue volume as well. Accordingly, this displays an ideal time for the implantation into the patient. Therefore, after 17 to 31 days of the liposuction depending on the size of the defect the patient can continue the treatment. Here, the composite scaffold could be transplanted subcutaneously into the patient to fill the defect. The patient should be accompaniment with regular check-ups, immediately after the operation and within 1 and 2 weeks up to 12 month to ascertain construct integration within the tissue, degradation of the biomaterial as well as wellbeing of the applicant (Howes et al., 2014).

5.4.6. Application as biphasic material for deeper contour defects including skin regeneration

The scaffold application developed in this study, was described for transplantation within the hypodermis of the skin presuming the intact bordering tissues. However, in case of deep injuries, such as those caused by traumatic accidents, not only the subcutaneous fat layer but also neighbouring tissue layers such as the skin can be affected (Tan and MacKinnon, 2006). In those settings the presented scaffold could be used as a biphasic material in combination with clinically applied skin substitutes to produce a composite material for the repair of soft tissue and skin regeneration.

Specifically, the utilisation of the scaffold for adipose tissue regeneration together with skin substitutes such as the synthetic bilayer material Integra or the natural derived Alloderm (Nathoo et al., 2014, Debels et al., 2015b) could be followed. Integra is a widely used dermal replacement with a top silicone layer with underlying porous matrix based on cross linked bovine collagen and shark derived GAG (Debels et al., 2015b). It is used for dermal regeneration and wound coverage. Alloderm consists of decellularised cadaveric skin and presents a natural derived dermis substitute (Nathoo et al., 2014). Generally these membranes are applied within the wound and accompanied by host epithelium infiltration from wound edges or
stimulate granulation tissue, therefore facilitate autologous grafting (Nathoo et al., 2014, Debels et al., 2015b). Here, it might be possible to produce a material, with the potential to combine a bottom layer of adipose tissue regeneration with an upper layer for reconstructing the overlying skin.

5.5. Future studies

Clearly, the scope of presented project was restricted. Specifically, the physical characterisation of the scaffolds was confined to cell based studies and the porosity, interconnectivity and liquid uptake were investigated. These features are essential for metabolite exchange, cell infiltration, attachment and proliferation (Zhong et al., 2012, Fan et al., 2015). Nevertheless, to cosmetically reconstruct adipose tissue along with the authentic feel, the stiffness of the replacement material is an important parameter (Choi et al., 2010a).

For the viscoelastic adipose tissue a wide range of stiffness depending upon its location and function have been recorded; from 1.6 kPa for visceral fat up to 22 kPa for heel pad fat, (Gefen et al., 2001, Samani and Plewes, 2004, Alkhouli et al., 2013). Therefore, to fully characterise the scaffolds for in vivo use in the reconstruction of adipose tissue, an investigation of the stiffness of the resultant construct needs to be addressed for future studies.

The modification of the gelatin concentration, cross linking concentration and freezing temperature are known parameters that influence gelatin stiffness (Bigi et al., 2001, Grover et al., 2012, Imani et al., 2013). Increased cross linker and gelatin concentration increases the stiffness of gelatin with superior effects of the gelatin concentration (Bigi et al., 2001, Imani et al., 2013). The higher the freezing temperatures, the larger the created pores become, and the softer the gelatin construct (Imani et al., 2013). Consequently, the use of more or less alginate beads in the composite would soften or stiffen the construct respectively. As a result, the constructed gelatin scaffolds provide a platform for additional modification of the stiffness according to the patient and site of injury requirements.
Additionally, the potential of scaling up the scaffold design size should be investigated to gain information about the physical as well as the biological restriction of the construct dimensions. Therefore, linear expansion of the biomaterials could be performed while analysing the stability of the physical scaffold properties such as porosity, pore size, interconnectivity, degradation rate and stiffness. This would ascertain the preservation of the related biological scaffold design features such as cell infiltration, migration, distribution, viability, natural feel and the time of construct degradation (Bellas et al., 2013b). Thus, the maintenance of the biological features have to be tested \textit{in vitro} as well to be moved forward to \textit{in vivo} studies.

Further, the preliminary degradation study as described in Chapter 3, section 3.3.3 should be repeated and expanded in future studies. Here, the scaffold degradation should be monitored not just by structural changes but also through scaffold weight and size change (Grover et al., 2012, Bellas et al., 2013a) to gain additional information about the degradation behaviour and stability of shape and weight of the constructs. This information is important for \textit{in vivo} studies with constructs that slowly degrade while the cellular component replaces the lost tissue. Following these studies, the ADSC proliferation and differentiation should be investigated as well during the course of this analysis (Korurer et al., 2014), due to the importance of these parameters for the reconstruction of the lost adipose tissue. This analysis would ideally yield quantitative knowledge about the degradation behaviour of the scaffolds not just in structural and shape degradation or maintenance but also of cellular proliferation and differentiation. Additionally, the cellular deposition of extracellular matrix components could be histological analysed (Vallée et al., 2009). Head to head tests including scaffolds with and without the artificial matrix could also be undertaken. From this investigation, the scaffold, which shows the most suitable profile for further \textit{in vivo} setting in terms of slow degradation while the cells are proliferating and differentiating to replace lost adipose tissue can be selected.

The next step should be the investigation of the scaffold under ischemic conditions to simulate the \textit{in vivo} setting of large contour defects (Yoshimura et al., 2011). This analysis should be performed to mimic the different time frames of clinical scenarios
and of varying dimensions of soft tissue defects. It is known that ADSCs undergo cell death after 3 days under hypoxic conditions (Yoshimura et al., 2011). Following, this analysis should be conducted within this time frame. Furthermore, ADSCs secrete angiogenic factors such as HGF and VEGF when subjected to ischaemic atmosphere (Rehman et al., 2004, Kilroy et al., 2007, Zuk, 2013). The support of vascularisation also represents an important factor to be addressed, which is still undermined and very important as it is seen as a cause of large fat graft failure in clinical settings (Nishimura et al., 2000). Therefore, cell viability should be monitored through metabolic assays. At the same time the secretion of ADSC angiogenic factors in hypoxic (1% O$_2$) compared to normoxic conditions (21% O$_2$) should be investigated, analysed through subsequent gene expression of HGF and VEGF (Rehman et al., 2004). This would likely yield information regarding the support of vascularisation under *in vivo* conditions via the ADSCs contained within the scaffold. This support could be further confirmed through extraction of conditioned medium and use as supplement for endothelial cell culture with subsequent monitoring of endothelial cell viability and proliferation (Rehman et al., 2004, Chung et al., 2015).

The follow up studies clearly demand *in vivo* evaluation of the scaffolds to fully evaluate the potential of the gelatin sponges for adipose tissue regeneration. Here, an *in vivo* rat model may be favourable to a mouse model due to the size of the scaffolds (Moioli et al., 2010). The scaffolds should be implanted subcutaneously whereby unseeded scaffolds would serve as controls (Wang et al., 2013a). Explants should be monitored over a time period of at least 2 months up to 12 months to be comparable to the performed *in vitro* degradation studies as well as gaining knowledge about the potential to regenerate adipose tissue over long term (Bellas et al., 2013b, Bellas et al., 2015). The *in vivo* investigation should include macroscopical and phenotypical as well as microscopical investigation of the construct integration and adipose tissue formation.

Macroscopic analysis should contain phenotypic imaging of the contour, size and shape measurement of the construct to evaluate the *in vivo* degradation (Bellas et al., 2015). Microscopic analysis should additionally include the evaluation of
inflammation. Histological staining for macrophage (CD68+) can be performed whereby it should be distinguished between pro-inflammatory CD180+ (M1) and remodelling CD163+ (M2) macrophages to gain detailed information about inflammation or remodelling function of macrophage infiltration (Bellas et al., 2015). The adipose tissue formation should be monitored visually utilising histological Oil-Red-O staining and H + E analysis of the appearance of lipid droplets and round adipocytes (Wang et al., 2013a, Lu et al., 2014). The vascularisation should be investigated as well with histological staining and gene expression analysis of endothelial cell marker such as von Willibrand factor (vWF) or CD31 to gain information of vessel infiltration and formation (Wang et al., 2013a, Aubin et al., 2015, Chung et al., 2015). Also human ADSCs would be used as a cell source and staining for human nucleolus (HuNu) can be performed which gives information about host cell infiltration and tissue reconstruction caused by delivered or host cells (Moioli et al., 2010, Wang et al., 2013a).

5.6. Contribution to knowledge

Despite the future studies that could be carried out, it is important to reemphasise the lack of useful bulk scaffolds for adipose tissue reconstruction and the restricted knowledge of individual extracellular components on the influence of ADSC differentiation. Therefore, this series of studies has contributed uniquely to knowledge in following ways:

1. This study presented a different approach for particulate leaching of alginate beads out of bulk scaffolds.

2. Additional knowledge about the influence of individual extracellular components (collagen I, III, IV, VI, laminin and fibronectin) in diverse concentrations on ADSCs morphology with indications to preserve stemness, to support or to suppress adipogenic differentiation was gained. Thus, this project delivered novel and detailed insights into the influence of extracellular matrix components on the morphological changes of ADSCs, providing a research platform for future studies on ADSCs with individual extracellular matrix constituents.
3. This project also delivered a platform of a novel range of bulk microporous macroporous constructs for adipose tissue reconstruction which have the potential to be modified in dimensions and stiffness according to the patient’s requirements and extent of soft tissue damage.

4. Further, insights in the degradation behaviour of the constructed microporous macroporous biomaterials was gained. Here, preliminary long-term studies comprising 2 months showed the ability of the scaffolds to support cell population of the porous structure, while the gelatin sponges started to degrade. Therefore, underpinning the favourable design features of pore size, porosity and interconnectivity of the presented materials for adipose tissue reconstruction.
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