Biofabrication of Alginate-Based Hydrogel Constructs and Hybrid Scaffold Structures for Tissue Engineering

Biofabrikation von Hydrogelkonstrukten auf Basis von Alginat und hybriden Gerüststrukturen für die Gewebezüchtung

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Tobias Zehnder

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Vorsitzender des Promotionsorgans: Prof. Dr.-Ing. Reinhard Lerch

Gutachter: Prof. Dr.-Ing. Aldo R. Boccaccini

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Abstract

Biofabrication encompasses the use of additive manufacturing (AM) techniques, biomaterials (hydrogels), cells and bioactive substances for tissue engineering or as 3D tissue models for drug-screening studies or for tumor research. Characteristic for biofabrication approaches is the exploitation of the freedom of design provided by AM techniques enabling the fabrication of defined, complex 3D structures together with the processing of hydrogel/cell mixtures, so called bioinks. This approach enables the defined positioning of different cell types and biomaterials in predefined positions of a construct and thus biofabrication appears as an important novel technology in the general field of tissue engineering and for investigating tumour development in 3D matrices.

In the present work, a biofabrication approach has been established using bioplotting and fused deposition modelling for the fabrication of 3D structures from a crosslinked hydrogel, termed ADA-GEL, based on alginate di-aldehyde (ADA) gelatine (GEL) crosslinked hydrogel (ADA-GEL) together with different bone tissue related cell types. It was shown that the ADA-GEL hydrogel is compatible with the bioplotting technique. Different parameters of the bioplotting process, namely speed, pressure and needle diameter were assessed regarding their interactions, their influence on the geometry of the created structure as well as on the viability of the immobilised cells in the bioink. In this way, a process chain was established which enables the fabrication of bioplotted ADA-GEL constructs with defined geometry. Moreover, the degradation behaviour and the mechanical properties of different modifications of the ADA-GEL system were evaluated. The stock solution concentrations of ADA and GEL as well as the molecular weight of the ADA-component were varied. It was revealed that ADA-GEL hydrogel degraded by the release of GEL and that the stiffness of the constructs decreased with ongoing incubation time depending on the used composition. Based on these results different cell types were used together with ADA-GEL as bioink and the cell development in bioplotted ADA-GEL constructs was assessed over incubation times of several weeks. MG-63 osteoblast like cells were used and it was shown that MG-63 cells proliferated in ADA-GEL constructs and covered the whole structure after 28 days of incubation. Furthermore, bone marrow derived stromal cells ST2 were
used as a cell model and it was shown that proliferation of ST2 cells could be dictated by the molecular weight of the ADA component and the stock solution concentrations of ADA and GEL. An elevated degradation of the ADA-GEL matrix showed faster cell proliferation. In a further approach, a co-culture of ST2 and osteoclast progenitor cells RAW 264.7 was used in an ADA-GEL modification with similar mechanical properties as the unmineralised osteoid bone phase. In this set-up, the interaction of the cell types in comparison to single cultures was investigated, revealing increased osteopontin expression and release of vascular endothelial growth factor (VEGF) in the co-culture group.

A sequential bioplotting process was used to create hybrid structures to overcome the limited mechanical properties of the ADA-GEL regarding application in bone tissue engineering. The hybrid constructs consist of ADA-GEL with immobilised ST2 cells and a phase of polycaprolactone/polyethylene glycol (PCL/PEG) blend, which showed superior properties regarding ST2 cell adhesion in comparison to pure PCL. The PCL/PEG phase acts as a support structure in the hybrid constructs and so enhances the mechanical properties in comparison to the pure hydrogel phase to a range, which is comparable with natural bone. Altogether, the ADA-GEL hydrogel system is a very promising material for use in biofabrication.
Kurzzusammenfassung


In der vorliegenden Arbeit wird ein Biofabrikationsprozess angewendet, welcher auf der Nutzung des Bioplottingverfahrens und der Technik des Fused Deposition Modellings zur Herstellung von 3D Konstrukten aus einem vernetzten Hydrogel, genannt ADA-GEL, zusammen mit verschiedenen für das Knochengewebe relevanten Zelltypen beruht. ADA-GEL Hydrogel basiert auf Alginat Dialdehyd (ADA) und Gelatine (GEL).


Um die limitierten mechanischen Eigenschaften des ADA-GELs hinsichtlich dessen Verwendung in der Knochengußbezüchtung auszugleichen, wurde ein sequentielles Bioplottingverfahren eingesetzt um Hybridstrukturen zu erzeugen. Diese Hybridstrukturen bestehen aus ADA-GEL mit immobilisierten ST2 Zellen und einem Polycaprolacton/Polyethylenglycol (PCL/PEG) Blendmaterial, welches vorteilhafte Eigenschaften hinsichtlich der Zelladhäsion im Vergleich zu PCL zeigte. Die PCL/PEG Phase dient in den Hybridkonstrukten als Unterstützungsstruktur und erhöht so deren mechanische Eigenschaften im Vergleich zur reinen Hydrogelphase, sodass diese in einem vergleichbaren Bereich wie natürlicher Knochen liegen. Insgesamt konnte gezeigt werden, dass das ADA-GEL Hydrogelsystem ein sehr vielsprechendes Material für die Anwendung in der Biofabrikation ist.
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1 Introduction

1.1 Motivation: Biofabrication – an evolving approach for tissue engineering (TE) and regenerative medicine (RM)

The emerging field of biofabrication has significant intersections with the fields of TE and RM as well as additive manufacturing (AM) [1]. Biofabrication has been defined by Mironov et al [2] in 2009 as: “the production of complex biological products using living cells, molecules, extracellular matrices, and engineered biomaterials”. As the young discipline is evolving the definition has been updated, so that it currently reads as follows: “the automated generation of biologically functional products with structural organization from living cells, bioactive molecules, biomaterials, cell aggregates such as micro-tissues, or hybrid cell-material constructs, through Bioprinting or Bioassembly and subsequent tissue maturation processes” [1]. Thus, as a similarity to conventional TE strategies, damaged tissue should be replaced by a scaffold/cell combination [3] and, like in RM approaches, self-healing of tissues should be stimulated by the delivery of cells, support structures and biomolecules [4],[5]. Besides their use as TE scaffolds, biofabricated products could have potential as tissue-equivalents for drug testing [6] and as in-vitro disease models [1], for example in cancer research as 3D tumor models [7]. Progress in the field of AM concerning improvement of the techniques as well as availability and sinking costs is a further important aspect in biofabrication [1] considering that novel AM techniques increasingly offer a high degree of automatization, reproducibility, accuracy and customization [8],[9]. Based on the layer-by-layer principle, AM techniques enable the creation of constructs with a relatively free design considering the positioning of different materials, cell types and growth factors [10]. The mentioned characteristics indicate that AM techniques are capable of overcoming some limitations of conventional scaffold fabrication techniques like particulate leaching, freeze drying or solution casting, where the design of the scaffolds, for example in relation to pore architecture, is usually limited by the process [11]. In contrast to the approach of scaffold fabrication with subsequent cell seeding, in biofabrication only a one-step fabrication process is necessary [6]. Cells are immobilised in hydrogel precursor solutions or hydrogels, resulting in so called bioinks [12],[13], before being processed
with one of the biocompatible AM techniques, as bioplotting, ink-jet printing or laser induced forward transfer [14]. Another complementary strategy is the automated assembly of prefabricated cell aggregates or cell containing building blocks, also named tissue spheroids [1],[15]. The advantages of this concept are a higher cell seeding efficiency, a more homogeneous cell distribution, the defined positioning of different cell types and the embedding of the cells in a three-dimensional (3D) extracellular matrix (ECM) like environment, which can be adjusted by the design of the hydrogels [6],[11],[16],[17]. The development of a 3D plotting technique using hydrogels as processing material was the pioneering work of Landers et al [18],[19],[20]. Characteristic for hydrogels is that they are water swellable crosslinked networks, which possess high content of water [16],[21]. Polysaccharides like alginate, agarose or chitosan as well as proteins such as collagen, its derivate gelatin (GEL), and hyaluronic acid are used in biofabrication [9],[11]. The difficulty in developing hydrogels for biofabrication approaches is to meet the demands of the immobilised cells as well as suitable processing properties that should lead to shear thinning behaviour and shape stability after the plotting process [22]. Hydrogel systems meeting both demands are an important objective of the biofabrication field [9]. Considering its very good processing properties alginate is widely used in biofabrication [12]. The modification of alginate with different proteins is investigated to combine the good processing properties as well as the possibility of ionic crosslinking of alginate with cell specific motifs provided by proteins like GEL [6],[12],[23],[24]. The limited degradability of alginate is a shortage for its use as matrix for cell immobilisation. Therefore, alginate (ALG) was oxidized to get alginate dialdehyde (ADA), which shows hydrolytic degradation [25]. ADA could be chemically crosslinked with GEL (= ADA-GEL) to improve the cell response, as GEL provides cell adhesive motifs. Additionally, ADA could function as a thermal stabilizer for GEL. This combination was developed and also optimized for injectable hydrogels by Balakrishnan et al [26],[27],[28]. The ADA-GEL hydrogel system was further modified considering parameters like solvents, crosslinking time and concentrations by Sarker et al [29] to adjust it for the fabrication of microcapsules. In a comparative study Grigore et al [30] showed that ADA-GEL microcapsules have beneficial properties compared to alginate and alginate modified with RGD peptide microcapsules for the behaviour of immobilised MG-63 cells. The cells showed a more spread morphology
and the cell viability was increased in ADA-GEL. In a following study Sarker et al [31] compared the development of MG-63 cells immobilised in ALG, ALG/GEL blend or ADA-GEL microcapsules. The results again indicated an improved cell development in the ADA-GEL matrix. Furthermore, subcutaneously implanted ADA-GEL microcapsules showed no significant immune reaction of the rats [32]. These studies indicated the great potential of the ADA-GEL system for use in cell encapsulation and confirmed ADA-GEL as an interesting system for biofabrication approaches.

1.2 Aim of the work

The aim of this work is to biofabricate and characterise hydrogel/cell constructs using ADA-GEL hydrogel, different cell types and the bioplotting technique for applications in bone tissue engineering and potential interest in cancer research. The hypothesis was that the ADA-GEL system, which is known to have favourable properties considering cell behaviour in 3D cell culture in comparison to pure alginate [30], could be also adapted for biofabrication to produce different 3D structures. The motivation was that using ADA-GEL as a bioink could be a substantial contribution to overcome the current lack of suitable bioinks, which limits the progress of the field of biofabrication [9]. Moreover, the use of oxidised alginate (ADA) offers the possibility of chemical crosslinking also with other proteins (in addition to GEL). Thus, the investigation of the ADA-GEL system in the context of biofabrication is proposed as the basis for the development of a whole novel group of advanced alginate-based hydrogels modified with different proteins. Several modifications of the ADA-GEL system were used for bioplotting and a series of in-vitro investigations with different cell types was carried out, also in a co-culture approach, to consider the application of the fabricated constructs in bone tissue engineering. The maturation phase of the plotted constructs is, besides the processing itself, a very important part of the biofabrication approach, requesting very specific material behaviour. Both mechanical characterisation as well as degradation behaviour analysis were performed, as these are decisive characteristics of the constructs regarding cell behaviour and ECM expression during maturation. Bioplotting process parameters were evaluated as well as the behaviour of immobilised cells considering their viability, migration, proliferation, differentiation and angiogenic factor release. Additionally, bioplotting
was combined with a fused deposition modelling process to create hybrid constructs with enhanced mechanical properties and structure. In Figure 1 a schematic overview of the main subjects covered in this work is presented.

![Figure 1: Schematic overview of the main subjects covered in this work using ADA-GEL hydrogel, different cell types and bioplotting technique to investigate different biofabrication approaches for bone tissue engineering. Adapted from [33] and [34].](image)

The thesis is organised in the following manner: Chapter 2 introduces the fundamentals and the current state of the research regarding this work, namely: biofabrication, additive manufacturing techniques, natural hydrogels, 3D cell culture, bone tissue engineering and cancer research. Chapter 3 contains the materials and methods description. In Chapter 4 the hydrogel characterisation and bioplotting process development is presented. The topic of Chapter 5 is the biocompatibility of the plotting process using MG-63 cells as well as the cell development during the maturation phase of 28 days. In Chapter 6 a further cell type, namely ST2 cells is used as a cell model to assess cell behaviour in biopotted constructs fabricated from different ADA-GEL modifications. Additionally, a co-culture of ST2 and RAW cells is investigated to assess the cell-cell interactions. Hybrid constructs with enhanced mechanical properties consisting of a hard phase (PCL/PEG) and a soft phase (ADA-GEL with ST2 cells) are presented in Chapter 7. Chapter 8 contains the overall
conclusions of this work and suggests possible future research areas based on the presented results.
2 Fundamentals and state of the art

2.1 Biofabrication and additive manufacturing in tissue engineering

AM techniques had been already used in TE before the upcoming of biofabrication to fabricate 3D scaffolds [35]. One important element in most TE engineering strategies is the 3D scaffold. Scaffolds are engineered biomaterial structures that provide support for the cells. In traditional TE, cells are seeded onto the scaffold after the scaffold fabrication process, for maturation and tissue development [6],[11]. Besides AM techniques, other methods like freeze drying, solution casting or particulate leaching have been used for scaffold fabrication [11]. As the AM techniques offer more control over scaffold design than other methods, AM techniques have become increasingly important in TE [6]. In spite of the beneficial aspects of AM techniques there are still unresolved issues related to the limitations of post-fabrication cell seeding, which the novel field of biofabrication could potentially solve.

2.1.1 The potential of biofabrication

Biofabrication is characterised by processing biomaterials (hydrogels) and cells in a one-step process [22],[35]. Thus, the post-fabrication cell seeding step becomes redundant. Beneficial aspects are a higher seeding efficiency and more homogeneous distribution of cells, which are both critical issues when using conventional cell seeding methods involving cell delivery by pipetting [11]. Moreover, controlled positioning of cells, also of different cell types, and even single cell like resolution, become possible [6],[36]. This capability of biofabrication is essential, especially considering the long term vision of printing whole organ structures exhibiting a complex hierarchical organisation of different cell types [6],[22]. The possibility to plan completely automated processing platforms including pre-process cell growing, processing and cultivation of fabricated constructs in bioreactors could be a major step forward in tissue engineering. Such automation could help, to handle for example, regulatory hurdles [37]. Another challenge is the fabrication of constructs with clinical relevant size dimensions [9]. One precondition for this in case of complex tissues and organs is the integration of a vascular system to ensure the supply with oxygen and nutrients of the developing tissue, for example in the case of bone and
muscle tissue engineering [38],[39]. This obstacle explains the successful engineering of tissues like cartilage or skin [40], as these tissues could be supplied with oxygen and nutrients by post-implantation neovascularisation from the host [38].

Besides these positive effects and possibilities the biofabrication approach offers, it is clear that immobilisation of cells limits the variety of materials which could be used mostly to hydrogels [6]. In addition, the options to modify the limited number of materials suitable for the plotting process is constrictive, e.g. using solvents for the often necessary crosslinking of hydrogels is not possible [41]. This fact enhances the challenge to develop optimised materials for biofabrication, the so called bioinks [12]. Indeed limitations associated to the availability of bioinks is seen to be one of the major obstacles in biofabrication and is hindering its faster progress [9]. In the following the challenge of developing bioinks for biofabrication is outlined.

2.1.2 Material optimisation: conflict of shape stability and cell response

The optimal hydrogel for biofabrication approaches must fulfil the demands of the used AM processing technique and must enable the required response of the immobilised cells in the fabricated construct. This is often a conflict. For example, using bioplotting technique an increase in shape fidelity by increasing the hydrogel concentration could negatively influence the immobilised cells as the network becomes stiff and dense, inhibiting cell migration and proliferation [9].

The demands of the bioplotting process on a hydrogel regarding its processability encompass rheological properties as well as crosslinking mechanism(s) [9],[22]. At first the hydrogel has to be printable under biocompatible conditions (processing temperature, no toxic solvents) [42]. The hydrogel should preferably show shear thinning behaviour to allow the flow through a thin capillary, which defines the printing resolution, and to reduce the shear forces as well as the pressure forces on the immobilised cells [22],[43]. The viscosity of the hydrogel has to be high enough and a rapid gelation after deposition is necessary to guarantee shape stability after the processing [22]. The modification of crosslinking mechanisms implemented in the hydrogel is important to adjust the viscosity, the gelation after processing as well as the mechanical properties of the hydrogel [9]. Thus, modifying the crosslinking
mechanism also influences the mechanical properties, which are decisive also regarding the cell response [24],[44]. The hydrogel has to mimic the physical structure of the relevant tissue, like an open fibrillar structure [44]. The network density and so the porosity have to be appropriate enabling cell migration and proliferation [16]. Moreover, the porosity influences the diffusion of nutrients, oxygen, waste products as well as growth factors and so the cell behaviour [45]. Cell adhesion motifs are necessary to facilitate cell-hydrogel interactions [44]. In addition, the hydrogel mimicking the extracellular matrix has to be degradable to provide space for the new extracellular matrix structure components expressed by the immobilised cells, which should take over and replace the hydrogel step by step during the maturation process [21]. Thus, spatiotemporal control over the hydrogel on a time scale from seconds up to weeks is necessary [16].

Considering all these criteria, it becomes obvious that the design of bioinks is a complex task [46],[47]. The ideas and approaches put forward to fulfil these requirements are manifold and emphasize different aspects. In general, it is possible to develop firstly an optimized material regarding the specific processing technique considered. Then the emphasis lies on the rheological optimization and the mechanical properties of the hydrogel. In a second step, the hydrogel must be tested for its performance considering cell immobilisation. Another alternative is to select hydrogel systems already established for cell immobilisation and to modify them for biofabrication processing, adjusting also their properties to enable favourable cell response [47].

2.2 Overview of additive manufacturing techniques used in biofabrication

Additive manufacturing is the most recent name given to a number of fabrication techniques also known as rapid manufacturing, solid freeform fabrication (SFF) or, originally, rapid prototyping [1],[48]. In general, these terms encompass process technologies that use sequential delivery of energy and/or material to create 3D structures directly [11] without the use of a prefabricated mould [48]. Objects are built up in a layer-by-layer process, whereas each layer reflects cross-section shapes, often based on computer-aided-design (CAD) data or imaging data from magnetic resonance imaging or computed tomography [11],[48]. Characteristics of AM are a
high degree of reproducibility, automation, free-design and patient customized implants [9],[22]. Amongst the huge variety of AM techniques only some are compatible with biofabrication as they have originally been invented for use in automotive industry and not for processing biological tissues or cells [49]. AM techniques used in biofabrication could be divided into nozzle-based systems like bioplotting/robotic dispensing, laser-based systems like stereolithography (SLA) as well as two-photon polymerisation (2PP) and printer-based systems, such as ink-jet printing [11]. Lately, Hözl et al [13] introduced the category of orifice-free bioprinting including laser-based methods, additionally mentioning a method based on surface acoustic waves, as energy source to deposit hydrogel/cell mixtures. Nevertheless, recently also non-biocompatible AM techniques such as fused deposition modelling (FDM) have been successfully integrated in biofabrication approaches [10],[50].

2.2.1 Printer based systems: ink-jet printing

The ink-jet printing technique is part of the printer-based systems category [11]. The first ink-jet printers used for cell printing were modified commercial office printers [6]. Most ink-jet printers follow the drop on demand (DOD) technique, ejecting material only after receiving a signal instead of continuously ejecting types [36]. There are two often used set-ups, which are presented in Figure 2, in cell printing with DOD systems: one is based on thermal control and the other one on piezoelectric control.

Figure 2: Scheme of two drop-on-demand (DOD) ink-jet printing systems based on a) thermal control to create a vapour bubble and b) mechanical actuation using a piezo device. Reprinted from [51] with permission from Annual Reviews.
Thermal control of ink-jet printing works by heating and vaporizing the ink to create a bubble which induces the force to create a drop. The piezoelectric method functions over electrical impulses, leading to a contraction of the piezo device and to drop formation [35]. One advantage of the ink-jet technique is the high resolution, which is possible by the precise control of the drop volumes varying between 1 and 100 pL [35],[36]. Thus, it is possible to process very small numbers or even single cells [52] on pre-defined spots as it has been demonstrated with endothelial cells dissolved in cell culture medium [36]. The dots had diameters of between 25 and 30 µm. The fact that ink-jet printing works without contact is beneficial as different substrates could be used [36]. Tirella et al [53] evaluated different substrates showing that soft and viscous bioinks, as GEL solution, improve cell viability as they allow force transmission of the kinetic energy after drop impact. Nevertheless, substrate and drop possibly coalesce, whereas stiff substrates do not show this effect. The use of liquid environments or hydrogels is also necessary to prevent the immediate drying, which could cause cell death as the drop volumes are very small [6],[54]. Therefore, cells could be printed on prepared hydrogel layers [55] or as a hydrogel/cell mixture in one step [56]. The possibility of processing hydrogels is limited as their viscosity is typically below 20 mPa s$^{-1}$ to avoid clogging of the ink-jet system [22]. Considering the biocompatibility of the ink-jet process there has been a study by Cui et al [57] using a thermally controlled ink-jet printer. The critical parameters are the applied heat for drop formation as well as the forces on the cell being pressed through a small capillary with a diameter of 48 µm. The results show cell viabilities of around 89 % and an apoptotic rate of 3.5 %. Thus, no additional influence of the printing process on cells compared to normal pipetting and cell handling could be observed. Damage to cell membrane could be detected, but the perforated membranes were restored by the cells 2 h after processing. The use of piezo-controlled systems is seen critical as some systems operate with frequencies in the range of 15-25 kHz, which is critical for cells [57]. Nevertheless, there are studies using piezoelectric controlled printers for cell printing showing high cell viabilities; of around 90 % [56],[58],[59]. The adjusted frequencies have been smaller than 10 kHz in these studies. Other important parameters to be adjusted in such systems are the voltage (40 V – 150 V) and the pulse width, whereas the voltage mainly influences the drop volume and the drop
velocity as it controls the piezo contraction [56],[59]. Higher voltages causing higher contraction and so higher speeds, mass flow and deposition volumes [59].

### 2.2.2 Nozzle based systems: bioplotting

Bioplotting also referred to as robotic dispensing [22], pressure-assisted microsyringe (PAM) [6] or dispense plotting [60] is basically an extrusion process. A cell/material mixture in a syringe reservoir, based in a moveable plotter head, is pressed through micro-capillaries to make contact to a substrate [11]. There are different set-ups as pneumatic systems (pressure), screws and pistons controlled by a stepper motor. Pneumatic systems have the advantage of a step-free pressure control in contrast to piston controlled set-ups and so they are very versatile. However, the volume between the material and the unfilled part of the cartridge has to be compressed before extrusion starts. This leads to delays in the pressure transmission [50]. In Figure 3 a scheme of the bioplotting technique is presented.

![Figure 3](image.png)

**Figure 3**: Scheme of the bioplotting technique, showing the extrusion of hydrogel/cell mixture through a microcapillary. Reprinted from [11] with permission from Elsevier.

Important parameters are the needle diameter as it mainly defines the resolution, the pressure and the used plotting speed [61]. All these parameters are interacting and are dependent on each other, which makes it necessary to define a set of parameters considering the needs of the process [55]. Besides the processing time and the resolution, also the cell viability, which could be influenced by shear stresses appearing on the wall of the micro-capillary as well as pressure forces, has to be considered [43],[62],[63]. The resolution of bioplotting approaches is in the range of
hundreds of μm [6]. The bioplotting process is characterised by high throughput of cells and materials, so enabling the fabrication of constructs in clinical relevant sizes (cm³ scale) [37]. Moreover, bioinks with high viscosities (30 – 6 x 10⁷ mPas) could be processed, offering improved cell viability after the deposition [13]. Using plotting heads with multiple cartridges provides scale-up conditions for the process as well as easy combination of different cell and/or materials in one construct [6],[37]. The multi-nozzle approach is also used to process sacrificial structures of dissolvable materials like GEL [64], PVA [65] or polyethyleneglycol (PEG) [66]. The loss structure serves as a stabilizer during the process, to overcome limited shape stabilities of the plotting solutions, before a stabilizing crosslinking step or to realize complex anatomical shape geometries with overhangs [65].

Bioplotting is similar to the classical approach of fused deposition modelling (FDM). FDM is based on the extrusion of melted thermoplastic polymers, thus involving high temperatures [67]. In the biomedical field poly-caprolactone (PCL) is a widely used material [68]. Scaffold structures plotted with PCL are investigated since 2000 with promising results in-vitro and in-vivo [3],[69],[70]. It was shown that scaffolds with adjustable geometrical features like porosity, pore size and shape as well as mechanical properties could be fabricated for potential use in bone or cartilage TE [3],[69]. Furthermore, PCL scaffolds containing bioactive glasses or coated with calcium phosphate showed an upregulation of osteogenic gene expression even with non-osteogenic media using sheep bone marrow stromal cells in vitro. In vivo these scaffolds showed host tissue infiltration, but no mature bone formation after 16 weeks [71]. PCL shows hydrophobic behaviour and semi-crystallinity. It has a relatively low melting temperature (59-64 °C) and shows slow degradation behaviour of 2 up to 4 years in the body [68]. The hydrophobicity is a disadvantage for its use in TE, as it results in poor cell attachment and proliferation [72]. Thus, several attempts have been done to overcome this limitation by coating plotted PCL scaffolds with proteins like fibronectin to improve the cell response [72],[73]. Bioactive glasses [71] and hydroxylapatite (HA) [70] were added to PCL to improve the cell-material interaction and the bioactivity. Furthermore, PCL was blended with PLA [74],[75] and copolymerised with PEG [76],[77] to adjust its wetting behaviour to the hydrophilic range as well as to adjust its degradation rate.
2.2.3 Laser based systems: stereolithography, laser induced forward transfer and 2-photon-polymerisation

Stereolithography (SLA) has been the first commercially available rapid prototyping technique in the 1980’s [11]. It is characterised by a high resolution down to 20 µm for commercial systems and it offers the possibility to fabricate constructs in cubic centimetre scale with complex architectures [11],[78]. There are also set-ups for laboratory use providing resolution in the submicron range [79]. The techniques principle is based on liquid photo curable resins, which are solidified using a moveable laser source or digital light projector combined with a digital mirror device to cure whole layers at once. After the curing of a layer in a predefined depth it sticks to a moveable platform dipped in a resin reservoir. The platform is moved down so that a new resin layer could be solidified on the top (bottom-up approach) or the platform is moved up when the light source is under the resin reservoir and so the next layer could be cured (top-down approach) [11]. Here the step-size in z-direction is important to ensure the connection of the different layers to each other. Thus, the curing depth is supposed to be larger than the platform moving step. As the conversion of reactive groups is often incomplete a post processing curing step is often applied to improve mechanical properties of the formed structure [78]. The application of this technique to biofabrication involves some critical issues as the necessity of using photo initiators at cytotoxic concentrations and high light intensities, which could possibly cause cell damage [78],[80]. Nevertheless, the successful encapsulation and long term viability up to 14 days of NIH/3T3 cells in polyethylene glycol diacrylate (PEGDA) was reported by Chan et al [81]. Also, natural biomaterials have been modified with RGD sequences and polyethylene glycol methacrylate (PEGMA) to make them appropriable for photo-patterning. Hippocampus neurons (HNs) and skeletal muscle myoblast cells (MCs) were encapsulated [82].

In this context two-photon polymerisation (2PP) is another upcoming technique, which provides excellent resolution down to 65 nm. Potential benefits in comparison to SLA are the use of light near the infrared spectrum, so milder processing conditions are given in comparison to UV-light [80]. Furthermore, 2PP technique is not limited to the surface [80], as absorption causing crosslinking of photopolymers is only
happening in the focus point of a laser with femto-second pulses [83]. Thus, actually 2PP is not strictly a layer by layer approach. Benefits are that the viscosity of the resins does not need to be considered and that also complicated structures with indentations can be realised [80]. Currently the processing of hydrogels containing cells is still critical using 2PP technique. Open issues are the need for biocompatible, water-soluble resins [84]. Also the processing is critical as the fabrication of mm-scale constructs could take several hours up to days [80]. Ovsianikov et al [83] carried out studies on cell viability during 2PP showing that laser radiation itself is not the cause of cell death, but it has possibly a chemical origin of species created during 2PP.

Another method among laser-based techniques, which could be deployed for cell printing, is laser induced forward transfer (LIFT), which shows high cell viabilities and no differences in cell phenotype expression [85]. Mostly two modified set-ups of LIFT are used for cell printing [22],[86]. One is absorbing film assisted laser induced forward transfer (AFA-LIFT), which is also referred to as biological laser printing and the other is matrix assisted pulsed laser evaporation direct write (MAPLE DW) [87]. The common principle is to use as a donor slide (ribbon) or target, which absorbs focused laser energy to create a drop, which is propelled on a CAD/CAM controlled target for the collection of the printed material [86],[88]. In MAPLE DW the biomaterial, cells or biomaterial/cells solution get mixed with a laser absorbing matrix material, whereas in AFA-LIFT an extra laser absorption layer is used in between the transparent support layer and the transfer layer with the biological material [86],[87]. The extra laser absorption layer is composed of Ti, TiO₂ or Au and has a thickness of around 70 nm [86]. The advantages of this set-up are the protection of the cells from the laser light and an increasing precision as the drop by drop variation is decreased by improvement of the energy conversion. In spite of these aspects MAPLE is more often the technique of choice for cell printing because of the contamination of samples with the absorption layer material [87]. The resolution of these techniques is in the range 10-100 µm and 2000-100000 cells/s could be processed [86]. Typical parameters which have to be adjusted for this set-up are the thickness of the biological layer, the laser fluence, the film to substrate distance, surface tension and the viscoelastic properties of the hydrogel [89],[90],[91].
Comparison of AM techniques used in biofabrication

The above presented common AM techniques used for biofabrication are difficult to compare, as they all have specific advantages and disadvantages depending on the planned application [6]. At present it seems that bioplotting is the most widespread technique [13]. It shows the best characteristics to fabricate constructs in clinical relevant sizes and with its potential for up-scaling, it is probably the most promising biofabrication method for creating whole organs structures in the future [9],[92].

2.2.4 Hybrid constructs as products of combined AM processing techniques

In biofabrication, hybrid constructs consisting of different materials are a strategy to overcome certain limitations of currently available hydrogel systems, such as limited mechanical properties or shape stability [9],[50],[93]. The different materials could be optimised for biological or for structural and mechanical functions [94]. To achieve the fabrication of hierarchical, complex tissue analogues, one trend is to combine different processing techniques, also AM techniques with conventional scaffold fabrication methods [95]. Cell-compatible processing techniques are also combined with cell “unfriendly” techniques [96]. The use of sequential bioplotting technique, as a combination of bioplotting and FDM, for the creation of mechanically enhanced 3D structures consisting of a thermoplastic hard phase (PCL) and a hydrogel (mostly alginate, but also a mixture of gelatin, hyaluronic acid, fibrinogen and glycerol) with immobilised cells has been reported [10],[50],[97],[98]. Shim et al [97] adapted their process to fabricate osteochondral constructs by using osteoblasts and chondrocytes in different areas of the scaffold. Another aspect is that the sequential bioplotting technique is superior to the conventional cell-seeding method of PCL scaffolds concerning seeding efficiency and cell distribution [17].

Another approach to achieve hybrid constructs is the fabrication of structures with cell incompatible AM techniques and their infiltration with a hydrogel/cell mixture in a second step [93]. For example, nanofibers fabricated by electrospinning have been infiltrated [99]. Visser et al [93] used melt electrospinning writing to fabricate highly porous 3D polycaprolactone (PCL) microfiber scaffolds, which were infiltrated in a second step with gelatin methacrylamide loaded with human chondrocytes. Such approaches have disadvantages in comparison to attempts of integrating different
techniques in a one-step processing method, which keeps all the beneficial design aspects from the layer-by-layer principle. These are the freedom for designing constructs with different materials and cell types in pre-defined positions [10]. In general the combination of AM techniques has tremendous potential in the field of biofabrication [37]. In Table 1 an overview of different attempts to create hybrid constructs containing hydrogels and cells by using AM techniques is presented.
Table 1: Overview AM-based fabrication approaches of hybrid constructs containing hydrogels and cells

<table>
<thead>
<tr>
<th>Techniques</th>
<th>Hydrogel</th>
<th>Cells</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Integrated Processing</td>
<td>Infiltration (2nd step)</td>
<td>Encapsulated</td>
</tr>
<tr>
<td>Melt electrospinning writing</td>
<td>-</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Electrospinning</td>
<td>Inkjet-printing</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Electrospinning</td>
<td>LIFT</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Fused Deposition Modelling</td>
<td>Bioplotting</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Two-photon polymerisation</td>
<td>LIFT</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Fused Deposition Modelling (FDM)</td>
<td>Electrospinning</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>FDM + Electrospinning</td>
<td>Bioplotting</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>FDM</td>
<td>Digital light processing based (DLP)-SLA</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Bioplotting</td>
<td>Ink-jet printing</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Proposed possible future combination in literature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bioplotting</td>
<td>SLA</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
2.3 Hydrogels as the material class of choice in biofabrication

Hydrogels are the material of choice in biofabrication [6]. They are described as three-dimensional physical or chemical crosslinked polymer networks with hydrophilic characteristics, as they have the ability to absorb large amounts of water [108]. Their network structure with a certain mesh size and the polymers they are made of are similar to the ECM of many human tissues. All these characteristics enable the immobilisation of cells in such hydrogels networks [45]. In the body the ECM provides a structural, 3D fibrous network as cellular environment with bidirectional complex physical and chemical signalling. Furthermore, different cell types are surrounded by specific microenvironments regulation cell behaviour [109]. Thus, engineering different hydrogels offers the possibility to adjust them to certain cell/tissue types. Following this approach, hydrogels are modified for example with proteoglycans and glycoaminoglycans, which are typical for the natural tissue. Mimicking the natural environment, the immobilised find an artificial, body-like surrounding to mature [110].

Classification of hydrogels is done in physically or chemically crosslinked hydrogels [22], as often several crosslinking mechanisms are mixed in complex hydrogel systems to match the manifold demands of biofabrication processes, this distinction is difficult [9]. In the following section, different crosslinking mechanism and their role for the biofabrication process will be explained. Furthermore, hydrogels are often divided in different categories considering the origin of the hydrogel forming polymers in natural hydrogels or synthetic hydrogels [111]. The class of semi-synthetic hydrogels describes materials of natural origin combined with synthetic polymers [16]. As this work is based in the field of natural occurring hydrogels, the focus lies on them in the later section.

2.3.1 Crosslinking mechanisms of hydrogels

The potential crosslinking mechanisms of a hydrogel precursor solution are very essential for its use in biofabrication, as they influence the viscosity, the stability and the mechanical properties of the construct [9]. Physical, also referred to as reversible, crosslinking mechanisms are often seen as beneficial before and during the
The reason is that chemical crosslinking implies the need for strict control of the crosslinking kinetics to avoid changes in the viscosity and so clogging of the needle as well as changes in the processing parameters [22]. Physical crosslinking often contains the problem of a low mechanical performance of the constructs. Thus, concepts of using physical and chemical crosslinking in a hydrogel system are investigated. Whereas the chemical crosslinking is often applied after the process to stabilize the constructs, simplify the handling and adjust the mechanical properties [9].

Physical crosslinking mechanisms are thermally induced gelation, ionic interactions or stereocomplexation. These mechanisms are based on molecular entanglements or secondary forces (hydrogen bonding, hydrophobic interactions, ionic forces) [45],[112]. Two classes of thermoresponsive polymers are named, such with an upper critical solution temperature (UCST) (e.g. GEL, agarose) or the group with a lower critical solution temperature (LCST) (e.g. elastin). Thus, they are not miscible below or above a critical temperature, leading to changes in the polymer structure and so causing gel formation. The driving forces for these changes are hydrophobic effects for LCST or enthalpy changes for UCST [45]. Polyelectrolytes form gels in the presence of electrically charged species, as e.g. negatively charged alginate does with di- or trivalent cations [45]. The formed complexes cannot be dissolved as their degradation is based on the exchange of the network forming ions [113]. Ionic gelation of alginate is done before [114] and after [115] bioplotting processes. A combined approach of thermally and ionic gelation has been used by Wüst et al [116] for bioplotting alginate/GEL blends. Heated solutions were plotted on a cooled template taking benefit of the thermal induced gelation of the GEL, enabling shape stability. In a second step Ca$^{2+}$ solution is added to the constructs to induce ionic gelation of the alginate phase and to guarantee stability of the constructs at 37 °C, when the GEL network is not stable.

Chemical crosslinking includes all mechanisms which induce gel formation by covalent bonds of the precursor solutions [9]. Here the main issue is to avoid toxic crosslinkers [117]. A very extensively used chemical crosslinking method is photocrosslinking, but often these gels have a limited biodegradability [118]. This method can be used also with chemically modified natural hydrogels using methacrylate chemistry and was
done for alginate [119] and GEL [120]. Oxidised alginate (ADA) was adapted for photocrosslinking by methacrylate chemistry [82]. Imine chemistry based on the reversible condensation between amines and aldehydes is used in hydrogel synthesis [121]. Several studies in the field of injectable hydrogels have exploited this mechanism in the last years. For example, Tan et al [122] used aldehyde hyaluronic acid crosslinked with chitosan and Balakrishnan et al [27] combined ADA and GEL as injectable hydrogel system. Moreover, membranes based on the Schiff’s base formation in between collagen and ADA were fabricated by Hu et al [123].

2.3.2 Natural hydrogels

In general hydrogels prepared from natural occurring polymers deal with the difficulty of reproducibility as the material properties show batch-to-batch variation [117]. Another issue, which has not been sufficiently investigated until now, is the contamination of hydrogels with endotoxins [124]. Such contamination could cause critical interactions with the human body like fever or endotoxin shock, when entering the blood stream [125]. Synthetic hydrogels have advantages considering this point and it is possible to exactly adjust the molecular weight or the degree of crosslinking. Nevertheless, natural hydrogels are widely used as they show a very good cell response [126]. There is a broad variety of natural polymers used for hydrogel synthesis, printing and biofabrication [94]. They can be classified as polysaccharides (agarose [19], alginate [61], chitosan [69], gellan gum [127]) and proteins (collagen [128], GEL [20], hyaluronic acid [129], fibrin [58], silk [46],[130]) [131],[132]). These polymers are derived from plants, animals or are produced biotechnologically using bacteria [12],[133]. Natural hydrogels mimic the natural ECM to a great extent or in some cases they are even a part of it like collagen or hyaluronic acid [134]. In Table 2 an overview of alginate, ADA as well as GEL and their use with different AM techniques in biofabrication approaches is presented, as they are relevant for the research in this work. The overview shows that alginate and GEL are already widely used in different biofabrication approaches.
### Table 2: Overview of hydrogel systems containing alginate, alginate dialdehyde or GEL used in biofabrication approaches

<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>Technique</th>
<th>Application</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate</td>
<td>bioplotting, ink-jet, LIFT</td>
<td>bone TE, osteochondral defects, cartilage TE</td>
<td>[63],[135],[97],[115], [54],[85],[136]</td>
</tr>
<tr>
<td>oxidised methacrylic alginate</td>
<td>SLA</td>
<td>multiple cell type approach</td>
<td>[82]</td>
</tr>
<tr>
<td>alginate / GEL</td>
<td>bioplotting</td>
<td>liver TE, aortic valves</td>
<td>[137],[99]</td>
</tr>
<tr>
<td>alginate / GEL / fibrinogen</td>
<td>bioplotting</td>
<td>tumor model</td>
<td>[7]</td>
</tr>
<tr>
<td>alginate / GEL / chitosan</td>
<td>bioplotting</td>
<td>liver TE</td>
<td>[138]</td>
</tr>
<tr>
<td>alginate / collagen I</td>
<td>Ink-jet printing</td>
<td>heterogeneous cell constructs</td>
<td>[139]</td>
</tr>
<tr>
<td>alginate / methylcellulose</td>
<td>bioplotting</td>
<td>TE</td>
<td>[140]</td>
</tr>
<tr>
<td>alginate / hyaluronic acid</td>
<td>bioplotting</td>
<td>Schwann cells encapsulation</td>
<td>[141]</td>
</tr>
<tr>
<td>GEL / hyaluronic acid</td>
<td>bioplotting</td>
<td>brain injuries</td>
<td>[142]</td>
</tr>
<tr>
<td>silk fibroin / GEL</td>
<td>bioplotting</td>
<td>TE</td>
<td>[143]</td>
</tr>
<tr>
<td>GEL / fibrinogen</td>
<td>bioplotting</td>
<td>liver TE</td>
<td>[144]</td>
</tr>
<tr>
<td>GEL methacrylate</td>
<td>bioplotting, SLA</td>
<td>TE</td>
<td>[62],[145]</td>
</tr>
<tr>
<td>GEL methacrylate / hyaluronic acid</td>
<td>bioplotting</td>
<td>TE</td>
<td>[146]</td>
</tr>
<tr>
<td>methacrylate</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Alginate (ALG)**

Commercially produced alginate is derived from brown algae (Phaeophyceae) [147]. Biotechnological routes using bacteria, as Azotobacter or Pseudomonas, are investigated to obtain alginate with a more defined chemical structure and improved physical properties, which is of high relevance for biomedical applications [22],[147]. Alginate is a polysaccharide with a linear block-copolymer structure, showing anionic charge [148]. The block-copolymer contains units of 1,4-linked $\beta$-D-mannuronate (M) and $\alpha$-L-guluronate (G), which show orders of sequential G-, M- or randomly ordered G-M-residues (schematically presented in Figure 4) [147].

![Figure 4](image)

**Figure 4:** Scheme of representative alginate structure: a) chain conformation and b) block distribution. *Reprinted from* [113] *with permission from Elsevier.*

Alginate solutions can be gelled in a mild way using different di- or trivalent cations [41],[149],[150]. Variation of the gelling ions or their concentration is a strategy to vary the mechanical properties of alginate [149],[151]. The used gelation ions could also induce the differentiation of encapsulated cells, e.g. using $\text{Sr}^{2+}$ or $\text{Zn}^{2+}$ in the context of bone tissue engineering [152]. Calcium ions are often used as they show low cytotoxicity [153]. The crosslinking of the alginate chains happens mostly by binding over the G-blocks resulting in a hydrogel structure described as an “egg-box” [147],[154]. Thus the M/G ratio is an important parameter considering the physical properties of alginate as well as the sequence of M- and G-blocks and the molecular weight, which defines the viscosity [148],[155]. Alginates containing a high amount of G-units show a more open pore structure, which is favourable for cell encapsulation considering diffusion of nutrients [148] and is also decisive parameter for cell
migration [16]. Commercially available alginates have molecular weight values between 32,000 and 400,000 g/mol [147].

Alginate is widely used for biomedical applications [156], for example as wound dressing material [157], in drug delivery systems [158], for cell encapsulation [159], [160] and for biofabrication [114]. Especially, the shear thinning properties [161] and ease of handling make it an attractive processing material for bioplotting [162]. Nevertheless, alginate does not provide cell adhesion sides [156]. Its degradation behaviour is rather uncontrolled as it depends on the exchange or removal of the crosslinking ions, which indicates that is a type of dissolution process [41]. To address this issue alginates have been modified with different proteins [23], [30] or peptides [163] to improve the cellular response. The cellular degradation of alginates has been controlled by modification with protease-sensitive peptides [164]. For the improvement of the degradation behaviour alginate was oxidized using sodium metaperiodate to achieve ADA, which is sensitive to hydrolysis [25], [165]. During this process the two secondary hydroxyl groups become oxidised, resulting in aldehyde groups [113]. Furthermore, the process leads to ring-opening cleavage of the carbon–carbon bond, an uronate residue is left, which is sensitive to hydrolytic degradation [29].

**Gelatin (GEL)**

GEL is an important material for cell encapsulation and 3D cell culture and so for biofabrication [45], [166]. GEL is derived from collagen, which is one of the main components building up the ECM and it is the organic structural component of bone tissue [134]. Collagen provides cell-adhesive motifs and its derivate GEL has a similar amino acid composition. It is mainly composed of glycine (33%), proline and hydroxyproline (22%). Further it contains arginine and aspartic acid and thus provides RGD-sequences for cell adhesion. Aspartic acid is only present in GEL derived from porcine skin [167]. Collagen is gained from cattle bones and pork skin. GEL is derived by physical and chemical treatment from the purified collagen. Depending on the treatment with acid or alkaline solution and the source GEL is labelled as type A (porcine skin) or type B (bovine). In contrast to collagen, which is practically insoluble in water, GEL can be dissolved [168]. The denaturation process of collagen to obtain
GEL, encompassing a high temperature treatment, has the beneficial side effect that GEL is non-antigenic, as opposed to collagen [167]. In water GEL forms thermoreversible gels with a gel point below 35 °C. This process is time dependent and causes an increase in viscosity [169]. The temperature initiated gelation leads to the formation of locally ordered regions through the partial random return of the dissolved GEL chains to collagen-like helical structures [170],[171]. Thus, one major drawback is the instability of GEL at 37 °C, which makes the use of crosslinkers necessary. There are different approaches for thermal stabilisation of GEL like using genipin [172], glutaraldehyde [173], transglutaminase [174],[175] or ADA [176] as crosslinker.

**Alginate dialdehyde gelatin crosslinked hydrogel (ADA-GEL)**

The reactive aldehyde groups of ADA offer the possibility of covalent crosslinking of GEL over Schiff’s base formation. This interaction is based on the ε-amino groups of lysine or hydroxylysine of gelatin and the aldehyde groups [29]. ADA-GEL has several parameters enabling its adjustment for flexible usage for different processing techniques and several applications in TE. For example, it has been used as an injectable system for cartilage TE [28] and wound dressings [177]. Further, it has been applied for microcapsule formation [29] and cell encapsulation [30]. The preparation of films consisting of ADA-GEL has been also established showing good compatibility with fibroblasts [178],[179]. Moreover, tubular vessel like structures have been prepared using ADA-GEL with endothelial and smooth muscle cells [180]. ADA-GEL induced no toxic or inflammatory reactions in subcutaneous in-vivo studies [32]. Moreover, ADA-GEL has been applied in the context of muscle TE [181]. This shows the high interest in this material system in TE. In Figure 5 in an overview different parameters and interacting properties of the ADA-GEL system are summarised.
2.4 Cell behaviour in a 3D hydrogel environment

It has been shown that differences in the behaviour of cells, which are cultivated in a 2D or 3D microenvironment are fundamental. Petersen et al [182] have demonstrated that breast epithelial cells behave like tumor cells in 2D, but revert to normal growth behaviour when cultured in a 3D environment which mimics their natural ECM. Thus, 3D cell culture models could bridge the gap between standard (2D) cell culture and living tissue [183]. The culture of cells onto a 3D microporous scaffold on the surface of the struts of a foam, for example is not seen as real 3D culture, as the pore size is often bigger than the cells, thus such cultures are related to a 2D culture on curved surfaces [15],[44]. On the other hand, 3D hydrogel matrices for cell encapsulation represent “real” 3D environments to mimic the actual 3D tissue topology [109].

In comparison to flat 2D cultures, where the cell has contact to the ECM/material only from one side, in a 3D set-up cells are completely surrounded by the matrix [109]. Thus, cell-material interactions occur from all sides and in multiple directions [184]. The cells can express, organize and restructure the ECM, but the matrix can also
regulate the behaviour of the cells [44],[184]. Cell-matrix adhesions are regulated over the connection between transmembrane surface molecules (integrins) and binding motifs of certain ECM proteins. Integrins connect ECM macromolecules with the cytoskeleton of the cells [185]. There are different categories of adhesions, which are also important for cell migration. They are named as nascent adhesions, focal complexes and focal adhesions [186]. 3D cell cultures enable also cell-cell interactions from all sides [44]. As the cell is completely surrounded by the matrix the supply with nutrients and oxygen can be characterised by a gradient like it occurs in living tissue. The gradient is dependent on the length of the diffusion pathway from the outside to the cell in the matrix. In 2D approaches one side of the cell is completely uncovered and in direct contact to the cell medium reservoir [44],[183]. The control of oxygen diffusion offers the possibility to create hypoxic conditions for the cells. This is an interesting feature in cancer research [187]. Hypoxia is also discussed in bone regeneration, as certain biomaterials could activate hypoxia inducible factor 1 (HIF-1), which accelerates bone regeneration [188]. Since articular cartilage is avascular, hypoxia and the HIF pathway play an important role in chondrocyte development and so are a useful tool in cartilage TE [189].

These parameters of the 3D set-up influence basic cell behaviour like adhesion, migration, proliferation, differentiation, signalling and morphology [16]. Considering cell migration in 2D cells face no or little resistance [44]. However, in a 3D environment the porosity and the physical structure of the hydrogel network take influence on cell migration. Hydrogels can have different physical structures as dense, porous, self-assembled and fibrous. Many of the used hydrogel matrices are in the categories dense and porous with pore sizes in the nm range, which is a hindrance to cell migration [16].

Cell migration in 2D is well described, whereas in 3D the situation is more complex and several aspects are still unclear, as the matrix and its mechanical and sterical properties play an important role in dictating cell behaviour [190]. In 2D cell migration is a cyclic process starting with the polarisation of the cell and stretching of protrusion in the direction of movement, followed by adhesion on the ECM to get the protrusions stabilized. By contraction the cell moves forward and the trailing edge gets detached from the substrate [185],[191]. Two types of cell migration for single
cells are described, named as amoeboid and mesenchymal cell migration [186]. Amoeboid cell migration is characterised by gliding and rapid migration based on low integrin-mediated traction forces, or it can take place completely without these interactions [183],[186]. Mesenchymal cell migration is dependent on integrin-mediated traction forces during interaction with the ECM. This is incident with reorganisation and local degradation of the ECM. The cells create space for their movement by secreting proteases like matrix metalloproteinases (MMPs), leading to proteolytic cleavage of matrix proteins [16],[185],[186]. Soft, fibrillar matrices could also enable non-proteolytic migration when cells squeeze through pores or deforming the fibers of the surrounding matrix [185]. This cellular behaviour shows the importance of the degradation behaviour of the surrounding hydrogel matrix, providing space for migration or for the expression of ECM by the cells to build up new tissue [94]. Fonseca et al [164] designed alginate with MMP-sensitive peptides to enable local proteolysis by the encapsulated cells. A similar approach was used with modified PEG hydrogels to control fibroblast invasion [192]. Tumor cells are able to switch between these different migration mechanisms adapting to their environment. This property is called plasticity of tumor cell migration [193].

2.5 Applications for biofabricated constructs

The ambitious long term goal of biofabrication is to fabricate complete functional organs for use in regenerative medicine. Another application of these constructs, which could be a by-product of these developments, is the use of the new 3D matrices as tissue models for drug screening or for investigating cell behaviour in diseased tissue or developmental biology [37]. This could reduce animal studies, which is beneficial from both ethical as well as economical viewpoints [6]. Nevertheless, considering the complex interactions in an organism, the biomaterial based 3D models will not completely replace animal studies, but could be an intermediate step from 2D in-vitro studies to animal studies and so to reduce and rationalise the number of animal studies [194]. The positive aspect is the reduced size of such disease models in comparison to whole organs as well as the much easier regulatory process [37]. Two examples are the use of biofabrication for bone tissue
engineering [8],[39] and for the development of tumor models in cancer research [195].

2.5.1 Biofabrication and bone tissue

As bone is after blood the most implanted tissue, there is a high need for bone replacement materials and several strategies are exploited to overcome the shortage of the “gold standard”, which is still the use of autologous bone material [8],[134]. Different kinds of scaffolds have been fabricated with different techniques (AM techniques, foam replica method, freeze drying) consisting of bioactive glasses [196], calcium phosphates [197], hydroxyapatite [198], thermoplastic materials [48] or composites of organic and inorganic materials [199],[200],[201]. Biofabrication is a recent approach to address unsolved issues of engineered bone grafts or conventional scaffolds including lack of vascularisation and appearance of tissue necrosis [8]. Further, the use of co-cultures to remodel the complex dynamic bone tissue is rather limited [8]. Biofabrication offers the possibility to directly fabricate a vascular system in the constructs and to place different kinds of cells in defined positions within a construct [39]. Luo proposed the plotting of scaffolds build up by hollow fibers, which could be filled with endothelial cells suspended in culture medium during the plotting processing taking advantage of core-shell nozzles [202]. Further co-cultures of osteo- and endothelial progenitor cells were bioplotted in alginate to improve the neovascularisation behaviour of the constructs [203].

Bone tissue is characterised by the remarkable interplay of osteoblasts and osteoclasts enabling the constantly reshaping of bone tissue while adapting to changing biomechanical forces or to remove damaged tissue, called remodelling [204],[205]. This crosstalk between osteoblasts and osteoclasts is described in Figure 6. Osteoblasts, which arise from mesenchymal stem cells, take the role of new bone formation process called ossification (or osteogenesis). Osteogenesis can be divided into three steps, namely: a) organic, unmineralised ECM synthesis (= osteoid phase), b) matrix mineralisation and c) bone remodelling (resorption and reformation) [205]. The organic bone phase consists mainly of collagen type I, but also of up to 10 % of non-collagenous proteins. The major ones are osteocalcin, osteonectin, osteopontin
and bone sialoprotein. These proteins play an important role in matrix mineralisation by accumulation of hydroxyapatite [206],[207],[208].

Figure 6: Scheme of the crosstalk between osteoblasts and osteoclasts regulating the dynamic, complex process of bone formation and resorption. Reprinted from [8] with permission from Elsevier.

Finally, the osteoblasts become encased in the mineralised matrix and become osteocytes [209]. Osteoclasts are responsible for bone resorption [210]. Osteoclasts develop from haematopoietic precursor cells by differentiation and fusion. Important cytokines for osteoclastogenesis are macrophage colony-stimulating factor (M-CSF) and receptor activator of the NF-κB ligand (RANKL) [211],[212]. Osteoclasts are multinucleated cells, which become polarized and adhere to mineralized bone surface before resorption. They create an acidic environment to dissolve the mineralised matrix components and express proteases to degrade organic elements, like collagen [211]. The role of the osteoclasts in bone remodelling is gaining increasing attention showing that the osteoblasts are not, as earlier believed, the only dominant factor in the cell crosstalk. The role of so called “clastokines” expressed by osteoclasts and their role in the regulation of bone formation is being also investigated [212],[213]. Also cell-cell signalling is possible between osteoblasts and osteoclasts [213]. In the first phase of ossification vascularisation is an important process to guarantee normal bone development, as bone is a highly vascularized tissue [205],[214].
2.5.2 Biofabrication in cancer research

One possible application of biofabricated constructs as tissue models lies in the field of cancer research to realise the step from 2D to 3D cell culture. The models can be used for testing cancer drugs or investigating tumor development as well as metastasis [7],[194],[215]. The beneficial aspects of such model systems in comparison to animal studies are their quick availability and the possibility for a high throughput of samples. Indeed, studies with a large amount of samples can be realized to obtain statistically significant results [216]. Biofabrication offers a better replica of natural tissue than simple cell encapsulation [216].

Invasion studies with breast carcinoma cells on collagen films mimicking natural tissue have been investigated by varying the pore size and stiffness of the samples to detect their influence on the invasion depth [217]. Cell encapsulation approaches have been used to simulate a diffusion gradient of oxygen, inducing a hypoxic state. Hypoxia is seen as an important parameter influencing tumor angiogenesis [218]. Skardal et al [216] build up a metastasis-on-a-chip platform consisting of an intestine model including cancer cells connected by a tube simulating blood stream to a liver construct. With this set-up cancer metastasis and also anti-cancer drugs can be tested.
3 Materials and Methods

3.1 Material synthesis and preparation

3.1.1 ADA-GEL

Sodium alginate (alginic acid sodium salt from marine brown algae, suitable for immobilisation of microorganisms, Sigma-Aldrich, Germany) with a guluronic acid content of 65-70 % and gelatin (Sigma-Aldrich) Type A, Bloom 300, derived from porcine skin, were used. Calcium chloride di-hydrate and sodium metaperiodate were purchased from VWR international, Germany.

Covalently crosslinked alginate dialdehyde-gelatin (ADA-GEL) hydrogel was synthesised similar to the description presented by Sarker et al [29]. In short, ADA was prepared by oxidation of alginate using sodium metaperiodate of different amounts. For this, 10 g of alginate dispersed in 50 ml of ethanol was mixed with 2.16 g (periodate equivalent 21.6 %(w/w)), 2.674 g (periodate equivalent 26.7 %(w/w)) or 3.21 g (periodate equivalent 32.1 %(w/w)) sodium metaperiodate dissolved in 50 ml deionized water. This suspension was stirred at room temperature for 6 h in the dark. The addition of ethylene glycol (equivalent to alginate) (VWR) quenched the reaction under continuous stirring for 30 min. For dialysis against ultrapure water (Direct-Q®, Merck Millipore, Germany) the resultant suspension was loaded into semipermeable membranes (MWCO: 6000-8000 Da, Spectrum Lab, USA). The water was changed several times during the 5 days of dialysis and in a final step the suspension was lyophilized for 4 days.

For the mixing of the ADA-GEL hydrogel, differently concentrated (5.0 %(w/v), 7.5 %(w/v), 10 %(w/v)) aqueous solutions of GEL were dropped slowly into an ADA solution (5.0 %(w/v), 7.5 %(w/v), 10 %(w/v)) in phosphate buffered saline (PBS, Life technologies, Germany) under stirring. In case of application in cell studies, the ADA as well as the GEL solutions were sterilised by filtration (0.45 µm and 0.22 µm pore size, Carl Roth, Germany).
3.1.2 Polycaprolactone (PCL) and Polyethyleneglycol (PEG)

PCL \((M_n = 40000 \text{ to } 50000)\) and PEG \((M_n = 7000 \text{ to } 9000)\) were purchased from Sigma-Aldrich, Germany. Granules of PCL and PEG were mixed in appropriate ratios in the cartridge of the plotter system and heated up to 110 °C and mixed with a spatula to get PCL/PEG blends.

3.2 Material characterisation

3.2.1 ADA-GEL hydrogel system

Flow rheology

The molar mass of ALG and ADA were determined using a viscosity method [178], [219]. ALG was dissolved in 0.1 M NaCl solution to get the final concentrations, namely 0.05, 0.1, 0.15 and 0.2 \%(w/v). For ADA, the concentrations of solutions were 0.1, 0.2, 0.3 and 0.4 \%(w/v). The experiment was carried out at 25 °C with an Ubbelohde viscometer (Schott-Geräte GmbH, Germany). The viscosity average molar mass \((M_\eta)\) of sodium ALG and ADA was calculated from their measured intrinsic viscosities \([\eta]\) according to the following Mark-Houwink equation, by adapting \(a\) and \(K\) values from Smidsrød [220]:

\[
[\eta] = 2.0 \times 10^{-5} \times M_\eta^{1.0}
\]

The intrinsic viscosities of the ALG and ADA solutions were determined by extrapolating the crossover point of their reduced and inherent viscosities, as shown in the Appendix (Figure A 1).

Shear rheology

A Discovery Hybrid Rheometer Series 3 (DHR-3) (TA Instruments, England) was used. The software was TRIOS supplied by TA Instruments. A cone plate geometry with a cone diameter of 40 mm and a cone angle of 2° was chosen. For environmental control, a peltier plate with a diameter of 40 mm combined with a thermostat were used. The measurement gap was set at 65 μm. The measurement temperature was 25 °C. The solutions were dosed on the plate with a plastic syringe. A solvent trap was used to prevent that the sample in the measurement gap dried out.
The shear rate range in the flow sweep experiment was varied from 0.01 s$^{-1}$ to 1000 s$^{-1}$. A conditioning step with a soak time of 180 s was set before starting the flow step to ensure the measurement temperature of the sample at 25°C.

The oscillation time sweep experiments were performed on the ADA-GEL with a ratio of ADA and GEL of 1:1. The warmed GEL (37°C) was dropped slowly in the ADA solution under stirring. They were stirred for 2 min. then filled into a syringe and dosed on the plate geometry. The time between dropping the GEL into the ADA solution and preparing the hydrogel in the rheometer was between 5 to 8 min. The oscillation frequency was set at 10 rad/s and the applied shear stress at 2 Pa.

**Nanoindentation**

For these measurements ADA, GEL or ADA-GEL films were prepared by pouring the solutions in a single petri dish. In case of samples containing ADA 0.1 M CaCl$_2$ solution was added for 10 min. Then the gelation solution was removed and the samples were washed and afterwards covered with Hank’s Balanced Salt Solution (HBSS, Sigma-Aldrich, Germany). The reduced Young’s modulus (rYm) of different ADA-GEL compositions at different temperatures was determined using a ferrule-top nanoindenter (PIUMA, Optics11, Amsterdam, Netherlands). Each sample was measured at different positions to receive an average value. The loading period was set to 2 s, the holding time to 1 s and uploading time to 2 s. The calculation of the rYm was based on the load-displacement curves using the Oliver-Pharr model [221]. The measurements were carried out at room temperature (RT) and at 37 °C as well as at different time points after incubation at 37 °C in HBSS (Sigma-Aldrich) solution.

**3.2.2 PCL/PEG**

*Fourier transform infrared spectroscopy*

A Fourier transform infrared (FTIR) spectrometer (Nicolet 6700, Thermo Scientific, Germany) was used to evaluate the material composition of plotted planar structures (10 mm x 10 mm) with different material compositions. A wave number range from 650 cm$^{-1}$ up to 4000 cm$^{-1}$ was used.
Contact angle

Contact angle measurements were done by the sessile drop method using ultrapure water. The resulting contact angle was determined by a drop shape analyser (DSA30 Kruess GmbH, Germany). Planar structures were plotted with the different material compositions.

3.3 Plotting Process

3.3.1 ADA-GEL (soft-phase)

For the processing of the ADA–GEL hydrogel a three axes moveable bioplotter (type BioScaffold 2.1, GeSiM mbH, Großerkmannsdorf, Germany) was used. The bioplotter was equipped with a pressurized air system, which was compatible with a syringe micro-nozzle system (Nordson EFD, Germany). The design and the dimensions of the plotting geometries were defined over the ‘ScaffoldGenerator software’ of the bioplotter. Simple lines and grid-like, square structures with an edge length of 15 mm were plotted to generate test specimens. The number of struts plotted over the edge length as well as the number of layers in z-direction was varied for the different investigations. Regarding the generation of hydrogel/cell constructs grid-like, cubic structures with an edge length of 15 mm were plotted. The geometry of these constructs was 10 struts in each direction of a grid per layer with an angle of 60° in between and 4 layers in total. The principal aim for this design was to gain height in z-direction by plotting 4 instead of only 1 layer while containing a grid structure with open pores. Thus, a grid with pores of rhombic shape was designed. The resulting construct after finishing the plotting process is shown in Figure 7.

A syringe, fitting with a holder in the plotter head, was filled with the hydrogel and processed into six well culture plates, which were placed in a holder on the static plotter platform. Ionic gelation was performed using 0.1 M CaCl₂ solution for 10 min after processing. In a next step, the samples were washed with deionised H₂O, DMEM culture medium (MG-63 cells) or MEM alpha Medium (ST2 and RAW cells) to eliminate the CaCl₂ solution.
Figure 7: Overview image of a typical ADA-GEL hydrogel construct used for cell investigations a) during the plotting process and b) after processing and CaCl$_2$ gelation.

The processing temperature was set in the range of 32 - 37 °C by heating the cartridge containing the hydrogel-cell mixture to prevent the gelling of the gelatin and to confirm a cell-friendly condition. As an exception from this set-up the experiment of grid-like constructs with varied number of struts to vary the pore size – was carried out at room temperature showing that this is also possible. For the plotting of the hydrogel a micro-nozzle with an inner diameter of 200 μm was chosen to reach a high resolution. The pressure and the plotting speed were adjusted considering the experimental set-up for the process parameter evaluation in a range between 55 and 100 kPa and 10–60 mm s$^{-1}$, respectively. For the fabrication of ADA-GEL/cell constructs a speed of 20 mm/s was chosen, which guarantees a fast processing, but also a run of the machine without disturbance. The pressure was adjusted in dependence of the used ADA-GEL concentration. The following pressure ranges were used for the processing: ADA-GEL of 2.5 % with 40 kPa to 80 kPa, ADA-GEL of 3.75 % with 120 kPa to 170 kPa and ADA-GEL of 5 % with 340 kPa to 410 kPa.

After processing, the hydrogel scaffolds with immobilised cells were stored in an incubator at 37 °C in a humidified atmosphere of 95% air and 5% CO$_2$. The DMEM culture medium supplemented with 10 %(v/v) FBS and 1 %(v/v) PS was changed every 3–4 days.

3.3.2 PCL and PCL/PEG (hard-phase)

For scaffold fabrication a bioplotting system (type Bioscaffold 2.1, GeSiM mbH, Großerkmannsdorf, Germany) was used. The scaffold design was generated with the “ScaffoldGenerator software” (GeSiM mbH). The scaffold geometry was set to 10 mm
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x 10 mm x 4.5 mm. Scaffolds were built of 18 double layers, means each layer consists of two struts plotted in reverse directions (as schematically shown in Figure 8). Subsequent layers were positioned in a 90° pattern with either 10 or 14 parallel lines with equal interspace. A conic aluminium nozzle (Vieweg GmbH, Kranzberg, Germany) with a diameter of 250 µm was used. The plotting speed was set to 4 mm/s and pressure to 410 kPa for all PCL or PCL-PEG compositions. The processing temperature was set at 120°C for PCL, 110°C to 100°C for PCL-PEG 80-20 and 90°C to 80°C for PCL-PEG 70-30. Further increase of PEG content resulted in scaffolds of inferior quality.

Figure 8: Scheme of a hard-phase (PCL/PEG) scaffold with typical double-strut structure in a) top view and b) side view.

3.3.3 Hard-soft plotting

The scaffolds of the sequential bioplotting process had two double layers in z-direction and the 10 lines design was used, to guarantee a high volume share of ADA-GEL with immobilised ST2 cells, which was plotted in the interspace (as schematically shown in Figure 9). The ADA-GEL was plotted with 10 mm/s, 170 kPa and 200 µm needle (Nordson EFD, Germany). Considering an improved cell response using a PCL-PEG 7030 blend, the hard-soft scaffolds were fabricated with this material composition. The plotting process of the ADA-GEL hydrogel was described in more detail earlier.
3.4 Plotted construct evaluation

3.4.1 ADA-GEL constructs

*Construct dimensions*

Construct geometry and structure were observed by bright field microscopy (Primo Vert, Carl Zeiss, Germany). The strut width and centre line length of pores of the scaffolds were determined from light microscope images using ImageJ software (open source Java image processing program). In Figure 10 a scheme of the construct geometry is shown, indicating the values of strut width and pore centre line length.

*Figure 9*: Scheme of a hard-soft phase scaffold with the hard thermoplastic phase (grey) and the soft hydrogel phase (blue) containing the cells.

*Figure 10*: Scheme of the construct geometry indicating the values of strut width and pore centre line length.
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Scanning electron microscopy

For SEM investigation of their morphology ADA-GEL constructs were placed in a fixing solution containing 3 % (v/v) glutaraldehyde (Sigma, Germany) and 3 % (v/v) paraformaldehyde (Sigma) in 0.2 M sodium cacodylate buffer (pH 7.4) after they have been rinsed three times with HBSS (Sigma-Aldrich). In a next step, the samples were incubated in a diluted ethanol series starting from a concentration of 30 % up to 99.8 %. Thus, the HBSS (Sigma-Aldrich) solution was replaced by ethanol. This step is necessary because water is, in contrast to ethanol, not miscible with liquid CO$_2$, which should be the transitional fluid during the critical point drying process. The critical point of liquid CO$_2$ is reached at a pressure of 74 bar and a temperature of 31 °C. These are conditions, which are sufficient for biological samples, whereas for the parameters of water or ethanol this would not be the case. Thus, the samples were critical point dried (EM CPD300, Leica) at the above named conditions after ethanol was replaced by liquid CO$_2$. Then scanning electron microscopy (SEM) was carried out (Auriga CrossBeam, Carl Zeiss Microscopy GmbH, Germany). Accelerating voltage was set in the range of 0.8 to 1.2 kV.

GEL release study

The concentration of GEL released from bioplotted ADA-GEL constructs with a concentration of 2.5 % (w/v), 3.75 % (w/v) and 5.0 % (w/v) after different time points of incubation in 2 ml HBSS (Sigma-Aldrich) at 37°C was determined by colorimetric protein assay using the Lowry method (Total Protein Kit, Micro Lowry, Sigma-Aldrich, Germany) [29]. The Lowry method is based on the biuret reaction in which the alkaline cupric tartrate reaction complexes with the peptides of the protein. In a second step the reduction of the Folin & Ciocalteu’s phenol reagent leads to a purple colour of the solutions [222],[223].

At selected time points the whole supernatant of the samples was removed and new solution was added. Deoxycholate solution (DOC) and trichloroacetic acid (TCA) were mixed with the supernatants to force the protein precipitation by eliminating the protein interference with other chemicals in the solution. Then the solutions were centrifuged to get protein pellets. The protein pellets were dissolved in deion. H$_2$O and mixed with Lowry reagent as well as Folin. The absorbance of each solution at
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750 nm was measured using a UV-Vis spectrophotometer (Specord 40, Analytik Jena, Germany). The release (%) of GEL from the constructs was calculated as follows:

\[
\text{Gelatin release } (\%) = \frac{[\text{Gelatin }_{\text{supernatant}}]}{[\text{Gelatin }_{\text{total}}]} \times 100
\]

(2)

where, \([\text{Gelatin }_{\text{total}}]\) is the initial concentration of GEL (in the constructs) and \([\text{Gelatin }_{\text{supernatant}}]\) is the final GEL concentration in the storage medium at different time points.

Electrophoretic Analysis

Protein patterns of GEL released from the ADA-GEL constructs with a concentration of 2.5 % (w/v) and 3.75 % (w/v) after different time points of incubation in HBSS (Sigma-Aldrich) were analysed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out using the omniPAGE mini vertical system (Cleaver Scientific, Rugby, UK). The separating gels were prepared by mixing 1.364 ml ultrapure water, 1.875 ml TRIS buffer (pH 8.8), 50 µl of 10 % SDS and 1.667 ml of 30 % acrylamide. Then 40 µl of 10 % APS and 4 µl of TEMED were added to initiate the polymerisation reaction. The solution was used to cast the gels. The collecting gels were prepared analogous to the separating gels with a slightly changes composition of 1.385 ml ultrapure water, 0.25 ml TRIS buffer (pH 6.8), 20 µl of 10 % SDS and 0.333 ml of 30 % acrylamide as well as 10 µl of 10 % APS and 2 µl of TEMED. The chemicals for the gel preparation were purchased from Carl Roth, Germany.

The supernatants with the released GEL were centrifuged, using Eppendorf tubes with an integrated filter (Roti®-Spin MINI-3, MWCO 3 kDa, Carl Roth, Germany). A volume of 500 µl of the supernatants was pipetted in these Eppendorf tubes and centrifuged for 20 min at 15000 rpm. The filtrate was resuspended in 20 µl HBSS (Sigma-Aldrich). 15 µl of the centrifuged supernatants was mixed with 5 µl loading buffer and vortexed. The samples were heated up to 100 °C for 15 min in a water bath and centrifuged at 5000 rpm. The samples were placed together with a blank, and a ladder, pre-stained molar mass markers (PageRuler™, ThermoFisher Scientific, Germany), in the predicted test chambers filled with running buffer. Electrophoresis was run by 120 V. Proteins were visualised using Coomassie Brilliant Blue R-250 (Sigma-Aldrich). The gels were imaged with a documentation system (omniDOC Gel Documentation System, Cleaver Scientific).
3.4.2 PCL and PCL/PEG scaffolds

Construct dimensions

Images were obtained using bright field microscopy (BF, Scope.A1, Carl Zeiss). The width of the struts from the top scaffold-layer in z-direction was measured using ImageJ (open source Java image processing program) software. In the same manner, the pore size was also obtained.

Porosity

Porosity was determined by measuring the density ($\rho_{Sc.}$) of the samples using the density kit of analytical balance (Kern, Germany). The weight of the samples was measured in air ($m_{Sc.}$) and in 99.8% ethanol, with ethanol density set to 0.800 g/cm³. Based on the results, the volumetric mass density was calculated. The exterior dimensions of the samples were measured using a calliper (accuracy of 50 μm), to calculate the total volume of the scaffold ($V_{tot.}$). The total porosity ($P$) was calculated [224], using the following equation:

$$P = 1 - \frac{m_{Sc.}}{\rho_{Sc.} \times V_{tot.}}$$

Weight loss

Scaffolds with 14-struts-design of each composition were incubated in Hank’s balanced salt solution (HBSS) (Sigma-Aldrich) for three days at 37 °C. After drying the samples for 24 hours, the weight was measured to examine the mass loss.

Scanning electron microscopy

For characterisation of the morphology of PCL and PCL-PEG scaffolds SEM (Auriga CrossBeam, Carl Zeiss Microscopy GmbH, Germany) was used.

Mechanical Testing

Mechanical properties of the samples were investigated using a compression testing machine (Zwick 2050, Zwick Roell GmbH, Ulm, Germany). The upper force limit was set to 1 kN, the compression rate to 1 mm/min and the maximum deformation to 3 mm. The compressive stiffness of the scaffolds was determined from the initial
linear region of the stress–strain curve. The dimensions of samples for these tests were 10 x 10 x 4.5 mm³.

3.5 Cell biology

3.5.1 Cell culture, cell immobilisation and cell seeding

Osteoblast-like cell line MG-63

MG-63 osteoblast-like cells (Sigma-Aldrich, Germany) as an adequate osteoblast model were used, for the first assessment of the plotting process and for investigating interactions with ADA-GEL. Advantages of the MG-63 cell-line for experiments, at this stage of the work, were that there are no interspecies differences as well as they show similar integrin subunits as human primary cells, which makes them attractive for cell adhesion experiments or cell–matrix interaction studies [225]. Culture medium DMEM (Dulbecco’s modified Eagle’s medium, Gibco, Germany) supplemented with 10 % (v/v) fetal bovine serum (FBS, Sigma-Aldrich) and 1 % (v/v) penicillin/streptomycin (PS, Sigma-Aldrich) was chosen. The culture flasks (Greiner-BioOne, Germany) were placed in an incubator at 37°C in a humidified atmosphere of 95% air and 5% CO₂ with the cells growing for 48 h to confluence. Cells were washed with PBS and detached from the flasks surface using Trypsin/EDTA (Sigma-Aldrich). For adjusting the cell concentration, they were counted with a hemocytometer (Carl Roth) and diluted with culture media. The cell concentration was set to 1 million cells per ml ADA-GEL. The incubation time of the experiments was usually set to 28 days as at this time point the ADA-GEL constructs are completely covered with cells.

Bone marrow stromal cell line ST2 and macrophage like cell line RAW 264.7

For further cell culture studies, murine bone marrow derived stromal cell line ST2 (German Collection of Microorganisms and Cell culture, Germany) and murine macrophage like cell line RAW 264.7 (Sigma-Aldrich) as osteoblast- and osteoclast progenitor cells, respectively, were used. As this study combines 3D plotting technique, 3D cell immobilisation and a co-culture approach, cell lines were chosen for these experiments to have a more stable cell source in this advanced set-up with multiple parameters. Cell culture medium MEM (minimal essential medium) alpha
(Life technologies) supplemented with 10 % (v/v) fetal calf serum (Sigma-Aldrich) and 1 % (v/v) penicillin/streptomycin (Sigma-Aldrich) was used. The culture flasks (Sarstedt, Germany) were placed in an incubator at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂ with the cells growing for 48 h. Cells were washed with PBS and detached from the flasks surface using Trypsin/EDTA (Sigma-Aldrich). The cell concentration was adjusted by cell counting using a hemocytometer (Carl Roth) and diluted with culture media. The cell suspension was centrifuged at 1200 rpm for 5 min to get a cell pellet. Cell culture medium was carefully removed. The remaining cell pellet was soaked up with a pipette and added to the hydrogel precursor solution. The total cell concentration was set to 2 million cells per ml ADA-GEL. The ratio of ST2 to RAW in the co-culture approach was set to 100:1. The incubation time of the experiments was usually set to 21 days as in this time frame the proliferation phase as well as the early stages of differentiation take place for osteoblasts [226]. Considering this fact, the studies concerning the osteogenic and osteoclastogenic differentiation were performed at the end of the incubation period, namely after 21 days.

Planar structures of PCL, PCL-PEG 8020 and PCL-PEG 7030 were plotted and seeded with ST2 cells in a concentration of 100000 cells/mL MEM alpha cell culture medium and incubated for a period of 21 days.

3.5.2 Analytical methods

Metabolic activity (alamarBlue)

Metabolic activity was measured through the reduction of resazurin (alamarBlue assay, Invitrogen, USA) to resorufin inside the viable cells after 1, 7, 14, 21, 28 days. The scaffolds were washed in Hank’s balanced salt solution (HBSS) (Sigma-Aldrich) before and after the incubation in the alamarBlue solution. A solution of 10% alamarBlue in DMEM was added in each well and the plates were incubated for 4 h. The absorbance at 570nm and 600nm was measured using a spectrophotometer (Specord 40, Analytik Jena, Germany). The percentage reduction of resazurin was calculated according to the manufacturers’ protocol.
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Cell viability (WST-8)

Cell viability was investigated through the enzymatic conversion of tetrazolium salt (WST-8 assay, Sigma-Aldrich) to formazan. A volume of 1 ml of a solution of 1% WST-8 assay in cell culture medium was added to each sample, which were incubated for 4 h. The absorbance at 450 nm was measured with a plate reader (type phOMO, Anthos Mikrosysteme GmbH, Krefeld, Germany). The same samples were measured during a specific study after different time points of the incubation phase.

For the study of ST2 cells seeded on PCL and PCL/PEG plates the incubation time with the WST-8 assay was reduced to 2 h.

Lactate dehydrogenase (LDH) activity

For LDH-activity analysis of cell lysates to determine the cell number, a commercially available LDH-activity quantification kit (TOX7, Sigma-Aldrich) was used. After 21 days of incubation samples were placed in a well-plate with Roswell Park Memorial Institute (RPMI, Life technologies) cell culture medium for 1 h at 37 °C under shaking conditions to dissolve the hydrogel. Afterwards remaining hydrogel pieces were manually destroyed by trituration. The dissolved hydrogel structures were centrifuged for 15 min at 3000 rpm. The cell culture medium supernatants were removed, 1 ml of lysis buffer added and the sample was vortexed. Then samples were incubated for 1 h in the lysis buffer. Lysates were centrifuged again at 3000 rpm for 15 min and 140 μl from the supernatant solutions was transferred to 1 mL cuvettes. 60 μl of master-mix (equal amounts of substrate solution, dye solution, and cofactor solution for LDH assay) was added to each cuvette and incubated for 30 min. The reaction was stopped with 300 μl 1 N HCl, and 500 μl of ultrapure water was added. The absorbance of each solution was measured at 490 nm and 690 nm using a UV-Vis spectrophotometer (Specord 40, Analytik Jena AG, Jena, Germany).

Alkaline phosphatase (ALP) activity

ALP expression was measured by an assay based on the change in absorbance of p-nitrophenolphosphate (pNpp) as it is enzymatically cleaved by ALP to p-nitrophenol (pNp). The lysates were centrifuged for 15 min at 3000 rpm. Then 250 μl of the supernatant was transferred to a cuvette and 100 μl of a 2.36 mg/ml pNpp solution
was added and incubated at 37 °C for 44 min. The reaction was stopped by adding 650 μl 1 M NaOH solution and the absorbance was measured at 405 nm by UV-Vis spectrophotometer. Bradford test (AppliChem GmbH, Germany) was used to determine the total protein content (Appendix Figure A 2: Bradford-test regarding Chapter 6 (Section 6.4.3)) of the samples containing the immobilised cells. The cell lysates were vortexed and 25 μl of the supernatants were mixed with 975 μl Bradford protein assay kit. After 10 min incubation in the dark, the absorbance was measured at 595 nm by UV-Vis spectrophotometer. The specific ALP activity was calculated from the ALP measurement and the Bradford test. It is presented as the activity, which hydrolysed a certain amount of nano molar para-nitrophenylphosphate (pNPP) per minute and mg of total protein.

**Osteopontin (OPN) quantification**

For determining the OPN expression of the immobilised cells, the cell culture supernatant after 21 days of incubation was used. A mouse OPN Elisa Kit (RayBiotech, Inc., Norcross, USA) was used by following the manufacturer protocol including a standard curve for the quantification of the OPN release. The optical densities were measured at 405 nm with a plate reader.

**Tartrate resistant acid phosphatase (TRAP) activity**

TRAP-activity was measured by the conversion induced by enzymatic hydrolysis of pNpp to pNp. 50 μl of cell lysate was pipetted into a 96 well-plate according to the study of Janckila et al [227]. An acetate-tartrate-buffer (pH = 6.1) was prepared with concentrations of 100 mM and 50 mM of sodium acetate and sodium tartrate, respectively. 7.6 mM pNpp acetate-tartrate-buffer solution was prepared and 150 μl was pipetted into each well. After incubation for 28 h at 37 °C the reaction was quenched by the addition of 50 μl 0.1 M sodium hydroxide solution. The absorbance at 405 nm was measured with a plate reader.

**TRAP staining**

A TRAP staining was done with the samples of the co-culture and the single RAW group after 21 days of incubation. An acid phosphatase kit (Sigma-Aldrich) was used. Briefly, 0.1 ml Fast Garnet GBC-Base solution was mixed with 0.1 ml sodium nitrite solution and kept in the dark for 2 min. Pre-warmed (37 °C) deionized water was
mixed with prepared 0.1 ml diazotized Fast Garnet GBC solution, 0.05 ml naphthol AS-BI phosphoric acid solution, 0.2 ml acetate solution and 0.1 ml tartrate solution. 1 ml of this mixture was added to each sample and incubated at 37°C for 1.25 h in the dark. Samples were washed with HBSS (Sigma-Aldrich) and dried at room temperature.

**Light microscopy**

During the whole incubation period of the ADA-GEL/cell constructs, cell distribution and morphology in the hydrogels were observed by light microscopy (Primo Vert, Carl Zeiss, Germany) to evaluate the development over time.

**Fluorescence microscopy**

The cell viability and morphology were revealed by calcein (Life Technologies, Germany) and the dead cells by propidium iodide (Life Technologies) staining after different time points of incubation in a plotted scaffold. The scaffolds were placed in a single Petri dish filled with 1.5 ml of HBSS (Sigma-Aldrich) mixed with 6 μl of calcein and 7.5 μl of propidium iodide and incubated for 30 min in the dark. The scaffolds were then placed in a fixing solution for 5 min. The fixing solution was prepared by adding 6.048 g piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPEC) (Merck, Germany), 0.076 g ethylene glycol tetraacetic acid (EGTA) (Sigma-Aldrich), 8 g polyethylene glycol (PEG) (Sigma-Aldrich) and 7.4 g paraformaldehyde (Sigma-Aldrich) to 200 ml HBSS (Sigma-Aldrich). The pH of the solution was adjusted to 7.4 with NaOH-pellets (VWR). Fluorescence images were taken using a fluorescence microscope (FM, Scope.A1, Carl Zeiss). For the study with ST2 cells in different ADA-GEL concentrations fluorescence microscope from KEYENCE (type BZ9000, Neu-Isenburg, Germany) was used. Automatic cell counting in the fluorescence images was performed using the ImageJ software (open source Java image processing program). In case of images taken with the Keyence microscope, BZ-II Analyzer (KEYENCE, Neu-Isenburg, Germany) was used.

For staining the actin filaments of cells and the cell nuclei phalloidin (red) (Life Technologies) and sytox (green) (Life Technologies) were used, respectively. The scaffolds were placed in a petri dish and washed with HBSS. Then cells were fixed, washed with HBSS and incubated in a permeabilization buffer (Triton-X 100) for 5 min. The fixing solution was prepared by adding 6.048 g piperazine-N,N′-bis(2-
ethanesulfonic acid) (Pipes) (Merck, Germany), 0.076 g ethylene glycol tetraacetic acid (EGTA) (Sigma-Aldrich), 8 g polyethylene glycol (PEG) (Sigma-Aldrich) and 7.4 g paraformaldehyde (Sigma-Aldrich) to 200 ml HBSS. The pH of the solution was adjusted to 7.4 with NaOH-pellets (VWR). After this treatment scaffolds were exposed to a solution of 1 ml HBSS and 8 µl phalloidin for 1 h in the dark, followed by a washing step with HBSS and incubated in a solution of 1 ml HBSS and 1 µl sytox for another 5 min. Fluorescence images were taken using a fluorescence microscope (FM, Scope.A1, Carl Zeiss) and confocal laser scanning microscope (CLSM) (DM6000, CFS, Leica, Germany).

For investigating cell morphology and distribution, cells were labelled with Vybrant (Dil) (Life Technologies) by adding 5 µL to 1 mL cell culture media and incubating for 30 min at 37 °C. Afterwards samples were washed three times with PBS. Fluorescence fixing solution was added for 15 min. For staining the cell nucleus DAPI was used with a concentration of 1 µL per mL cell culture medium. Fluorescence microscope (FM, Scope.A1, Carl Zeiss, Oberkochen, Germany) was used for imaging.

Scanning electron microscopy

For the SEM investigation ADA-GEL samples with immobilised cells were fixed with a solution containing 3 % (v/v) glutaraldehyde (Sigma, Germany) and 3 % (v/v) paraformaldehyde (Sigma) in 0.2 M sodium cacodylate buffer (pH 7.4) after they have been rinsed three times with HBSS. In a next step, the samples were incubated in a diluted ethanol series starting from a concentration of 30 % up to 99.8 %. Afterwards the samples were critical point dried (EM CPD300, Leica). Then scanning electron microscopy (SEM) was carried out (Auriga CrossBeam, Carl Zeiss Microscopy GmbH, Germany).

VEGF Release

MG-63 cells

For investigating the VEGF-A release from the immobilised cells, the VEGF concentration in the surrounding cell culture medium was measured for each 4 days period during the 28 days incubation time. This means the VEGF expressed in the period from day 3 to 7, day 10 to 14, day 17 to 21 and day 24 to 28 was analysed using a human VEGF Elisa Kit (RayBiotech; Norcross, USA) by following the
manufacturers’ protocol including a standard curve for the quantification of VEGF release. For the optical density measurements at 450nm a plate reader was used (type phOMO, Anthos Mikrosysteme GmbH).

**ST2 and RAW cells**

For investigating the VEGF-A release of the immobilised cells, the VEGF concentration in the surrounding cell culture medium was measured. The last medium exchange was at day 18 of the incubation period. Thus, the final VEGF release during the period between day 18 and day 21 was analyzed. A mouse VEGF Elisa Kit (RayBiotech, Inc.) was used by following the manufacturer protocol including a standard curve for the quantification of VEGF release. For the optical density measurements at 450 nm a plate reader was used.

3.6 **Statistical analysis**

**MG-63 cell studies**

For statistical analysis of the differences of the metabolic activity, the one-way analysis of variance (ANOVA) was used, which is implemented in SPSS statistics 22 software (IBM). The number of samples was N=3 for the metabolic activity and N=4 for the mitochondrial activity studies as well as for the VEGF measurements. The significance level was set as p<0.05=*, p<0.01=** and p<0.001=***. For the comparison of the mean values the Games–Howell test was used.

**ST2 and RAW cell studies**

For statistical analysis of mean value differences, the one-way analysis of variance (ANOVA) or Student’s t-test were used, which are implemented in Origin 9.0 G (OriginLab Corporation, Northampton, USA) software. The significance level was set as p < 0.05= *, p < 0.01= ** and p < 0.001= ***. For the comparison of the mean values with ANOVA the Tukey post-hoc test was used. Student’s t-test was applied in cases for comparing only two independent samples.

**Mechanical testing**

For statistical analysis of mean value differences, regarding the mechanical testing of the PCL and PCL-PEG scaffolds, the one-way analysis of variance (ANOVA) was used,
which is implemented in Origin 9.0 G (OriginLab Corporation, Northampton, USA) software. For the comparison of the mean values with ANOVA the Tukey post-hoc test was used.
4 Hydrogel characterisation and process development

4.1 Introduction

In this part of the study, the rheological characterisation of the ADA-GEL system was carried out, starting with the precursor solutions ADA and GEL. The effect of the oxidation process on the viscosity of ADA was investigated in comparison to pure ALG. Moreover, the gelling time of the ADA-GEL system was evaluated with regard to its use for the bioplotting process. Different bioplotting strategies were assessed in connection to the shape fidelity of the deposited hydrogels and thus to the geometry of the fabricated constructs. Furthermore, the mechanical properties of the ADA-GEL system were investigated as well as its degradation behaviour during incubation times of several weeks. Thus, these investigations are the basis for the interpretation of the behaviour of different cell types in the ADA-GEL matrix, which is investigated in the Chapters 5 and 6. Parts of this chapter contain results and discussion published in reference [33].

4.2 Rheological characterisation of ADA-GEL precursor solutions

In this section, the rheological properties of the ADA-GEL precursor solutions ADA and GEL as well as the ADA-GEL system before the ionic gelation are discussed. The rheological properties of the solutions determine their processability and the shape fidelity of the bioplotted structures [9]. In addition the dynamic covalent crosslinking process of ADA and GEL has to be considered with regard to the processing window of the bioplotting procedure [22].

4.2.1 Oxidation process and molecular weight

The oxidation process using sodium metaperiodate results in a decreased molar mass of the ADA in comparison to the original alginate. This decrease is dependent on the amount of the oxidising agent [27],[228],[229]. The oxidation reaction leads to scission at the vicinal glycols in the alginate chains, preferentially at the G-units [27],[229]. As two different batches of alginate (different Lot-numbers) were used in this work, the influence of the oxidation process on the molar mass depending on the
original alginate (using comparable values for batch 1 from Sarker et al [178]) as well as on the used amount of sodium metaperiodate was evaluated. The intrinsic viscosity and the molar mass of hydrogels were determined using an Ubbelohde viscosimeter as described in Chapter 3 (Section 3.2.1). Table 3 shows the intrinsic viscosity and the molar mass of the original alginates in comparison to ADA prepared with different periodate equivalents.

Table 3: Intrinsic viscosity and calculated molar mass of ALG and ADA of different batches and varied periodate equivalents.

<table>
<thead>
<tr>
<th>alginate – batch</th>
<th>periodate equivalent [wt%]</th>
<th>intrinsic viscosity [dL/g]</th>
<th>molar mass [kDa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 0</td>
<td>8.5</td>
<td>422 [178]</td>
<td></td>
</tr>
<tr>
<td>2 0</td>
<td>7.5</td>
<td>375</td>
<td></td>
</tr>
<tr>
<td>1 32.1</td>
<td>3.7</td>
<td>186 [178]</td>
<td></td>
</tr>
<tr>
<td>2 32.1</td>
<td>2.3</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>2 26.7</td>
<td>2.4</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>2 21.6</td>
<td>3.7</td>
<td>185</td>
<td></td>
</tr>
</tbody>
</table>

The molar mass of the two alginate batches was slightly different with values of 422 kDa [178] and 375 kDa. Using these alginate batches for oxidation with a periodate equivalent of 32.1 % resulted in ADA with a molar mass of 186 kDa and 115 kDa, respectively. Thus, the difference in the molar mass of the original alginate implicated different values of the molar mass of ADA oxidised with the same periodate equivalent. Decreasing the periodate equivalent to 21.6 % led to an increased molar mass of up to 185 kDa for the alginate-batch 2. This value was in the same range of ADA derived from alginate-batch 1, but with a periodate equivalent of 32.1 %. The molar mass and the intrinsic viscosity of ADA prepared by using a periodate equivalent of 26.7 % and 32.1 % was in a close range in comparison to the one with a lower periodate equivalent of 21.6 %. Gomez et al [229] showed that the interaction of the intrinsic viscosity and the degree of oxidation is not linear, but it follows an exponential relationship. The degree of oxidation is dependent on the periodate equivalent and the reaction time, whereas an increase of these parameters decreases the intrinsic viscosity and the molecular weight. If the degree of oxidation is overstepping a certain point the alginate loses its ability for ionic gelation as the
number of G-units is reduced. Thus, the interaction with the calcium ions is limited. Processes implying ionic gelation are working with ADA of lower degree of oxidation. The ADA used by Sarker et al [29] for the fabrication of microcapsules had an oxidation degree of 33 % using a periodate equivalent of 32.1 %. The microcapsule fabrication includes an ionic gelation reaction. ADA-GEL, which is extruded through a micro-capillary, drops in a beaker filled with 0.1 M CaCl\(_2\) solution to form stable microcapsules. On the other hand Balakrishnan et al [28] developed an injectable ADA-GEL system for cartilage repair using ADA with an oxidation degree of 58 %. This system is based on the covalent crosslinking between ADA and GEL without any ionic gelation. Hence, the covalent crosslinking reaction is advanced by a high oxidation degree along with a high number of reactive aldehyde groups. Thus, as the bioplotting process also includes an ionic gelation step with Ca\(^{2+}\) ions, the extent of oxidation is an important parameter to ensure that there are enough G-units left, which are necessary for the egg-box formation and so for ionic gelation. Furthermore, the molar mass of the original ALG has to be considered regarding the reproducibility of the preparation of ADA with a certain molar mass.

### 4.2.2 Dynamic viscosity measurements

Besides the concentration, the molecular weight is a critical parameter regarding the viscosity of polymer solutions. Increased concentrations and molecular weights enhance the viscosity. High viscosities are favourable considering shape fidelity of the hydrogel after extrusion using bioplotting techniques [9]. In this study viscosity of different ADA solutions was measured using shear rheology. The viscosity of ADA solutions of different concentrations, prepared with a periodate equivalent of 32.1 %, in comparison to an alginate solution of 2 % (w/v) is presented in Figure 11. ADA with a periodate equivalent of 32.1 % was successfully applied to microcapsule fabrication out of ADA-GEL [29]. Moreover, encapsulated MG-63 cells showed a spread morphology and proliferation in this ADA-GEL matrix with a final ADA concentration of 2.5 %, whereas the reference group of MG-63 cells immobilised in a 2 % ALG or an ALG/GEL blend matrix, with a 2 % ALG concentration, showed an undesired round morphology [31].
Figure 11: Flow curves of alginate and ADA solutions with different concentrations showing the decreased viscosity of ADA in comparison to ALG and the concentration dependence of the viscosity of ADA solutions.

Alginate and ADA both with a concentration of 2 % (w/v) had at low shear rates viscosity values of 1.0 Pa*s and 0.05 Pa*s, respectively. Rezende et al [230] measured similar viscosities of 2 % alginate solutions, showing also shear thinning behaviour. The concentrations of alginate solutions in biofabrication approaches are usually in a range of 1.5 %(w/v) up to 3.0% (w/v) [61],[63],[231]. This range, described as the “traditional” biofabrication window, is a compromise between shape fidelity and cell response influenced by the alginate network density [9]. Higher alginate concentrations like 6 % (w/v) [114] or 16.7 % (w/w) [201] were used in approaches emphasizing structural purposes without cell immobilisation. The drop of the viscosity of alginate after the oxidation process could be explained by the decrease in the molar mass, as described in the previous section. Jia et al [232] also reported decreased viscosities depending on the oxidation degree and evaluated the oxidation process for optimising the viscosities of ADA solutions for ink-jet printing. It is seen that the viscosity of ADA was as expected dependent on the concentration. The viscosity of 5 %(w/v) ADA solution was in the range of the 2 %(w/v) alginate solution. The alginate and the ADA solutions show shear thinning behaviour [9]. Thus, the molecular weight of the ADA is still high enough to form entanglements causing shear thinning behaviour. Otherwise ideal viscous behaviour would be expected [233]. The decrease in the zero shear viscosity of ADA is unfavourable considering the shape
fidelity after the extrusion process. The viscosity could be increased as shown by enhancing the ADA concentration or by adding a second phase in our case GEL.

In Figure 12 the flow behaviour of GEL and ADA-GEL is shown, indicating shear thinning behaviour, similarly to the results on ADA shown in Figure 11. The ADA-GEL 5050 composition has shown beneficial results regarding immobilisation of MG-63 cells [30],[31]. A GEL solution with 2 % was chosen as a reference, as it is in the range of the GEL concentration in the ADA-GEL system.

![Figure 12: Flow curves of ADA-GEL 5050 and GEL (2 %) showing a similar behaviour for ADA-GEL and pure GEL at 25 °C.](image)

The flow curves of ADA-GEL and GEL showed a flow point [169]. The viscosity at a shear rate of 0.2 s\(^{-1}\) of ADA-GEL was around 20 Pa*s, which was higher than that of ADA of 2.5 % (w/v) concentration (around 0.1 Pa*s). The 2.5 % ADA concentration was comparable to the ADA concentration in the measured ADA-GEL 5050 system. This indicates that the GEL compensated the drop in the viscosity and even increased it in comparison to a 2 % (w/v) alginate solution. GEL exhibits of a temperature induced gel formation under 35 °C, which possibly explains the increase in the viscosity. Ouyang et al [234] measured increased viscosities for a blend consisting of 1 % ALG and 7.5 % GEL in comparison to pure ALG (1 %). The difference in viscosities decreased when the measurement temperature was increased to 37 °C and so exceeded the gel point of pure GEL. Furthermore, the crosslinking reaction between ADA and GEL leads to gel
formation and thus to higher viscosities [235]. Therefore, the GEL is an important component of the ADA-GEL system considering the plotting process.

For the fabrication of more complex structures with overhanging parts higher viscosities would be necessary. Müller et al [47] evaluated Pluronic F127 gels for plotting applications showing zero shear viscosities higher than 5000 Pa*s, providing good shape fidelity after plotting.

### 4.2.3 Time-sweep studies to evaluate the crosslinking time

The crosslinking time of ADA and GEL is of interest considering changes in the material properties during the bioplotting process, which may lead to a limited processing window if the hydrogel is not printable after a certain amount of time. The time of the covalent crosslinking between ADA and GEL both with a stock solution concentration of 5 % (w/v) and a 5050 ratio mixing composition, as this ADA-GEL composition showed promising results for cell immobilisation [30], was analysed. Time-sweep studies were performed, measuring the development of the storage modulus (G’) and the loss modulus (G’’) over time using a shear rheometer. The crossover point of G’ and G’’ marks the point of gel formation [236]. In Figure 13 a typical time-sweep study of ADA-GEL 5050 is shown.

![Figure 13: Gelation time obtained by an oscillation time-sweep study measuring storage modulus (G’) and loss moduli (G’’) of ADA-GEL 5050 under constant shear rate at 25°C.](image-url)
The crossover point could be detected after 4 min. As the preparation of the experiment took also 4 min, the crosslinking time for this hydrogel could be set around 8 min [29]. Test tube inverting method was used by Sarker et al [29] to analyse different mixing ratios of ADA and GEL besides the 5050 composition. The increase of GEL content decreased the crosslinking time in a range of 19 to 4 min using ratios of ADA-GEL 7030 to 3070 in 10 % steps. The crosslinking reaction is accelerated using higher concentrations of ADA and GEL or ADA with a higher degree of oxidation. Also the pH could influence the crosslinking reaction, as the use of borax along with an alkaline pH shortened the reaction [27],[235].

4.3 Evaluation of the bioplotting process

As a next step, the processability of the ADA-GEL system using a bioplotting system is evaluated. The influence of processing parameters like plotting speed and crosslinking time of the hydrogel was investigated. Furthermore, the geometry of the hydrogel constructs was evaluated. In a further step, different approaches for adjusting the shape stability were assessed.

4.3.1 Evaluation of plotting parameters

*Interaction of speed and pressure: influence on the strut width*

In Figure 14 the general dependency of the strut width on the plotting speed under constant pressure of 100 kPa using ADA-GEL 5050 is presented. The pressure was set to 100 kPa to ensure that also at high plotting speeds of 60 mm s⁻¹ the deposition of a regular strut is possible. High plotting speeds enable fast processing and so shorten the time of the cells being suspended in the hydrogel in the reservoir cartridge of the bioplotter without cell culture medium supply.
It is seen that with increasing plotting speed in the selected broad range between 10 and 60 mm s\(^{-1}\) the strut width decreased. The difference in the strut width values at 10 and 60 mm s\(^{-1}\) was more than double. It was also observed that the decrease in strut width in the plotting speed steps between 30 and 60 mm s\(^{-1}\) was lower as compared to the difference between 10 and 20 mm s\(^{-1}\). This result indicates that the plotting speed is an important parameter affecting the dimensions of the plotted structure and that the investigated plotting rates were higher than reported in literature. For example Fedorovich et al [237] used plotting speeds up to 30 mm s\(^{-1}\) and Song et al [238] plotted alginate (1%(w/v)) with 9.7 mm s\(^{-1}\), which was the speed limit of the used plotter. Indeed, the plotting speed must be considered in connection to the used pressure. The dependency of strut dimensions in high concentrated alginate solutions from the plotting pressure and speed was investigated by Luo et al [239]. A high plotting speed requires a higher pressure compared to the use of lower plotting speeds if the strut width should be kept constant. The pressure can be kept constant if a narrower strut can be tolerated. The pressure defines the flow rate of the hydrogel. Thus, a high speed requires an elevated flow of hydrogel, otherwise no constant deposition of material is possible. A too low flow rate results in single hydrogel dots instead of a constant strut. Tirella et al [55] stated that the use of high speeds and low pressures defines a processing window to fabricate alginate (4%(w/v)) scaffolds with high fidelity using a pressure-assisted microsyringe.
In this investigation, also grid-like structures were plotted. These structures were used to create test scaffold geometries. The creation of grid structures or meshes provides the possibility of cell immobilisation in a defined geometry to form scaffolds of tailored shape for improved vascularization. For vascularization a pore size >300 μm is proposed by Fedorovich et al [39]. In Figure 15 the pore center line length (as described in Section 3.4.1) is presented for the variation of the number of struts over a constant edge length of 15 mm.

![Figure 15: Pore center line length of grid-like two layered scaffolds in dependence of the number of struts on a constant edge length (= 15 mm) [33].](http://iopscience.iop.org/article/10.1088/1758-5090/7/2/025001)

From this study, it followed that by increasing the number of struts the pore size can be decreased from ~600 μm down to ~400 μm. These measurements were done on two layered structures. Plotting was carried out in a set of parallel lines at 90° to each other with an increase in the z-height. In Figure 16 light microscopy images of such a scaffold structure made of ADA-GEL 5050 (without cells) is shown. The image in Figure 16 (left) indicates that the struts of layer 1 and layer 2 were fused. The same behaviour has been observed when plotting blends of alginate and GEL [240]. At higher magnification in the image of Figure 16 (right), the texture of the hydrogel can be seen, indicating that the extrusion process during the plotting resulted in the alignment of ADA-GEL structure, what possibly could direct the orientation of immobilised cells.
Figure 16: Light microscope images of plotted ADA–GEL grid-like scaffolds as prepared. Overview image (left) and detailed spot image (right). The images give a representative impression of the plotted structures and the texture of the hydrogel [33]. ©IOP Publishing. Reproduced with Permission. All rights reserved. http://iopscience.iop.org/article/10.1088/1758-5090/7/2/025001

Influence of ADA-GEL crosslinking reaction on strut width

The chemical crosslinking between the ADA and the GEL solutions changes the characteristics of the hydrogel, as described earlier in Section 4.2.3. Thus, with ongoing crosslinking time the plotting solution becomes more gel-characteristics, meaning that the elastic modulus (G’) is increasing. Therefore, a process window was defined between 20 min and 60 min after the start of the crosslinking reaction. The beginning of the plotting after 20 min considers the time for the mixing of the ADA and GEL as well as the cells. Moreover, the hydrogel/cell mixture has to be poured into the cartridge, which has to be fixed in the plotting head. In steps of 5 min, as an arbitrary scale, the same geometry under constant conditions was plotted and the strut width was measured to evaluate if there was an effect of the crosslinking reaction on the plotted struts. It is seen in Figure 17 that there was no distinctive trend between the ongoing crosslinking time and the strut width in the defined processing window.
The strut width values were between 200 µm and 400 µm while using constant processing parameters. An explanation of this quite broad range of strut width values could be the inhomogeneity of the hydrogel or more obviously the changes in the distance between the plotting needle and the template. The struts were plotted directly on cell culture plates. This was beneficial considering the following maturation phase of the hydrogel/cell constructs. Nevertheless, the cell culture plates were not exactly plane so that the distance between needle and substrate surface changed. The correlation of this distance with the strut width is that with a decreasing distance the material is pressed on the template leading to broader struts. In case of an increasing distance, the needle and the material develop no contact to the substrate and no material is deposited at all [61].

Considering the time required for the preparation of the ADA-GEL, which was even increased when cells were involved, an extended processing window is beneficial for an economical process. As reported by Leite et al [24] the addition of bioactive glass particles to the ADA-GEL reduced the processing window to a range of 5 – 20 min. Hence, after this time frame new ADA-GEL had to be prepared and loaded into the cartridge.
4.3.2 Assessment of enhanced processing strategies

Besides modifying the bioink to adjust the shape stability of the extruded hydrogel struts, there are several approaches reported to adapt the process flow. The classical bioplotting concept defined by Landers et al [19] describes the plotting into a liquid with an ideally identical density as the plotting solution to make use of the buoyancy compensation of the liquid to prevent the floating of the plotted struts and overcome the gravitation forces. Rajaram et al [141] plotted into a solution containing CaCl$_2$ to start immediate gelation after deposition and additionally polyvinyl alcohol to prevent the floating of the deposited struts. The use of cooled templates, when plotting thermosensible hydrogels like GEL, was also reported [116]. In another attempt the deposition of a cell/hydrogel mixture was done directly into a self-healing support hydrogel matrix based on guest-host complexes offering reversible crosslinking of the gels [242]. All these approaches give the possibility of plotting scaffolds with an increased z-height and improved geometry with changing the properties of the cell containing hydrogel. However, there are also several problems like clogging of the needle when the gelling solution diffuses in the plotting solution during the extrusion. Thus, for the processing of alginate and the immediate crosslinking with CaCl$_2$, Ahn et al [243] developed a humidifier constantly fuming CaCl$_2$ on the already deposited struts. Moreover, coaxial needles were used for plotting the hydrogel in the inner core and gelation solution through the outer ring. So immediately at the moment of the deposition the hydrogel gets gelled from the outside and the shape stability is enhanced [13].

In Figure 18 images of ADA-GEL constructs are shown. These were fabricated using two different gelation strategies with CaCl$_2$ solutions. Gelling after the processing a) and gelling integrated in the plotting process b) by simultaneous plotting of the ADA-GEL and the CaCl$_2$ solution. Therefore, an additional cartridge was filled with CaCl$_2$ and the solution was dropped in between the deposited ADA-GEL struts, causing immediate crosslinking and prevents the struts from floating.
Figure 18: Light microscope images of plotted ADA-GEL scaffolds of different gelling concepts with CaCl\(_2\). Gelling after the processing a) and gelling integrated in the plotting process by simultaneous plotting of the ADA-GEL and the CaCl\(_2\) solution b).

Image a) indicates that the struts of layer 1 and layer 2 are fused and so build up more like a one layered structure. The same behaviour was observed by plotting blends of alginate and GEL by Detsch et al [240]. In contrast to this structure, in image b) a two layered construct is shown, where the single struts are still visible and the shape of the pores is cubic and not round. Besides this improvement regarding the shape fidelity, immediate gelation after the deposition, inhibits the connection of the struts of layer 1 and layer 2, as the gelation potential of the ADA chains seems to be already saturated. Thus, the constructs were unstable and lost their grid structure during handling.

In another approach investigated here, the CaCl\(_2\) solution was replaced with GEL. The concept was that the GEL acts as a sacrificial phase, which could be removed while incubating the constructs at 37 °C exposed to CaCl\(_2\) solution. In Figure 19 images at different processing stages of these constructs are presented.
Figure 19: Light microscopy images of hydrogel constructs processed using GEL as a sacrificial phase. One layer (a, b) and two layers (c, d) pattern of ADA-GEL and GEL after the processing and a two layer pattern after incubation phase at 37 °C (e, f).

In Figure 19a,b one layer and in Figure 19c,d two layers of the hydrogel constructs are shown before the incubation at 37 °C with the two phases remaining still intact. The phase boundaries were clearly defined. The images showing the second layer indicate that there was no fusion of layer 2 with layer 1. In Figure 19e, f images of a construct after the incubation at 37 °C are shown. It was visible that the GEL phase is leached out and that the ADA-GEL phase was still intact. Despite this positive result, again the constructs were not very stable, caused by a limited connection between layer 1 and 2 in some intersection point of the construct. The use of GEL as a sacrificial phase for the creation of complex channels in alginate constructs was also reported by Wüst et al [64]. In this study, massive block structures instead of grid-like structures were fabricated.
Considering the limited stability and the increased processing time using the integrated CaCl₂ gelation approach or the GEL spacer approach, the processing with CaCl₂ gelation after the plotting process was used to evaluate cell behaviour in ADA-GEL constructs. To adjust the ADA-GEL constructs for potential use in bone TE approaches requiring enhanced mechanical properties a sequential bioplotting process was chosen. Therefore, ADA-GEL was combined with a thermoplastic phase acting as a mechanical support as well as a mould considering the shape stability (presented in Chapter 7).

### 4.4 Characterisation of the ADA-GEL hydrogel

In the following section studies on different modifications of the ADA-GEL system, after the ionic gelation with CaCl₂ solution, are presented regarding their mechanical, morphological and degradation properties. The named properties are known to have a major impact on the behaviour of immobilised cells in hydrogels [16],[44].

#### 4.4.1 Mechanical analysis

Nanoindentation technique was applied to investigate the influence of single components and crosslinking mechanisms of the ADA-GEL system on its mechanical properties. Therefore, ADA and GEL as well as ADA-GEL samples of different concentrations were prepared and measured at different preparation steps (with and without (w/o) CaCl₂ gelation) as well as measurement conditions (room temperature (RT) and 37 °C). In Figure 20 the reduced Young’s moduli (rYm) in dependence of the above listed samples and parameters are presented.
The rYm of ADA after CaCl₂ gelation was enhanced from around 15 kPa to around 68 kPa by increasing the concentration of the ADA solution. The increasing rYm values indicate that the oxidation process was only partially implemented, considering that the oxidation process results in a scission of the uronate groups, which are necessary for the ionic gelation [25]. Nevertheless, the used ADA still had enough intact uronate groups enabling the ionic gelation known from alginate. Alginate, even with a lower concentration of the precursor solution of only 2 % (w/v) has a rYm of 33 kPa [179]. Thus, the oxidation process reduced the efficiency of the ionic gelation mechanism leading to a weakened hydrogel network [179]. Besides the mentioned oxidation of the uronate groups, also the reduced molecular weight of ADA (as shown in Section 4.1.1) could be the cause for the reduced mechanical stability [25]. The reduced molecular weight possibly reduced the physical entanglements of the alginate chains.

The results of pure GEL samples indicated an increasing trend with higher concentrations. The rYm values of less than 5 kPa were lower for all evaluated concentrations in comparison to the ADA samples. The ionic gelation process of ADA and thermally induced gelation of GEL are competing reactions [176]. Thus, ADA-GEL samples without CaCl₂ gelation were measured to evaluate if the presence of ADA
and so the crosslinking process between ADA and GEL over the Schiff’s base formation could hinder GEL network formation [29]. GEL monomer interactions leading to gel formation are based on hydroxyl- and amino-groups forming hydrogen bonds. These sensitive groups could be blocked or getting masked as reported for methacrylation of GEL using methacrylic acid anhydride [244]. Thus, ADA interacting with the amino-groups of GEL [29] could have a similar effect. The results corroborate this hypothesis. All assessed concentrations of ADA-GEL w/o CaCl$_2$ gelation had lower rYm values than pure GEL.

In a next step, the significance of the ionic gelation of the ADA-GEL system should be evaluated. The rYm of ionically gelled ADA-GEL samples is significantly increased for all modifications in comparison to the untreated ones. Thus, ionic gelation is the dominating mechanism to adjust the rYm of the ADA-GEL hydrogel system.

The stiffness values of the ADA-GEL measured at RT are relevant considering the handling of the samples after the processing step. Referring to the maturation phase of the constructs for tissue development the measurements at 37 °C are more relevant considering realistic in-vivo conditions. The rYm values of ionically gelled ADA-GEL dropped down slightly at 37 °C as GEL is a thermo-reversible gel, switching from gel to liquid state at this temperature [176].

Nevertheless, the ionic gelation of the ADA network in the ADA-GEL stabilises the system at 37 °C [176]. Interestingly, the rYm values of the ionically gelled ADA-GEL samples increased from a relative concentration of ADA and GEL of 2.5 %(w/v) to 3.75 %(w/v), but balanced for a concentration of 5.0 %(w/v). This effect could be possibly explained by the already mentioned competition between the GEL network formation and covalent crosslinking reaction and the influence on the ionic gelation mechanism of ADA. Higher stock solution concentrations of ADA and GEL decrease the crosslinking time between them [27] and so possibly suppress the ionic gelation mechanism [179]. It could be that an accelerated network formation is hindering the ionic gelation of ADA and so no further increase of the rYm is possible by enhancing the ADA-GEL concentration from 3.75 % to 5.0 %. Decreased mechanical properties of scaffolds consisting of highly concentrated alginate solutions blended with polyvinylalcohol (PVA) were reported by Luo et al [202]. The blending seemed to
disrupt the physical entanglements of the alginate chains as well as to hinder the ionic gelation with CaCl$_2$ ions.

The present study shows that the stiffness of the ADA-GEL system with its different modifications matches that of many soft tissues, ranging from 0.1 kPa to 100 kPa. In this context, it could be especially interesting for engineering cell microenvironments for adipose (~3 kPa), muscle (~9-15 kPa), cartilage (~20 kPa) and pre-calcified bone tissue (~30-40 kPa) [245].

### 4.4.2 Morphological analysis

SEM was used to analyse the morphology of ADA-GEL. In Figure 21 SEM images of the surface of a hydrogel construct are shown. The sample was plotted of ADA-GEL with a relative concentration of 3.75 %(w/v) of the ADA and GEL component.

![SEM images of the surface of a hydrogel construct](image)

**Figure 21**: SEM images of the surface a single strut of an ADA-GEL construct consisting of ADA-GEL with a relative concentration of 3.75 %(w/v) of ADA and GEL. a) Overview image of a twisted strut and b),c) detailed spot images of the border region between lower (in contact to the substrate during processing) and upper (in contact to air during processing) surface of the strut.

The upper part of a plotted strut shows a different morphology than the lower part. This could be explained by the ionic gelation of the hydrogel construct. While the construct was fabricated, the lower part was fixed on the tissue culture plate, which was used as a substrate. The upper part was exposed to air before the CaCl$_2$ solution was added and covered the structure. During the ionic gelation, the upper part was immediately in contact with the solution and the gelation reaction started. The excess to the lower part of the constructs was hindered. As a result, the upper part showed a dense ruffled surface and the lower part a more open porous structure. The inhomogeneous, ruffled surface morphology was also reported for microcapsules fabricated from ADA-GEL [29]. The difference in the surface morphology was not described, as the microcapsules are exposed to CaCl$_2$ solution from all sides during
the gelation, possibly avoiding the formation of a diffusion gradient. In Figure 22 cross-section images at different magnifications of an ADA-GEL construct are shown.

![Figure 22: SEM images of the cross-section of a single strut of an ADA-GEL construct consisting of ADA-GEL with a relative concentration of 3.75 %(w/v) of ADA and GEL. a) Overview image of a broken strut and b),c),d) detailed spot images of the porous inner strut morphology at different magnifications.](image)

In Figure 22a an overview image of a broken strut is shown. In contrast to the dense surface of the upper part of the construct, the inner structure shows a porous morphology, which is favourable for the immobilised cells. In Figure 22c the very thin, dense surface is visible, in the lower half of the image, which changes into the porous bulk morphology. A heterogeneous distribution of the alginate concentration, decreasing from the surface to the inner part of alginate capsules gelled with different ions with varying concentrations was reported by Mørch et al [150]. Thus, possibly an increased concentration of ADA-GEL at the surface of the struts caused the dense outer layer observed in the present study.

Considering migrating cells, the pore size distribution is a significant parameter, enabling or inhibiting cell migration. The SEM images can not be used to analyse the pore size as the preparation via critical point drying caused a significant, undefined shrinkage of the hydrogel network [246]. For ADA-GEL with 5%(w/v) stock solution concentration cryo-SEM analysis was performed [247]. The cryo-step inhibits the hydrogel network from collapsing, while enabling SEM observation. This investigation
revealed pore sizes of the ADA-GEL surface of 20 - 1600 nm. Hydrogel networks made of pure alginate have pore sizes of around 5 - 20 nm [248],[249]. The pore size distribution is very heterogeneous and so also pore sizes up to 200 nm were reported [155],[250]. It is hypothesized that the ADA-GEL has also pores in the narrow nm range, which could not be imaged as the samples were sputtered. Thus, the ADA-GEL seems to have a hierarchical pore size distribution [247].

4.4.3 Degradation behaviour

When developing hydrogels for TE, encompassing cell immobilisation, the control of the mechanical properties and the degradation kinetics of these materials is significant for tissue formation [251]. The forming free voids in the degrading hydrogel network could be filled with ECM, expressed by the immobilised cells, gradually replacing the hydrogel matrix and so building up new tissue [21],[252]. The ADA-GEL hydrogel system was shown to partially release the un-crosslinked GEL [178], leading to an amended cellular behaviour in comparison to pure alginate [31]. ADA is sensible to hydrolytic degradation [25]. Thus, the ADA-GEL possesses two material based degradation mechanisms, which could be adjusted to control the degradation kinetics and so the mechanical properties over time. This aspect makes it a valuable material system [253]. Engineering these spatiotemporal aspects, which influence cell response, is challenging [16]. Considering this issue, different modifications of the ADA-GEL system are investigated in this section regarding their GEL release behaviour and their mechanical properties in dependence of the incubation time.

GEL release

The GEL release has been measured for ADA-GEL modifications with different stock solution concentrations of 5 %, 7.5 % and 10 % of ADA and GEL, resulting in relative concentrations of ADA-GEL of 2.5 %, 3.75 % and 5.0 % after mixing in a 1:1 ratio. The cumulative protein release of these ADA-GEL modifications over an incubation period of up to 35 days is shown in Figure 23.
Figure 23: Cumulative protein release in dependence of the incubation time in HBSS from bioplagotted ADA-GEL constructs of different concentrations.

All three ADA-GEL modifications showed a similar release kinetic, as there was a burst release in the first hours of the incubation period. Nevertheless, the percentage of the protein release was extenuated with an increasing ADA-GEL concentration from 2.5 % up to 5.0 %. The fast release in the beginning could be explained by the release of the GEL component, which is not covalently crosslinked with the ADA [178]. There is a distinct difference in the total percentage of released GEL. The ADA-GEL with 2.5 % concentration showed a GEL release of up to almost 50 %, whereas the release from ADA-GEL 3.75 % was around 15 % and that from 5.0 % ADA-GEL was slightly lower at around 13 %.

The following reasons could explain the differences in the GEL release. There are electrostatic interactions between the GEL and ADA. Furthermore, the molecular weight of the GEL could be too high, so that the diffusion controlled release process is hindered by the increased density of the hydrogel network, which is related to an increase in the concentration of the ADA component [41]. The diffusion of GEL through the ADA-GEL hydrogel network could be accelerated by its degradation, which is related to the hydrolysis of the ADA network. Furthermore, the GEL could degrade so that its molecular weight is reduced and the molecules could pass through
the pores of the ADA-GEL network. As reported by Sarker et al.[178] the released percentage of GEL was lower for ADA-GEL films containing higher amounts of GEL. This phenomenon might be explained by an increased extent of physical interactions due to high GEL content. The GEL renaturation is intramolecular at low concentrations, and becomes intermolecular at high concentration [254]. Thus, it is also possible that the GEL network is stabilising itself.

SDS page analysis was performed for clarifying if there is a difference in the molecular weight of the released GEL over time. This would encourage the mentioned theory of elevated GEL release by GEL degradation leading to enhanced diffusion rates with ongoing incubation time. Therefore, the supernatants of ADA-GEL 2.5 % and ADA-GEL 3.75 % were analysed, considering the distribution of the molecular weight of the released GEL molecules in comparison to a pure GEL solution. The results are presented in Figure 24.

**Figure 24:** SDS-PAGE results of protein released in the supernatant from bioplotted ADA-GEL 2.5 % and ADA-GEL 3.75 % constructs over an incubation time of 14 days. A pure GEL solution was used as a reference. The SDS Page gels indicate the protein patterns according to the molecular weight of the analysed samples.

The pure GEL showed a broad distribution of bands with values in the range of 40 kDa to 180 kDa. This result is concuring with previous measurements done by Sarker et al.[178]. The 2.5 % ADA-GEL composition showed a distribution of bands from 10 kDa to 180 kDa after 1 h of incubation. Even though GEL molecules with a high molecular
weight were released, there were low weight molecules especially for the time points 3 h and 5 h. After 8 h hardly any bands were visible. Thus, in comparison to the pure Gel solution the GEL released from the incubated constructs indicates molecules with a lower molecular weight, which is a sign for their degradation during incubation. This finding of the SDS page analysis is fitting with the protein release kinetic, which is presented in Figure 23, showing the highest release during the first 8 h of incubation. The 3.75 % ADA-GEL sample showed bands during the 24 h after the start of the incubation time. This outcome is also consistent with the protein release kinetic, which indicates the highest release during this time frame. The protein pattern of the 3.75 % ADA-GEL sample is similar to the 2.5 % ADA-GEL sample showing a broad range of bands from 10 kDa to 180 kDa. Thus, there is no strict distribution pattern of the molecular weight of released GEL molecules from low to high, considering ongoing incubation and hydrolysis of the ADA. Previous studies showed bands of released GEL from ADA-GEL samples (2.5 %) up to 7 days of incubation [178]. Nevertheless, the investigated samples had been films with a high bulk component. In this study plotted grid like patterns with a high surface area were investigated. Thus, the diffusion pathways were much shorter. This possibly explains the accelerated GEL release.

\textit{Degradation kinetic: Change of rYm over incubation time}

In addition to the GEL release analysis, the variation of the rYm over time was investigated. As the stiffness of the hydrogels is an important parameter regarding the cell development in the maturation phase [251]. The degradation kinetic of varied ADA-GEL compositions was investigated by monitoring the rYm during the incubation time. In one study, the ADA-GEL was modified by using ADA prepared with different periodate equivalents and therefore varied molecular weights (as shown in Section 4.2.1) in a 2.5 % ADA-GEL (Figure 25). Furthermore, an ADA-GEL series was evaluated, which was prepared with stock solution concentrations of ADA and GEL of 5 %, 7.5 % and 10 % (Figure 26), resulting in ADA-GEL compositions of 2.5 %, 3.75 % and 5.0 %.
In Figure 25 it is shown that the $rY_m$ of all three ADA-GEL modifications decreased at 37°C in comparison to RT at the beginning of the incubation period. This could be explained with the change from gel to liquid state of GEL as already mentioned in Section 4.4.1. Interestingly, the ADA-GEL made of ADA, which was prepared using a periodate equivalent of 32.1 %, indicates the highest percentage decrease of the $rY_m$ (around 50 %). It was shown in Section 4.4.1 that the ionic gelation mechanism, which is based on the ADA component, is the dominant factor regarding the mechanical properties. Possibly this mechanism is hindered by a reduced molecular weight of the ADA chains, which is reduced with increasing periodate equivalents, and so $rY_m$ is lower. Moreover, a lower amount of unreacted G-units, which are necessary for the ionic gelation, in comparison to the ADA synthesized with lower periodate equivalents, is likely the reason for the lower values of $rY_m$ [229]. The decrease of the periodate equivalent from 32.1 % to 21.6 % causes an increase of the absolute values of $rY_m$ from 4 kPa to 18 kPa measured at day 0 of the incubation period at RT. Hence, it could be stated that the ionic gelation of the ADA in the ADA-GEL system is hindered or less effective using ADA prepared with a higher periodate equivalent. After three days of incubation all three modifications of the ADA-GEL showed an almost equal percentage reduction of the $rY_m$ of around 90 %. This extensive loss in
the mechanical properties could be related to the release of 50% of the GEL component after three days of incubation, as shown in Figure 23. Moreover, ADA is sensible to hydrolysis leading to chain scissions, which could cause a weakening of the ADA network. It was shown by Boontheekul et al [253] that the degradation rate of ADA depends on the degree of oxidation and on the molecular weight. An increased oxidation degree could accelerate the degradation behaviour. In their study ADA hydrogels with a bimodal molecular weight distribution were used to adjust the degradation behaviour. A further degradation mechanism is the dissociation of the calcium ions, whereas in the case of ADA networks, the hydrolytic chain scission is the dominant factor [253]. In this context also the surrounding medium and its ionic composition has to be considered [255]. It could be further demonstrated that oxidised alginates with a higher degree of oxidation degrade faster also in combination with a photo-crosslinking process instead of ionic gelation [165].

At day 7 of the incubation period only the ADA-GEL modification synthesized with a periodate equivalent of 23.1% could be measured as the other two hydrogel compositions were beyond the resolution of the measurement device. This one composition could be measured also at day 14 and day 21 of the incubation period showing no further disintegration of the network and so constant values of around 500 Pa were determined. These results indicate that ADA-GEL compositions using ADA with a higher molecular weight seem to extend the long-term stability of the hydrogel.

As shown in Figure 20 (Section 4.4.1) the increase of the concentrations of the ADA and GEL stock solutions leads to an increase of the rYm of the resulting ADA-GEL being thus possible to tailor the mechanical properties of the ADA-GEL system for certain applications. The rYm could be adapted to reach the mechanical properties of certain tissues depending on the used cell model. Thus, degradation kinetics of different ADA-GEL concentrations were measured by evaluating the rYm over time, as presented in Figure 26. Based on the results of the previous study ADA synthesized with a periodate equivalent of 26.7% was used to guarantee a fast degradation of the ADA component. This fast degradation should be favourable for improved cell response [31],[165].
Even though the higher concentrations of 3.75 % and 5 % ADA-GEL have higher absolute values in comparison to 2.5 % ADA-GEL, they still show similar degradation behaviour. As discussed above, increase of temperature from RT to 37 °C, decreases the rYm. In this study, the percentage decrease of rYm of the 2.5 % ADA-GEL is with around 75% higher than that of the other two concentrations, which show a decrease of around 20 %. Analogous to the degradation study of ADA-GEL presented in Figure 25, after three days of incubation there is a massive percentage loss of the rYm for all concentrations in the range of 80 % to 90 %. This could be explained considering that even the higher concentrated ADA-GEL hydrogels (3.75 % and 5.0 %) showed a release of GEL of around 20 % in the first three days of incubation (as presented in Figure 23), which was lower as for the 2.5 % ADA-GEL with around 50 % but is still significant. After this rapid degradation phase a plateau is reached up to 14 days of incubation. Thus, considering the elevated absolute rYm values of ADA-GEL hydrogels with concentration of 3.75 % and 5 %, it is apparent that they keep higher absolute values during the incubation phase. This is possibly caused by a more stable ionic gelation of the ADA network, caused by a higher concentration of ADA chains, and thus a higher content of available G-units in the hydrogel network. Jeon et al [165] reported that the increase in the concentration of photo-crosslinked ADA could delay
the degradation process. Thus, similar to an increased molecular weight, also a higher concentration of ADA can improve the ionic gelation of the ADA-network in the ADA-GEL system. As a result, higher stiffness values could be maintained during the degradation process.

It had to be mentioned that the hydrogel was difficult to measure at the later time points of the incubation period, especially the 2.5 % concentration after three days. At some positions, the stiffness seemed to be out of the working range of the measuring method. Thus, the system appeared to be inhomogeneous showing parts of advanced degradation. These studies show that higher concentrated ADA-GEL modifications enable a better handability after processing, indicating a fast degradation kinetic in the maturation phase. Nevertheless, experiments with ST2 cells immobilised in ADA-GEL with a concentration of 3.75 % and 5.0 % (presented in Chapter 6) showed the ADA-GEL is not completely degrading in a period up to 6 weeks.

It could be shown that the mechanical properties and the degradation behaviour of the ADA-GEL system could be modified by the variation of the periodate equivalent or the concentrations of the stock solutions of ADA and GEL. This is an important outcome considering that they are decisive parameters dictating the behaviour of immobilised cells [118]. In this way, the ADA-GEL system could be adapted to meet the requirements of each cell type. In the following chapters, the development of different cell types in different ADA-GEL modifications is presented.
5 Cell biology characterisation of hydrogel/cell constructs using MG-63 cells

5.1 Introduction

Biopotted cell-loaded ADA-GEL hydrogels were tested in vitro to determine the biocompatible character of the whole bioplotting process chain, which has been introduced in the previous chapter. Therefore, ADA-GEL (ADA from alginate batch 1 with periodate equivalent 32.1 %, 5 % (w/v) stock solution concentration) was mixed with MG-63 osteosarcoma cells and grid-like structures were plotted. This particular ADA-GEL composition has been used for the fabrication of microcapsules with immobilised MG-63 cells [30],[31]. The immobilised MG-63 cells have shown promising results regarding cell spreading and proliferation. The biopotted ADA-GEL/MG-63 cell constructs, which are investigated in this chapter, are interesting in the context of bone TE as the MG-63 cells are an established osteoblast cell model [225]. Furthermore, as already described in Chapter 2, the immobilisation of cancer cells in hydrogel systems is highly attractive, as there have been a growing number of publications using 3D cell culture approaches to establish in vitro cancer models as drug-testing systems [194]. Considering that MG-63 cells are derived from an osteosarcoma [256] the presented approach is potentially also relevant for this field of research. Parts of this chapter contain results and discussion published in reference [33].

5.2 Cell viability studies

In the following section, the influence of different process parameters on the cell viability was investigated. Furthermore, the long-time cell behaviour regarding cell metabolic activity as an indication of cell viability was assessed. The cell biology study involves the use of MG-63 cell culture as discussed in Chapter 3 (Section 3.5).

5.2.1 Live-dead staining

Live-dead staining method was used to determine the cell viability in dependence of the chosen process parameters and the whole bioplotting process chain in a short
time after the processing. In Figure 27 a-d fluorescence microscope images of a live/dead (green/red) staining of MG-63 osteoblast like cells immobilised in scaffold structures bioplotted with varied process parameters are shown. In Figure 27e a quantitative analysis of the fluorescence images is presented.

**Figure 27:** Representative fluorescence microscope images of live (green) and dead (red) staining of immobilised MG-63 cells in ADA-GEL matrix after the plotting process: a) 5 h of incubation, needle diameter 200 µm b) 5 h of incubation, needle diameter 400 µm c) 48 h of incubation, needle diameter 200 µm d) 48 h of incubation, needle diameter 400 µm e) Quantitative analysis of the fluorescence images: Percentage of live cells dependent on the needle diameter (200 µm and 400 µm) and the incubation time (5 h and 48 h).
The percentage of live cells in relation to the total cell number as well as in dependence of the used needle diameter and for a variation in plotting pressure, at incubation times of 5 h and 48 h is shown. The needle diameters of 200 µm and 400 µm were chosen as they both enable a high resolution in the hundreds of µm range, which is typical for the bioplotting process [6]. The high percentage of live cells in the representative images in Figure 27 a-d indicated that the plotting process and the used parameters were compatible with the used cell-line MG-63. The plotting parameters, i.e.: needle diameter and shape (conical/cylindrical), plotting speed and thus pressure, as well as the shear forces in the needle, influence the cell viability [61],[62]. In Figure 27 b high percentages over 90 % of live cells with no significant differences are shown for the two investigated needle diameters and time points. The change of the needle diameter from 200 µm to 400 µm led to a decrease in the pressure from 85 kPa to 40 kPa to guarantee an open pore grid-structure, while compensating the flow rate. Thus, in our case the range of variation of both pressure and needle diameter causes no difference on the cell viability. In a study of Chang et al [63] cell viability increases, when the pressure decreases and/or the needle diameter increases. Moreover, the decrease of the pressure was reported to have a more significant effect in comparison to the increase of the needle diameter. It is important to consider that the cells are dispensed in the hydrogel and so they are protected from the direct impact of shear forces. For example, experiments on endothelial cells have shown that their viability is not influenced by six times higher shear forces (up to 1150 kPa) during plotting in comparison to the force needed to detach a cell from a surface [9]. Thus, also the rheological properties of the hydrogel influence the cell viability [63]. Using a needle of 200 µm diameter and cylindrical shape and a pressure of 100 kPa, Billiet et al [62] calculated shear stresses of maximum 400 Pa at the needle wall using finite element modelling. Cell viabilities around 90 % for HepG2 cells were found. Fedorovich et al [237] found no differences in cell viability of printed and unprinted samples of alginate containing bone marrow stromal cells. They reported high cell viabilities, namely up to 80 %, by performing a live dead staining and counting the number of live and dead cells immobilised in alginate 2 % (w/v). The evaluation of the cell viability after 5 h and 48 h was done to assess if a recovery of cells damaged by the influence of mechanical forces during the plotting process occurs, as it was reported by Chang et al [63], especially during the
first 24 h of the incubation phase. The mechanical forces could perforate the cell membrane, which makes it permeable for the dead staining dye. A recovery effect and thus a healing of the perforated membrane would lead to an increase of the cell viability from 5 h to 48 h. The intact cell membranes would not be permeable for the dead staining dye anymore and so the number of dead cells would be decreased. In the presented investigation, the cell viability did not increase from 5 h to 48 h and thus no recovery effect could be detected. The results of cell viability in the present study are in accordance with the outcomes typically reported for the bioplotting technique. This shows that the developed bioplotting process as well as the chosen process parameters and the bioink (ADA-GEL) are compatible with MG-63 cells during the first 48 h of incubation.

5.2.2 Metabolic assay kinetics

After proving the biocompatibility of the bioplotting process, in a next step the long-term development of immobilised MG-63 cells in bioplotted ADA-GEL constructs should be assessed during a maturation phase of up to 28 days. Two assays were used in combination with different microscopy techniques (presented in Section 5.3) to obtain information about cell viability, proliferation and morphology. In Figure 28 the percental reduction of resazurin to resorufin via the reduction reactions of metabolically active cells in dependence of the incubation time is presented. At day 1 after processing the metabolic activity is seen to increase and this rise continues to day 14. The increase in metabolic activity could be explained by the proliferation of the cells leading to a higher cell number and thus resulting in a higher metabolic activity. After day 14 there was a decrease of metabolic activity to day 21, but the activity at day 28 was at the same level than after 14 days of incubation. It seems that the highest level was reached after 14 days. A possible explanation for the balancing of the values in the incubation period of 14 days up to 28 days and the drop of the metabolic activity after 14 days could be the transition from the cell proliferation phase (day 1 until day 14) to a phase of cell differentiation [226].
Another assay (WST-8) was used, which is based on the enzymatic conversion of tetrazolium salt to formazan by the immobilised cells, to back up the results presented in Figure 28. The results of the WST-8 assay can be seen in Figure 29, showing the development of the mitochondrial activity of immobilised MG-63 cells over an incubation period of 28 days. The progression shown in Figure 29 indicates an increase of the assay signal from day 1 to day 14, followed by a plateau until day 28. Thus, the results of the two assays are consistent.
5.3 Cell morphologies and cell distribution

In this section, the cell morphology at different time points of the incubation period was analysed using different microscopy techniques. Imaging at different time points enables also an investigation of the cell distribution over time.

5.3.1 Light microscopy

To demonstrate cell development, in Figure 30 light microscope images of ADA–GEL constructs with immobilised MG-63 cells after 1 day (a,b), 7 days (c,d) and 14 days (e,f) of incubation are presented.

Figure 30: Light microscope images of plotted ADA–GEL grid-like scaffolds with immobilised MG-63 cells after different time points of the incubation phase: a,b 1 day, c,d 7 days and e,f 14 days.
The images in Figure 30 show the open pore structure of the constructs geometry as well as the cells, which are homogeneously distributed over the whole structure. At day 1 the cells have a round morphology, which changes to a more elongated and spread morphology at day 7 and day 14 of the incubation phase. Moreover, these images visualise that the cell density obviously increased over time. Thus, these images confirm the results from the metabolic activity assays shown in Figure 28 and Figure 29, which indicated a proliferation phase during the first 14 days of the maturation phase.

5.3.2 Fluorescence microscopy

In Figure 31a,b fluorescence microscope images at different magnifications of an ADA-GEL/MG-63 cells construct after 28 days of incubation are shown. An actin cytoskeleton staining of the cells was used, to visualise the cell-material interactions at the end of the incubation phase. The images clearly show that the cells are attached to the ADA-GEL and are able to spread during the period of incubation. Furthermore, the MG-63 cells are densely grown in the whole ADA-GEL construct and so concurring with the results of the investigations using the metabolic assays (presented in Section 5.2.2). The chosen hydrogel matrix should provide the cells the ability to spread as well as to migrate and so to form connections with neighbouring cells [118]. In contrast, Shim et al [97] published a study about bioplotting of MG-63 osteoblast-like cells in a 4% (w/v) alginate solution. Although the cell viability increased up to 7 days of incubation, the cells exhibited almost a round shaped morphology, which indicates that the cells are alive, however exhibiting low cell-material interaction. On the other hand, in a different study, plotting mouse MC3T3-E1 cells in an alginate solution of 3.5% (w/v) resulted in cells spreading after 42 days of incubation [243]. Furthermore, applying an alginate solution of 1% (w/v) for bioplotting of MG-63 cells showed no significant cell migration and spreading after ten days of cultivation [257]. Thus, the cellular behaviour inside non-modified alginate hydrogels has been discussed in the literature with different outcomes. In general, most recent studies in this field have demonstrated the need to modify the alginate network using cell-binding peptides or proteins in order to enhance cell-material interaction. Fonseca et al [164], for example, investigated the actin filaments of mesenchymal stem cells immobilised in alginate, which was modified with different
peptide sequences, describing the shape of the spread cells. This study also emphasized the need to modify alginate with peptide sequences to achieve improved cell adhesion and cell-material interaction in a 3D environment.

**Figure 31:** Fluorescence microscope images of an bioplotted ADA–GEL construct, which is seen to be overgrown with MG-63 cells showing actin cytoskeleton (red) and cell nuclei (green) staining after 28 d of incubation. Overview image a) and detailed spot image b). (Image b: [33] ©IOP Publishing. Reproduced with Permission. All rights reserved. [http://iopscience.iop.org/article/10.1088/1758-5090/7/2/025001](http://iopscience.iop.org/article/10.1088/1758-5090/7/2/025001))

### 5.3.3 Scanning electron microscopy

In Figure 32 SEM images of ADA–GEL constructs after one day of incubation are shown. The images present the macroscopic surface structure and indicate the porosity of the hydrogel network in the spot image. In this phase of cultivation period, no cells on the surface can be detected. They seem to be encapsulated in the bulk of the ADA-GEL matrix.

**Figure 32:** SEM images of ADA–GEL grid-like scaffolds with MG-63 immobilised cells after one day of incubation. Overview image (a) and detailed spot image (b) [33]. ©IOP Publishing. Reproduced with Permission. All rights reserved. [http://iopscience.iop.org/article/10.1088/1758-5090/7/2/025001](http://iopscience.iop.org/article/10.1088/1758-5090/7/2/025001)

Figure 33 shows SEM micrographs of a construct surface with MG-63 cells after 28 days of cultivation at two magnifications. The SEM images in Figure 33 confirm the
information provided by the fluorescence images in Figure 31 and indicate that after 28 days, cells grew out of the scaffold structure and were distributed over the whole surface. In Figure 33b spread MG-63 cells are visible on the scaffold surface indicating excellent cell adhesion and migration on the hydrogel surface.

The SEM observation indicates that there was a high movement and migration of the initially encapsulated cells as visualised by the comparison of Figure 32 and Figure 33. The cells were seen to grow out of the ADA-GEL and to cover the scaffold structure. In Chapter 4 the degradability of the ADA-GEL system was outlined, which is a likely explanation for the cell proliferation during the maturation phase. The results presented in this section indicate the permeability of the internal architecture of the ADA-GEL for the immobilised MG-63 cells, as a result of the degradation process. Pure alginate does not provide this property for immobilised MG-63 cells, as shown by Sarker et al [31] in a comparative study with ADA-GEL.

5.4 VEGF-A release

The angiogenic potential of the hydrogel-cell constructs was investigated by measuring the VEGF-A release from the immobilised cells as this growth factor plays a major role in the neovascularization process [187]. The experiment was designed as explained in Section 3.5.2. In Figure 34 the VEGF-A concentration measured in the cell culture medium surrounding the hydrogel-cell constructs is shown for a four days period in an ongoing incubation process. With increasing incubation time, the VEGF-A concentration expressed in 4 days rises from the first period to the third period and
reaches a plateau. Considering the results of the investigation of both the metabolic and mitochondrial activities, it seems that the increase of cell number leads to the upregulation of the VEGF-A expression. Interestingly, the increase of the VEGF concentration from the second to the third period is very distinctive. The third period, which is measured from day 17 to day 21, is set after the time point of 14 days, when the metabolic assays have shown a maximum as previously discussed. Thus, possibly the high cell number potentiated the hypoxic conditions leading to an upregulation of the VEGF release. In accordance with this finding is that during the fourth period (day 24 to day 28) no increase of the released VEGF was measured and no increase of the metabolic assays was detected. The increased concentration of released VEGF-A also indicates that the ADA–GEL matrix has enabled VEGF expression of immobilised MG-63 osteoblast-like cells. The upregulation of VEGF expression in alginate matrices has been investigated previously. For example, hypoxia mediated upregulation of VEGF expression by tumor cells immobilised in alginate disks was reported by Verbridge et al [218]. In a previous study it was shown that immobilised MG-63 cells synthesise more VEGF in ADA–GEL compared to pure alginate after two days of incubation [30].

Figure 34: Concentration of human VEGF-A released from immobilised MG-63 cells over different time periods lasting 4 days during a total incubation phase of 28 days [33].

The evaluated process parameters and the plotting setup were compatible with MG-63 osteoblast like cells. It was shown that immobilised cells were able to adhere and to proliferate on the plotted structures, which was confirmed by the results of the
metabolic assays and the microscopy studies. Furthermore, the angiogenic potential of this hydrogel matrix was confirmed by showing the upregulation of VEGF-A during incubation up to 28 days. Thus, the ADA–GEL hydrogel is a promising material for the immobilisation of cells as well as for use in bioplotting technology and thus for biofabrication.

Based on the developed bioplotting approach with ADA-GEL and MG-63 cells presented here, studies with human colorectal tumor cell line (HCT116) have been carried out in comparison to pure ALG [258]. The biopotted ADA-GEL constructs with immobilised HCT116 cells have shown superior behaviour regarding cell attachment, spreading and viability in comparison to pure ALG constructs. This study indicates the relevance of the presented approach in the context of cancer research. In the following chapters, the further investigation of the ADA-GEL system for bone TE by using other bone related cell models (Chapter 6) as well as by adapting the plotting process (Chapter 7) will be presented.
6 ADA-GEL cell constructs as tissue scaffolds using ST2 cells

6.1 Introduction

In this chapter, the influence of ADA-GEL with varied preparation parameters on the behaviour of immobilised bone marrow stromal cells (ST2) in biopotted ADA-GEL constructs (as developed in Chapter 4) is discussed. MG-63 cells, which have been successfully implemented in the bioplotting process as shown in the previous chapter, were replaced by ST2 cells. ST2 cells are a suitable cell model for bone tissue engineering. For example Schumacher et al [197] used ST2 cells to evaluate different bone substitute materials and investigated their potential for osteoblastic differentiation. In previous studies, the behaviour of immobilised cells in ADA-GEL microcapsules, as a 3D cell culture model, has been assessed in comparison to pure ALG, ALG modified with RGD sequences or a blend material of ALG and GEL [30],[31]. In this study, the ADA-GEL system itself was modified and taken as a reference system to investigate the influence of certain parameters of this material system on the cell development. The varied parameters in the ADA-GEL preparation were the amount of sodium metaperiodate used for the oxidation of ALG, as it defines the molar mass of the ADA [229]. It has been described in Chapter 4 that in this manner the mechanical properties of ADA-GEL could be tailored. Furthermore, the stock solution concentrations of ADA and GEL were increased from 5.0 % (w/v) [29],[33] to 7.5 % (w/v) and 10 % (w/v). In that way, the mechanical properties as well as the degradation behaviour of the ADA-GEL could be adjusted, as presented in Chapter 4.

Co-cultures represent a superior model for mimicking the cellular structure in natural tissue [259]. Indeed, bone tissue is very complex and comprises the dynamic crosstalk of osteoblasts and osteoclasts for bone formation and resorption [212]. Thus, a further approach, which is discussed in this chapter, is the co-culture of osteoblastic progenitor cells (ST2) with osteoclastic progenitor cells (RAW 264.7) in ADA-GEL constructs, mimicking pre-calcified bone tissue (=osteoid) to evaluate the influence of the osteoblast/osteoclast crosstalk on cell development. The investigation of co-culture systems in biofabricated constructs is an important step to mimic natural tissues in their full complexity. Xu et al [139] used ink-jet printing technology for the precise positioning of stem cells with an osteogenic lineage, smooth muscle cells and
endothelial cells in alginate-collagen constructs. It was shown that the cells kept their cell specific functions in their predefined positions and that fabrication of heterogeneous constructs is possible. The constructs matured to functioning tissue with adequate vascularization in vivo. The study showed the great possibilities of co-culture systems in biofabricated constructs and emphasized the importance of angiogenesis for the success of implants with clinically relevant size. Parts of this chapter contain results and discussion published in reference [260].

6.2 ADA-GEL prepared from ADA with different molar mass: Influence on ST2 cell behaviour

In the following section, the influence of ADA-GEL prepared with ADA of a different molar mass on the behaviour of immobilised ST2 cells is presented. It has been shown in Chapter 4 that by varying the periodate equivalent for the synthesis of ADA and so the molar mass, the mechanical properties and the degradation kinetics of the ADA-GEL could be tailored. The interaction of these properties and their effect on ST2 cell development are discussed.

6.2.1 Cell viability

The influence of ADA-GEL hydrogels with constant stock solution concentrations of 5%, but mixed with ADA synthesised of different periodate equivalents (21.6%, 26.7%, 32.1%), on the cell viability of immobilised ST2 cells during an incubation period up to 21 days was investigated. The WST-8 assay was used. The results of this study are presented in Figure 35. The periodate equivalents were chosen to get ADA with molecular weights in a similar range to the ADA used in Chapter 5 for the studies with MG-63 cells.
Figure 35: Cell viability of ST2 cells immobilised in plotted constructs of ADA-GEL consisting of ADA prepared with different periodate equivalents (21.6 %, 26.7 %, 32.1 %) over 21 days of incubation.

The viability of ST2 cells in all three ADA-GEL modifications shows a significant increase from day 7 to day 21. Interestingly, only the cell viability using ADA-GEL of the 26.7 % periodate equivalent group, increased already significantly from day 7 to day 14 of the maturation phase. All three set-ups indicated a significant increase from day 14 to day 21. The ADA-GEL groups with 26.7 % and 32.1 % periodate equivalents revealed a relative increase of the cell viability of around factor 3 from day 7 to day 21. The ADA-GEL group with 21.6 % periodate equivalent showed a relative increase of around factor 2 during this period.
The increase of cell viability indicates an increase of cell number and so proliferation of the cells in the ADA-GEL matrix. This result is consistent with the experiments immobilising MG-63 cells in bioplotted ADA-GEL constructs as discussed in Chapter 5, also showing increasing signals of the WST assay with a sharp increase between day 7 and day 14 of the incubation phase.

6.2.2 LDH-activity

For corroborating the higher cell number in the ADA-GEL groups of 26.7 % and 32.1 % periodate equivalent in comparison to the ADA-GEL group of 21.6 % periodate equivalent, LDH-activity measurements after 21 days of incubation were performed. The results are presented in Figure 36. The higher cell number of the groups with 26.7 % and 32.1 % periodate equivalent indicate also a higher proliferation rate in these hydrogels, as the cell concentration was equal during the fabrication step.

![Figure 36: LDH-activity of ST2 cells immobilised in plotted constructs of ADA-GEL consisting of ADA prepared with different periodate equivalents (21.6 %, 26.7 %, 32.1 %) after 21 days of incubation.](image)

The LDH-activity after 21 days of incubation for the ADA-GEL groups of 26.7 % and 32.1 % periodate equivalent ADA in comparison to the 21.6 % group was significantly enhanced. Hence, the LDH measurements are consistent with the WST-8 results, which indicated also a higher cell number for the groups with higher periodate equivalent.
In Chapter 4 it has been discussed that ADA prepared with higher periodate equivalents led to ADA-GEL hydrogels with reduced stiffness and faster degradation behaviour. Possibly this is the reason for the higher proliferation rate of ST2 cells in these ADA-GEL groups. This result confirms also the fact that hydrogel degradation is a significant parameter affecting tissue formation [251]. A faster proliferation rate of MG-63 cells immobilised in ADA-GEL microcapsules in comparison to pure ALG microcapsules was presented by Grigore et al [30]. The stiffness of the ALG (2 % (w/v)) was with values of 33 kPa also higher in comparison to the stiffness of ADA-GEL with 8 kPa [179]. Furthermore, films prepared from ALG showed no degradation up to 42 days, whereas ADA-GEL films degraded in the same period [179].

6.2.3 Cell morphology and cell development

For evaluating the cell morphology and the cell development during the incubation phase different microscopy techniques were used. In Figure 37 and Figure 38 light microscopy images of all ADA-GEL/ST2 cell groups with the varied periodate equivalents are presented after 2 days and 21 days of incubation, respectively. The mentioned time points were chosen as they reveal potentially a visual difference considering the time lag.
Figure 37: Light microscopy images of ST2 cells immobilised in plotted constructs of ADA-GEL consisting of ADA prepared with different periodate equivalents (a,b 21.6%; c,d 26.7%; e,f 32.1%) after 2 days of incubation. (images a,c,e scale = 200 µm and images b,d,f scale = 100 µm)

The images of the ADA-GEL groups of 26.7% and 32.1% periodate equivalent showed ST2 cells with a round, but also exhibiting an elongated morphology at day 2 of the incubation phase. The ST2 cells of the ADA-GEL group with 21.6% periodate equivalent showed a different morphology. The ST2 cells were solely round shaped at this time point. In general, an elongated morphology indicates an enhanced cell-material interaction, as cells are reshaping while trying to migrate through the ADA-GEL network. A rounded morphology usually appears if there are no adhesion sites provided by the surrounding matrix [261]. In the present case, the GEL provides attachment cues for cells. The improved cell adhesive properties of ADA-GEL in comparison to pure ALG were demonstrated in a 2D culture with fibroblasts [178]. Thus, the decisive factor was more obviously the increased network density expressed by the increased rYm of the group with a periodate equivalent of 21.6%, as discussed in Chapter 4.
In Figure 38 images after 21 days of incubation are presented. The same distinctive ST2 cell morphology in the different ADA-GEL groups is observed. The samples with a higher periodate equivalent (images c-f) showed large cell agglomerates of elongated and spread cells, whereas in the group with the 21.6 % periodate equivalent (images a,b) no such big agglomerates were visible. Moreover, only some single cells presented an elongated morphology. This result is in agreement with the results on degradation kinetics presented in Chapter 4, showing that there was no significant decrease of the rYm for ADA-GEL with 21.6 % periodate equivalent from the incubation time points of day 3 up to day 21. Furthermore, the presented images confirm the development of ST2 cells as measured by the cell viability kinetic and the study of the LDH-activity.

**Figure 38:** Light microscopy images of ST2 cells immobilised in plotted constructs of ADA-GEL consisting of ADA prepared with different periodate equivalents (a,b 21.6 %; c,d 26.7 %; e,f 32.1 %) after 21 days of incubation. (images a,c,e scale = 200 µm and images b,d,f scale = 100 µm)

Fluorescence microscopy and LSCM were used for further studies regarding the cell morphology of the ST2 cells by staining their actin cytoskeleton after 21 days of
incubation. In Figure 39 e,f the ADA-GEL group with a periodate equivalent of 32.1 % is presented. The ST2 cells had formed dense agglomerates, showing spread morphology. Moreover, the cells seemed to form cell-cell contacts throughout the ADA-GEL network. In Figure 39 c,d the ADA-GEL group with a periodate equivalent of 26.1 % is shown. The ST2 cells behave analogous to the group with 32.1 % periodate equivalent. The images in Figure 39 a,b indicate a lower cell density in accordance with the WST-8 and LDH-activity studies. Furthermore, only few cells seem to interact with the ADA-GEL matrix as it was also observed in the light microscopy images.

**Figure 39:** Fluorescence microscopy images of ST2 cells immobilised in plotted constructs of ADA-GEL consisting of ADA prepared with different periodate equivalents (a,b 21.6 %; c,d 26.7 %; e,f 32.1 %) after 21 days of incubation; staining of the cell nucleus (green) and the actin cytoskeleton (red).

In Figure 40 additional fluorescence images, which have been taken with an LSCM, of the ADA-GEL groups with 26.7 % (images a,b) and 32.1 % periodate (images c,d) equivalent are presented. The images emphasize the impression of enhanced interaction of ST2 cells with the selected ADA-GEL matrices. The actin-filaments of
single cells and their morphology in the agglomerates are visible, indicating an effective cell-cell interaction.

Figure 40: LSCM images of ST2 cells immobilised in plotted constructs of ADA-GEL hydrogels consisting of ADA prepared with different periodate equivalents (a,b 26.7 %; c,d 32.1 %) after 21 days of incubation; staining of the cell nucleus (green) and the actin cytoskeleton (red).

SEM images of the ADA-GEL group with 26.7 % periodate equivalent after 21 days of incubation are presented in Figure 41.
As expected from the previous microscopy investigations the ST2 cells, cultivated in the ADA-GEL hydrogel prepared with ADA of a sodium periodate equivalent of 26.7 %, showed a spread morphology, and areas of high cell density on the typically folded surface of the ADA-GEL constructs (image a,b). Image c shows flattened ST2 cells with a high surface area indicating a superior cell adhesion and exhibiting an osteoblastic morphology [198],[262]. Moreover, cell-cell interactions are visible as the cells had formed a dense layer. In image b it is visible that ST2 cells have migrated out of the ADA-GEL matrix. The covering of the construct surface was also observed for MG-63 cells bioplotted in ADA-GEL hydrogels as presented in Chapter 5. This behavior could possibly be explained by a diffusion gradient of oxygen and nutrients, making the outer parts of the constructs more attractive for the immobilised cells. In previous
studies it was seen in images of MG-63 cells immobilised in ALG/GEL blend microcapsules that the cells are forming elongated agglomerates only at the margin areas of the capsules, possibly caused by a better supply of nutrients [31].

The ALP-activity of the immobilised ST2 cells as an indication for osteoblastic differentiation was measured. The results of this study are presented in Figure 42.

![Figure 42: Specific ALP activity of ST2 cells after 21 days of incubation in biopotted ADA-GEL constructs prepared with ADA of different periodate equivalents.](image-url)

It is seen that the specific ALP-activity of ST2 cells cultivated in ADA-GEL hydrogels with increased periodate equivalents (26.7 % and 32.1 %) was also enhanced in comparison to the group with a periodate equivalent of 21.6 %. Taking into account that the groups with enhanced periodate equivalents also showed increased cell numbers, this could be the reason for the increased ALP-activity. Interestingly, the ST2 cells seem to differentiate in the ADA-GEL matrix even without the addition of osteogenic differentiation factors like ascorbic acid or β-glycerolphosphate. Experiments with ST2 cells seeded on ADA-GEL scaffolds prepared by freeze drying have also revealed the expression of ALP without the addition of osteogenic stimulating agents [263].

Summarising the results of the different characterisation techniques regarding the development of ST2 cells, it is obvious that the periodate equivalent and so the amount of sodium metaperiodate used for the oxidation of ALG is a decisive parameter regarding the control of the cell response. It seems that ADA prepared
with higher periodate equivalents and so characterised by a decreased molar mass, is favourable for ADA-GEL synthesis regarding an increased degradation behaviour, as shown by nanoindentation studies as reported in Chapter 4. The enhanced degradation seems to result in an increased cell proliferation and also into a better cell-material interaction. Nevertheless, ADA with a too high oxidation degree or too low molar mass could lead to an unstable ADA-GEL network, when the ionic gelation process is not active. Thus, for following studies ADA with a periodate equivalent of 26.7 % was used.

Comparing the results of ST2 cells and MG-63 cells as reported in Chapter 5, where ADA with higher molar mass was used and showed no hampering of the cell proliferation, it becomes apparent that this parameter has to be adjusted considering the used cell type [232].

6.3 The influence of different ADA-GEL concentrations on the ST2 cell behaviour

In Chapter 4 it was shown that the reduced Youngs’s modulus of ADA-GEL could be increased by enhancing the stock solution concentrations of ADA and GEL. A higher stiffness of the ADA-GEL is beneficial regarding the handling of the fabricated constructs considering a potential implantation. Furthermore, it is interesting to evaluate the influence of the elevated stiffness and the reduced degradation of higher concentrated ADA-GELs, as shown in Chapter 4, on immobilised ST2 cells.

6.3.1 Live-dead staining

The increase of the ADA-GEL concentrations resulted in an elevated plotting pressure, which is known to be a critical factor regarding cell viability [63]. Therefore, live/dead staining was done to analyse the percentage of live cells after the plotting process. In Figure 43 the results of the quantitative analysis of the fluorescence images are shown, presenting the percentage of live cells in connection to the applied plotting pressure.
In general, for all set-ups the percentage of live cells > 80 % is high. Thus, as already discussed in Chapter 5 (Section 5.1) the bioplotting process is biocompatible and is not the limiting factor for the progress in the field. Interestingly, the percentage of live cells is even significantly higher for elevated concentrations, even though the pressure is around 8 times higher for the ADA-GEL prepared with stock solutions of 10 % in comparison to the 5 % one. This result shows that the hydrogel acts as a protecting shield for the immobilised cells [9]. Thus, the negative influence of the plotting pressure on cell viability has to be seen in connection to the used hydrogel and its composition. Aguado et al [264] showed in comparative study that ionically gelled alginate solutions containing cells have a protective influence on the cells. These solutions showed higher cell viabilities after extrusion through a capillary in comparison to using non-gelled alginate solutions or PBS solutions as a cell carrier.

Besides the negative influence of the different mechanical forces applied to the cells during the printing process, also positive aspects of these mechanical stimuli on cell behaviour can be considered. For example Snyder et al [265] reported increased ALP-activity of mesenchymal stem cells by applying enhanced plotting pressures from 0 kPa to 276 kPa printed in alginate solutions.
6.3.2 Concentration dependence: ADA-GEL 2.5 % and ADA-GEL 3.75 %

*Cell viability*

In the previous section, it was shown that the ST2 cells are compatible with the adapted parameters of the bioplotting process for ADA-GEL hydrogels with increased stock solution concentrations of ADA and GEL. The enhanced stock solution concentrations of 7.5 % in comparison to the previously used 5 %, resulted in 3.75 % ADA-GEL instead of 2.5 % ADA-GEL. The viability of ST2 cells bioplotted in 3.75 % ADA-GEL in comparison to 2.5 % ADA-GEL is presented in Figure 44 over an incubation period of 21 days.

![Figure 44](image)

*Figure 44:* Cell viability of ST2 cells immobilised in plotted constructs of ADA-GEL consisting of ADA-GEL prepared with different stock solution concentrations of ADA and GEL, resulting in ADA-GEL 2.5 % and ADA-GEL 3.75 %, over 21 days of incubation.

As expected, the 2.5 % ADA-GEL showed an increase of cell viability from day 6 up to day 21 of the incubation period. It is seen that there was a significant enhancement of the assay signal in between day 14 and day 21. The comparative group of 3.75 % ADA-GEL showed also a significant rise from day 6 to day 14 as well as from day 14 to day
After 21 days of incubation the absolute signal of the WST-8 assay was in the same range for both groups.

**LDH-activity**

For corroborating a comparable cell number in the bioplotted constructs of the two ADA-GEL groups (2.5 % and 3.75 %) a LDH-activity study was performed after 21 days of incubation. The results are presented Figure 45.

![Figure 45: LDH-activity of ST2 cells immobilised in plotted constructs of ADA-GEL consisting of ADA prepared with different stock solution concentrations of ADA and GEL, resulting in ADA-GEL 2.5 % and ADA-GEL 3.75 %, over 21 days of incubation.](image)

There was no significant difference between the LDH-activity of the two investigated ADA-GEL hydrogels. Thus, this result was in accordance with the WST-8 assay results. The increase of the stock solutions of ADA and GEL from 5.0 % to 7.5 % enabling higher rYm values in the early stages of the maturation phase as well as an improved handability of the hydrogel constructs after the processing, did not create a restrictive matrix environment, suppressing cell proliferation. It has been shown in Chapter 4 that the 3.75 % ADA-GEL had enhanced mechanical properties, but still showed rapid degradation behaviour.

**Light microscopy**

Light microscopy images were taken after 21 days of incubation, as shown in Figure 46, to compare the cell morphology in the ADA-GEL matrices of 2.5 % (Figure 46 a) and 3.75 % (Figure 46 b) ADA-GEL.
Figure 46: Light microscopy images of ST2 cells immobilised in plotted constructs of ADA-GEL consisting of ADA prepared with different stock solution concentrations of ADA and GEL, resulting in a) ADA-GEL 2.5 % and b) ADA-GEL 3.75 %, over 21 days of incubation.

In both ADA-GEL groups ST2 cells with a rounded as well as an elongated and spreaded morphology were visible. The 3.75 % ADA-GEL group gave the impression of containing a greater number of dense cell agglomerates. This result was possibly caused by the stiffer ADA-GEL network even after 21 days of incubation.

6.3.3 Concentration dependence: ADA-GEL 3.75 % and ADA-GEL 5.0 %

Cell viability

The influence of ADA-GEL hydrogels prepared with enhanced stock solution concentrations of 10 % in comparison to concentrations of 7.5 % (resulting in ADA-GEL 5.0 % and ADA-GEL 3.75 %) on the cell viability of immobilised ST2 cells during an incubation period up to 42 days was assessed. The WST-8 assay was used. The results of this study are presented in Figure 47.
The 3.75 % ADA-GEL group exhibits an increase of cell viability during the incubation period. In contrast to the previous study presented in Figure 44, the most decisive increase of the assay signal was seen between day 21 and day 28 in comparison to day 6 and day 14. Nevertheless, there was an increasing trend during the incubation time of 42 days. The incubation time was increased up to 42 days, because the reference group of 5.0 % ADA-GEL did not indicate a significant increase of the WST-8 assay signal until 21 days of incubation. In between day 28 and day 35 the 5.0 % ADA-GEL group showed significantly increased cell viability, which further increased from day 35 to day 42. Both ADA-GEL groups showed increased cell viabilities, but enhanced stock solution concentrations of ADA and GEL shifted the increase of the assay signal backwards. A possible explanation of this trend is a reduced degradation kinetic. The values of the \( r_{ym} \) of the two ADA-GEL groups and their degradation kinetic were similar, at least over an incubation period of 14 days, as discussed in Chapter 4. Nevertheless, the percental GEL release of the 5.0 % group was around 5 % lower than that of the 3.75 % group (presented in Chapter 4). This behaviour and the
increased stock solution concentration of ADA could possibly cause a denser network with more physical interactions of the components and a decreased porosity, which likely hampered cell proliferation. Only at the very late time point of 35 days, when the network was potentially more degraded, the cells could proliferate and the assay signal was increased. The concept of a releasing phase to improve the cell response has been considered also by Müller et al [47], while developing a synthetic hydrogel of Pluronic F127. In this approach acrylated Pluronic was mixed with non-modified Pluronic. After UV crosslinking the unmodified phase could be selectively released. Pure ALG is also only suitable for cell encapsulation at low concentrations, namely up to a maximum of 3 % (w/v) [140], but preferentially also at lower concentrations like 2 % (w/v) [61]. Interestingly, this study with the modified ADA-GEL hydrogels showed how the cell response could be dictated over a wide time period by varying the ADA-GEL composition, which could be potentially interesting for the development of gradient materials with tailored degradation kinetics.

6.4 ST2/RAW co-culture model in an osteoid-like hydrogel matrix

6.4.1 Concept

In this section, the biofabrication of an osteoid-like construct containing a co-culture of osteoblast (ST2) and osteoclast (RAW 264.7) progenitor cells to investigate cellular interaction in this 3D cell culture set-up is presented. In an early stage of bone regeneration, the formation of the non-mineralized osteoid phase [208], which has a share of 25% in calcified bone matrix [205], must be considered. The osteoid phase is built up by osteoblastic progenitor cells and is mineralized by mature osteoblasts [208]. The osteoid consists of collagen type I and glycosaminoglycans [266]. Chicatun et al [266] fabricated an osteoid-like tissue model based on collagen/chitosan hybrid gels that could be used as an early stage bone tissue model for bone tissue engineering. The two used cell types have been previously applied in a co-culture approach on hydroxyapatite ceramics by Detsch et al [267]. Furthermore, co-cultures of osteoblasts and osteoclasts have been seeded on different biomaterials (polylactic acid (PLA) nanohydroxyapatite (nanoHA) [268], silk fibroin chitosan [269], composite xerogel [270]) for tissue engineering studies or in the context of bone remodelling investigations. ADA-GEL with a 5050 ratio of ADA and GEL was chosen, as this
composition showed promising results regarding the immobilisation of MG-63 cells (presented in Chapter 5). Thus, ADA-GEL contains 50 % GEL, which is a derivate from collagen and the main phase of the osteoid [271]. The stiffness values, as presented in Chapter 4, of the 3.75 % (w/v) concentration ADA-GEL (36 ± 8 kPa) are more suitable for mimicking the osteoid bone phase than 2.5 % (w/v) concentration ADA-GEL (9 ± 3 kPa), considering that Engler et al [272] reported the osteoid bone to have a stiffness of 27 ± 10 kPa.

The ST2/RAW co-culture system was investigated in vitro in comparison to single cultures considering cell viability and proliferation by using WST-8 assay as well as measuring the LDH activity. The ALP-activity and the OPN expression were determined to evaluate the osteogenic differentiation of the ST2 cells. The osteoclastogenic differentiation of the RAW cells was investigated by measuring the TRAP-activity and using a TRAP staining. Furthermore, different microscopy techniques were applied to evaluate the cell morphology. The release of VEGF is determined, which is an important growth factor promoting potential angiogenesis of the constructs.

6.4.2 Cell viability and LDH activity

Figure 48 shows the result of the WST-8 assay kinetic as a measure of the cell viability over incubation time (Figure 48 a) and of LDH activity as an indication of cell number after 21 days of incubation (Figure 48 b).

![Figure 48: Cell viability (a) of different cell type set-ups during 21 days of incubation and lactate dehydrogenase activity (b) at day 21 of single-culture osteoblasts (ST-2), single-culture osteoclasts (RAW) and osteoblast/osteoclast co-culture (ST-2/RAW) [260]. ©IOP Publishing.]
The cell viabilities of the ST2 single culture and the co-culture show higher values compared to the RAW single culture until day 7. A sharp increase of the cell viability in the co-culture as well as in the RAW single culture after 14 days of incubation could be detected, whereas the values for the ST2 single culture decreased slightly from day 1 to day 21. The results of the LDH study at day 21 of the incubation period showed increased values for the RAW single culture and the co-culture in comparison to the ST2 single culture. The kinetic of cell viability showed a slightly decreasing trend for the ST2 single culture. However, the RAW single culture and the co-culture exhibited a sharp increase after 14 days of incubation. In previous experiments with immobilised MG-63 cells in ADA-GEL also a sharp increase of the mitochondrial activity as an indication of cell viability after 14 days of incubation was detected [33]. Considering both, the results of the cell viability study and the lactate dehydrogenase activity values showing lower values for the ST2 single culture in comparison to the RAW single culture and the co-culture, the lower cell viability could possibly be explained by a lower cell number in the ST2 group. These results indicate that RAW cells proliferate much faster than ST2 cells in the ADA-GEL matrix. A faster proliferation rate of RAW cells in comparison to ST-2 cells was reported by Detsch et al [267] in the context of co-culture experiments and hydroxyapatite.

In general, the proliferation rate of immobilised cells in the ADA-GEL is possibly connected to the hydrogel degradation and the related decrease in stiffness [31]. It has been shown by Sarker et al [32],[178] that the ADA-GEL system degrades by the release of un-crosslinked GEL molecules. This has also been confirmed in Chapter 4 for ADA-GEL with 3.75 % concentration, which is the hydrogel used in the present study. Besides this degradation mechanism, there could be an increased degradation of the ADA-GEL matrix, especially when it contains RAW cells, as macrophages are known to promote phagocytosis of collagen [273]. The influence of osteoclasts on the degradation of biopolymers has been investigated also by Torres et al [274]. Chitosan and chitosan modified fibrinogen were used for the study emphasizing the role of osteoclasts also in the context of organic bone tissue engineering materials. The degradation of silk biomaterials by osteoblasts and especially osteoclasts, which degraded the silk films the most, was reported by Sengupta et al [275]. In the case of
alginate an initial accelerating influence of fibroblasts on the rate of degradation was discovered by Hunt et al [276], but also no influence on alginate degradation of CAC cells was reported [248]. All these studies indicate that cell mediated degradation of biomaterials is an important issue, which has to be considered in future studies. Thus, the results of the cell viability and LDH-activity studies could be explained by a combination of an enhanced proliferation rate with the influence of the ADA-GEL matrix degradation or it could be just related to the proliferation rate itself. This behaviour shows the difficulty of developing a suitable hydrogel matrix for two different cell types.

6.4.3 Osteogenic differentiation

ALP activity

The measurement of ALP as an early marker for osteoblastic maturation [266], and so as an indicator of the differentiation of ST2 stromal cells into osteoblasts [277], is presented in Figure 49. The pNpp-conversion measured at 405 nm as indicator of the ALP activity is presented in dependence of the cell type after 21 days of incubation. The ST2 single culture shows a significant higher ALP activity than the co-culture set-up.

Figure 49: ALP-activity at 405 nm of the different cell type set-ups after 21 days of incubation [260]. ©IOP Publishing. Reproduced with Permission. All rights reserved. http://iopscience.iop.org/article/10.1088/1758-5090/aa64ec
The total protein concentration of each cell culture set-up measured by Bradford-test is shown in the Appendix (Figure A 2). It was observed that the protein contents of ST2 single culture and ST2/RAW co-culture are very different, which is possibly caused by the high number of RAW cells in the co-culture. Thus, the protein content has not been taken into account for the calculation of the ALP activity. Although no osteogenic differentiation factors like β-glycerolphosphate or ascorbic acid were added to the cell culture medium, a distinct ALP expression of the ST2 cells could be detected. Interestingly, the ST2 single culture showed a higher rate of ALP expression in comparison to the co-culture. After 21 days of incubation the ST2/RAW co-culture caused no higher ALP expression. As the level of expression of ALP is time dependent [277], it could be possible that at a different time point during incubation the co-culture shows a higher ALP expression. Increased ALP activities in co-cultures of osteoblasts and osteoclasts in comparison to single osteoblast cultures on PLA and PLA nanoHA scaffolds were reported by Morelli et al [268] using osteogenic as well as osteoclastogenic differentiation factors. Using mineralized collagen as template for osteoblast/osteoclast co-culture, Bernhardt et al [278] reported the same trend. Heinemann et al [270] reported also increased ALP activities for a co-culture of osteoblasts and osteoclasts on polystyrene. This effect was only significant in the group of osteogenic media induced osteoblasts before the seeding of human monocytes and not for the group without this pre-treatment. Besides the pNpp conversion also the total protein concentration in the different cell-cultures was determined (Appendix Figure A 2). The higher protein concentration in the co-culture and in the RAW single culture is a sign of a higher cell number caused by the elevated proliferation of RAWs in comparison to ST2 cells [267].

**OPN synthesis**

The concentration of OPN, as a marker for osteogenic differentiation [268], synthesized by the different cell type set-ups is shown in Figure 50.
The concentration of OPN expressed by the co-culture set-up is significantly higher in comparison to the ST2 single culture set-up. OPN is an important non-collagenous glycoprotein associated with bone formation. It is involved in the process of bone mineralization and bone cell adhesion [206],[207],[279] and is an important mediator in the crosstalk of osteoblasts and osteoclasts during bone remodelling [280],[281]. OPN is expressed by osteoblasts [206] and thus is a marker of early osteoblastic differentiation [266],[281]. The higher OPN concentration of the co-culture in comparison to the ST2 single culture indicates that the osteoblastic differentiation of the ST2 cells has increased in the co-culture. It is known that OPN can be also expressed by macrophages [280]. The expression of bone related proteins like OPN or osteocalcin by foamy macrophages at the interface of aseptic loosened prosthesis has been reported by Zreiqat et al [282]. However, in our study, osteoclast-like cells were identified (Figure 51 a,b). The higher OPN concentration of the co-culture indicates possibly a positive effect by the crosstalk between ST2 and RAW cells on bone related protein expression.

6.4.4 Osteoclastic differentiation

Besides the osteoblastic differentiation of the ST2 cells, also the osteoclastic differentiation of the RAW cells is of interest for understanding the behaviour of cells
in co-culture and the cell-cell interaction. In Figure 51 a) the TRAP-activity and in Figure b) a TRAP staining of the co-culture in comparison to the RAW single culture after 21 days are presented.

Figure 51: TRAP-activity (a) and TRAP staining (b) of the co-culture in comparison to the RAW single culture after 21 days of incubation [260].

The TRAP-activity as an indicator of the differentiation of the RAW macrophages into osteoclasts is significantly higher for the co-culture set-up than for the RAW single culture. The images of the TRAP staining show positive results for both groups, namely the RAW single culture and the ST2/RAW co-culture. In both set-ups dense cell agglomerates appear rather black in comparison to the typical red colour of the
TRAP staining, but single cells show the typical red staining in the co-culture. A significantly increased TRAP activity of the co-culture in comparison to the RAW single culture could be detected, even considering that no differentiation factors like RANKL (receptor activator of NF-κB ligand) or M-CSF (macrophage colony stimulating factor) were used in this study. In general, TRAP is highly expressed by osteoclasts [212] being a marker for osteoclastic differentiation, the co-culture set-up seems to support the differentiation of RAW cells. This result is in contradiction to experiments previously reported using the same ST2/RAW co-culture model on HA platelets [267]. In the previous experiments the non-stimulated group (without M-CSF and RANKL) showed no TRAP positive cells, which could be detected only in the stimulated one. Udagawa et al [283] reported that ST2 cells induced TRAP positive osteoclast-like multinucleated cells in co-culture with mouse spleen cells by addition of 1α, 25-dihydroxyvitamin D3 and dexamethasone to the media. Increased TRAP activity in an osteoblast/osteoclast co-culture was also reported by Morelli et al [268]. Differences could be possibly explained by the set-up using 2D instead of 3D cell culture environment and by the influence of the used biomaterial [259]. The images of the TRAP staining show positive results for both investigated groups and confirm the results of the TRAP activity measurements. Dense cell agglomerates appear rather in black colour and not in the typical red colour characteristic of TRAP staining [269]. This result could be possibly explained by the very high density in these agglomerates and the overlapping of cells, because TRAP staining positive single cells appeared in red colour. Besides the positive TRAP staining also multinucleated cells were present in both groups, as shown in the Appendix (Figure A 3). This is a further sign for osteoclastic differentiation caused by the fusion of cells [212],[274].

6.4.5 VEGF-A release

The concentration of VEGF released from the different cells type set-ups after 21 days of incubation is shown in Figure 52.
The VEGF concentration of the co-culture group is significantly increased in comparison to the ST2 and RAW single groups, which show no difference to each other. Vascularization and angiogenesis are recognized as challenging aspects in tissue engineering and they represent strategic directions for further research in the field [284] in order to develop bone tissue constructs of useful dimensions [40],[285]. VEGF is one of the important growth factors upregulating the angiogenic process [40]. Approaches to establish drug-delivery systems in scaffolds for VEGF supply have been successfully implemented [38],[40]. In order to improve the angiogenesis ability of biofabricated constructs, Fedorovich et al [203] cultured spatially separated multipotent stromal cells and endothelial progenitor cells immobilised in matrigel/alginate/bi-calcium phosphate hydrogels. The constructs were tested in-vivo and the results showed that in the predefined areas heterogeneous extracellular matrix formation occurred. Grigore et al [30] showed in a comparative study of human osteoblast-like cells (MG-63) encapsulated in alginate, RGD-modified alginate and ADA-GEL that VEGF expression was significantly increased in the ADA-GEL group after 48 h of incubation. Furthermore, it has been shown for MG-63 cells immobilised in plotted ADA-GEL structures that the VEGF concentration was increased with incubation time for up to 28 days [33]. In the present study, the VEGF concentration of the co-culture group was shown to be significantly increased compared to that of the two mono-cultures. Thus, the use of the co-culture could have positive effects for
the vascularization of the biofabricated ADA-GEL constructs by attracting endothelial cells [40] in case of implantation or by adding endothelial cells to the system [203]. It is known that osteoblasts and osteoblastic progenitor cells express VEGF [285]. There is no significant difference between the RAW and ST2 single cultures. The increase of the VEGF concentration in the co-culture could possibly be explained by the high number of RAW cells, which may have created a hypoxic state for the ST2 cells in the construct. The hypoxic state causes the upregulation of VEGF [38].

6.4.6 Cell morphology

In Figure 53 optical microscope images of the different cell type set-ups at incubation time points of day 1 and day 21 are presented. These images present the cell development over the incubation time.

![Figure 53: Optical microscopy images of different immobilised cell type set-ups at incubation times of 1 day and 21 days showing the cell development during cultivation [260].](http://iopscience.iop.org/article/10.1088/1758-5090/aa64ec)

Different cell morphologies of the RAW and the ST2 cells are visible Figure 53. At day 1 in the ST2 single culture mostly separated round cells (image a: arrow) and only some agglomerates (image a: dotted line) are visible. These cell morphologies could be also detected in the co-culture group (image g). At day 21 more dense
agglomerates of ST2 cells as shown in image c become visible. There are still separated round ST2 cells, but also elongated cells in separated position as well as in cell formations (image b). Moreover, cells formed dense agglomerates, both in the single culture and in the co-culture. Such cell formations are also visible in image f after 21 days showing the co-culture group. The RAW cells are round shaped and separated single cells are visible at day 1 in the single culture group (image d). Large, dense colonies have developed indicating cell proliferation after 21 days of incubation in the RAW single culture (image e) as well as in the co-culture (image h).

In Figure 54 LSCM images of the actin cytoskeleton of the three different cell type set-ups are presented after 21 days of incubation. The ST2 cells in the single culture group attached to the ADA-GEL matrix, show partially spread morphology and are forming large agglomerates (image a, b). The overview images (Figure 54 a, c, e) for each cell type set-up indicate a good cell distribution and a qualitatively higher cell density for the cultures containing RAW cells (image c, e), confirming the results of the LDH measurements (Figure 48 b). Image d shows dense agglomerates of RAW cells in the single culture. Similar structures are visible in the co-culture (image f), which could be also the RAW cells. Additional images are presented in the Appendix (Figure A 4) showing comparable spread cell morphologies possibly of ST2 cells as in Figure 54 a,b presenting the ST2 single culture. This finding confirms and reinforces the results from Figure 53 showing the partial spreading of ST2 cells and the formation of dense cell layers in some areas on the surface of the hydrogel structure. Furthermore, it is also visible that a part of the cells stays round shaped (Figure 54 a,b). RAW cells remain round and form dense colonies (Figure 54 c,d). In the co-cultures the described morphologies for each cell type could be found (Figure 54 e,f).
ADA-GEL cell constructs as tissue scaffolds using ST2 cells

In Figure 54: LSCM images of the actin cytoskeleton (red) and the cell nuclei (green) of a) b) ST2, c) d) RAW, e) f) ST2/RAW cells after 21 days of incubation [260].

In Figure 55 SEM images at different magnifications of an ADA-GEL construct with ST2/RAW co-culture are shown after 21 days of incubation. Based on the previous cell morphology investigations the images could indicate that the ST2 cells are forming large and very dense cell layers in some areas of the surface of the hydrogel structure as can been seen in the overview image (Figure 55a) (marked dotted line) and detailed spot image (Figure 55d). The ST2 cells show spread morphology (arrow in...
116 ADA-GEL cell constructs as tissue scaffolds using ST2 cells

Figure 55c). The RAW cells could be also detected all over the surface exhibiting a round shape and dense agglomerates (image b and arrow in image c in Figure 55).

Figure 55: SEM images of an ST2/RAW hydrogel construct after 21 days of incubation with different magnifications a) 250x, b) 1000x, c) 2500x, d) 4000x [260]. ©IOP Publishing. Reproduced with Permission. All rights reserved. http://iopscience.iop.org/article/10.1088/1758-5090/aa64ec

In the SEM images both cell types could be detected on the surface of the construct. This proves the cell mobility in the ADA-GEL network as immobilised cells can migrate from the inside to the surface. This result is consistent with the behaviour of immobilised MG-63 cells also biofabricated with ADA-GEL [33]. The SEM images confirm the fluorescence microscopy results showing RAW colonies in high number and dense layers of ST2 cells in some parts of the structures. It is also worthwhile that cell morphologies are similar to the RAW and ST2 cells on hydroxyapatite [267].

The ADA-GEL system has been mechanically adjusted to fit with the osteoid matrix. It could be shown for the first time that a ST2/RAW co-culture set-up biofabricated within an osteoid-like ADA-GEL hydrogel, has beneficial aspects considering the cell response. The ADA-GEL hydrogel was compatible with both cell types so that cell migration and proliferation were observed. Especially, the synthesis of OPN and VEGF is increased using the co-culture set-up. A future challenge will be the transfer of this system using a primary cell model and the evaluation of differentiation factors, which
constitutes the next step for the in vitro evaluation. For further adaptation of the
developed system to bone tissue engineering approaches, considering enhanced
mechanical properties, the bioplotting technique offers the use of sequential
processing together with a thermoplastic phase. The investigation of this kind of
process is presented in the following chapter. In general, such an osteoid-like model
could be used for further more detailed investigations, with longer incubation times,
of the mineralization process induced by the osteoblast and osteoclast crosstalk and
for studies considering therapeutic drugs for bone healing.
7 Plotting of hard-soft constructs

7.1 Introduction

Despite their numerous advantages hydrogels lack mechanical integrity for use in bone or cartilage tissue engineering [39],[93]. So, in this chapter a sequential bioplotting approach using a novel material combination of PCL-PEG blend material and ADA-GEL is presented to fabricate advanced 3D cell containing constructs. In a first step the material and plotting process parameters of different PCL-PEG blends in comparison to pure PCL were evaluated as well as the degradation behaviour, mechanical performance and cell attachment. In a second step, the pre-evaluated PCL-PEG blend was used for the sequential bioplotting with ADA-GEL containing stromal cells (ST2 cells). In vitro characterisation of the fabricated constructs was done considering cell viability, cell distribution and cell interaction with the PCL-PEG blend by migration from the hydrogel phase. Parts of this chapter contain results and discussion published in reference [34].

7.2 Hard-phase evaluation

7.2.1 Material characterisation

FTIR

In Figure 56 the FTIR spectra of PCL, PCL-PEG blends and PEG plotted plates are presented. The PCL-PEG ratios were chosen considering their suitability for the later application in the sequential bioplotting process.
Figure 56: FTIR spectra of PCL, PCL-PEG blend and PEG plotted plates. (The relevant peaks are discussed in the text.) ©2016 by [34]; licensee MDPI, Basel, Switzerland. http://www.mdpi.com/1996-1944/9/11/887

It is shown that typical PCL absorption bands attributed to the C-O and C-C stretching in the crystalline phase (at ~1295 cm\(^{-1}\)) [286], the C=O carbonyl stretching (at ~1730 cm\(^{-1}\)) [286],[287] are visible for pure PCL and the PCL-PEG blends, but not for pure PEG. The peak at around 1287 cm\(^{-1}\) is sharper for pure PEG and PCL-PEG blends [288]. The peak at around 1158 cm\(^{-1}\) attributed to ether groups [289] is sharp for the pure PEG and it is also present in the spectra of PCL-PEG blends. For pure PCL there is a slightly shifted peak at 1171 cm\(^{-1}\), which could be ascribed to the C-O and C-C stretching in the amorphous phase [286]. Peaks corresponding to both pure materials were found in the blend compositions, but there are no shifts or new peaks visible indicating possible intermolecular interactions [287],[290].

Contact angle measurements
The contact angle of the PCL-PEG blends (ratio 8020 = 58°±3°, ratio 7030 = 65°±1°) was reduced compared to pure PCL (78°), which is comparable for values reported by Won et al [73] (80°). This could be explained by the hydrophilic properties of PEG [291] compared to the hydrophobic PCL [68]. The increase of contact angle for blends 8020 to 7030 is possibly caused by not completely homogenous mixing. Hoque et al [76] reported that a PCL-PEG copolymer had a contact angle of around 40° in comparison to 90° for pure PCL. The measured difference in the contact angle has a very positive effect on the wettability of the samples, as shown Figure 57. When scaffolds made of pure PCL or PCL-PEG blend with a 7030 ratio were placed in a
beaker filled with deionised water, the PCL scaffolds swam on the surface, while the one made of the blend material sank to the bottom of the glass.

![Image](image_url)

**Figure 57**: Effect of PCL blending with PEG (30 %) on the wettability of plotted scaffolds. PCL scaffold is on the surface and PCL-PEG 70-30 scaffold on the bottom of the glass.

The hydrophobic properties of the PCL scaffold inhibit the infiltration of the water into the porous scaffold structure, so that the remaining air causes buoyancy forces, keeping the scaffold on the top. On the other hand, the more hydrophilic blend scaffold was infiltrated and sank to the bottom.

### 7.2.2 Cell adhesion and cell viability

PCL has been modified in form of PCL-PEG diblock and triblock [76] copolymers and by using blends of PCL/PLA [75] and PCL/PLGA [10] to successfully improve the cell response by overcoming the hydrophobic properties of PCL. In this study, ST2 cells were chosen to characterise the cell adhesive properties of PCL/PEG materials. ST2 cells are a bone marrow stromal cell line [197] and thus are an adequate cell model studying materials for potential use in bone TE. In Figure 58 fluorescence microscopy images of ST2 cells on PCL and PCL-PEG blends with different composition are shown after two days of incubation. The cells are attached on all three materials. On pure PCL (Figure 58 a) a higher number of single cells is visible in comparison to the blends, whereas on the 7030 (Figure 58 c) composition the cells show a more dense and more homogenous distribution than on the 8020 (Figure 58 b) composition. This result indicates that cell adhesion is possible on all compositions and is consistent with previous results for PCL-PEG copolymers [76].
In Figure 59 the viability of ST2 stroma cells seeded on PCL and PCL-PEG plotted plates is presented. The results of cell viability kinetic at several time points during the 21 days of incubation indicate that the PCL-PEG blend of 7030 composition is superior to pure PCL and to PCL-PEG 8020 blend. One possible explanation, regarding the contact angle measurements, is the improved wetting behaviour compared to pure PCL. Nevertheless, the similar contact angle of the two blend materials does not explain the cell viability of the 8020 composition in comparison to pure PCL. Thus, additionally topographic structure changes in the surface could explain the improved behaviour of the 7030 composition considering the degradation behaviour of the materials (data shown later). Patrício et al [74] showed that the blending method of PCL/PLA had an influence on the surface roughness of plotted scaffolds and so possibly on the cell performance.
Figure 59: Cell viability of PCL and PCL-PEG plates seeded with ST2 cells over an incubation period of 21 days. ©2016 by [34]; licensee MDPI, Basel, Switzerland. http://www.mdpi.com/1996-1944/9/11/887

7.3 Hard phase scaffold characterisation

7.3.1 Scaffold design data

Scaffolds consisting of pure PCL and PCL-PEG blends with a composition of 8020 as well as 7030 and with two different design approaches by varying the number of struts per layer were plotted. The process parameters were adjusted to achieve scaffolds within a comparable range considering strut width, pore size and porosity. Therefore, the processing temperature was adjusted, whereas plotting speed and pressure as two further defining processing parameters, which influence the size dimensions of a plotted strut, were kept constant [76],[77],[97]. The temperature was constantly decreased from 120°C for pure PCL, 110°C to 100°C for PCL-PEG 8020 and 90°C to 80°C for PCL-PEG 7030. The temperatures for the PCL-PEG blend are in the range of the PCL-PEG copolymer processing done by Hoque et al [76]. The temperature was decreased instead of increasing the speed or decreasing the pressure for adjusting the strut width. This was because for the later approach (discussed below) of sequential processing of PCL/PEG together with the hydrogel/cell solution, a low temperature is beneficial to ensure cell viability [96]. Images of a fabricated scaffold with a 14 lines per layer design are shown in Figure 60 a,b.
Figure 60: Stereomicroscope images of a plotted PCL-PEG (7030) scaffold as fabricated topview (a) and side view (b) (scale bar = 2 mm). ©2016 by [34]; licensee MDPI, Basel, Switzerland. http://www.mdpi.com/1996-1944/9/11/887

In Table 4 scaffold geometry data for the different scaffolds produced is listed.

Table 4: Scaffold geometry data for PCL and PCL-PEG blend scaffolds for two different design set-ups using 14 or 10 lines per layer.

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<tbody>
<tr>
<td>14</td>
<td>PCL</td>
<td>358 ± 15</td>
<td>401 ± 20</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>14</td>
<td>PCL-PEG 8020</td>
<td>425 ± 118</td>
<td>318 ± 123</td>
<td>33 ± 13</td>
</tr>
<tr>
<td>14</td>
<td>PCL-PEG 7030</td>
<td>313 ± 73</td>
<td>434 ± 67</td>
<td>52 ± 9</td>
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<tr>
<td>10</td>
<td>PCL</td>
<td>313 ± 72</td>
<td>801 ± 67</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>10</td>
<td>PCL-PEG 8020</td>
<td>380 ± 79</td>
<td>712 ± 76</td>
<td>46 ± 5</td>
</tr>
<tr>
<td>10</td>
<td>PCL-PEG 7030</td>
<td>307 ± 84</td>
<td>794 ± 104</td>
<td>65 ± 10</td>
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With the chosen processing parameters scaffolds of all material compositions had an average strut width (nozzle diameter 250 µm) in the range of 307 µm - 425 µm. The pore size depending on the strut width was between 318 µm and 434 µm for the 14 struts per layer design. The porosity of these scaffolds is between 33 % and 52 %. In addition, the 10 struts per layer design was evaluated to increase the porosity and so the volume which can be filled with the cell/hydrogel solution for the later sequential bioplotting process. For this design the pore size increased for all material compositions, which is consistent with the constant strut width. Pore sizes were between 712 µm and 801 µm. The porosity increased from 41 % to 64 % for pure PCL, from 33 % to 46 % for PCL-PEG 8020 and from 52 % to 65 % for PCL-PEG 7030. The strut width, pore sizes and porosity are in the typical range for PCL and modified PCL scaffolds fabricated by bioplotting reported in literature [75],[76].
7.3.2 Degradation study: Mass loss

The degradation of the scaffolds was investigated by measuring the mass loss during 3 days of incubation at 37 °C in a physiological buffer solution. Previous experiments have shown that the mass loss remains constant after an initial drop. The results of the mass loss study are shown in Figure 61.

![Figure 61](http://www.mdpi.com/1996-1944/9/11/887)

The pure PCL, as expected, showed no mass loss during 3 days of incubation as PCL is known to have a total degradation time up to 2 years depending on its initial molecular weight [68]. The PCL-PEG blends showed a fast degradation and a mass loss tending to be almost equal with the corresponding content of PEG being ~14 % for the PCL-PEG 8020 and ~23 % for the PCL-PEG 7030 compositions. Thus, it is obvious that most of the hydrophilic PEG is released within the first three days causing the rapid mass loss. The results are consistent with a study of Cheng et al [291] using PCL-PEG blends to establish a drug delivery system. Degradation studies on PCL-PEG and PCL-PEG-PCL copolymers showed first mass loss after 9 weeks and up to 3.3% and 7.5% weight loss at week 60, whereas pure PCL showed no weight loss at all [292]. Therefore, blending enables a fast early degradation. Lam et al [293] showed that PCL blending with TCP increases the degradation of the composite. TCP particles act as “defects” in the polymer matrix causing enhanced water absorption and so increasing the effective surface area for degradation. The increased surface area by the PEG release could possibly have the same effect and increase the long-term degradation of PCL. The accelerated degradation of the PCL-PEG support structure
will reduce the volume occupied by it in early stages and so it will give space for tissue formation [93].

### 7.3.3 Scanning Electron Microscopy

For illustrating the influence of the rapid mass loss on the external and internal morphology of the scaffold struts, SEM images are presented in Figure 62 and Figure 63. In Figure 62 a), d) overview images of the scaffold structures and in b) and e) detailed spot images of pure PCL and PCL-PEG 7030 scaffolds after fabrication are shown. The surface of PCL appeared smooth, whereas the blend showed a rougher surface. The images c) and f) indicate the differences after the three days incubation period at 37°C. The pure PCL surface is still smooth, but the blend surface shows pores and an even rougher structure.

![Figure 62: Electron microscopy images of the surfaces of PCL scaffolds as fabricated (a, b), after 3 days of incubation in HBSS (c) and PCL-PEG 7030 scaffolds as fabricated (d, e), after 3 days of incubation in HBSS (f) at 37°C. ©2016 by [34]; licensee MDPI, Basel, Switzerland.](http://www.mdpi.com/1996-1944/9/11/887)

This enhances the results of the mass loss study and the fast release of the PEG content. The increased surface roughness could possibly influence the increase in the cell viability shown in Figure 59. The development of an interconnected pore structure after the PEG release from PCL-PEG blends is also reported by Cheng et al [291]. The morphology of the pore structure in this study was dependent on the PEG content, causing discrete pores with lower PEG content and an interconnected pore structure with an increased PEG content of up to 10-30%. Further images showing cross-sections of the struts are presented in Figure 63. The cross-sections of PCL and PCL-PEG 7030 scaffolds are shown after fabrication and after 3 days incubation in
HBSS at 37 °C. There is no apparent difference in the internal structure of the PCL scaffolds before (image b) and after (image c) the incubation. The cross-section shows a homogeneous and dense morphology. However, the cross-section of the blend appears inhomogeneous, showing two phases before the incubation (image e). The morphology shows distributed round drops, possibly PEG, surrounded by a main phase, which is the PCL matrix. After the incubation, the round drops are almost not visible and a porous internal structure remains (image f), which correlates with the measured mass loss.

Figure 63: Electron microscopy images of the cross-sections of PCL scaffolds as fabricated (a, b), after 3 days of incubation in HBSS (c) and PCL-PEG 7030 scaffolds as fabricated (d, e), after 3 days of incubation in HBSS (f) at 37°C. ©2016 by [34]; licensee MDPI, Basel, Switzerland. http://www.mdpi.com/1996-1944/9/11/887

7.3.4 Mechanical testing

The influences of the rapid mass loss and the differences in the material composition on the mechanical properties, of the scaffolds in compression are shown in Figure 64 a).
Figure 64: Compressive stiffness of PCL and PCL-PEG blend scaffolds with different compositions in dependence of a) storage time in HBSS at 37°C and b) different scaffolds design. ©2016 by [34]; licensee MDPI, Basel, Switzerland. http://www.mdpi.com/1996-1944/9/11/887

The trend of a decreasing average stiffness for incubated scaffolds in comparison to as fabricated ones for both blend compositions was not significant. The PCL structure showed no decline in stiffness with values of 53 ± 13 MPa before and 60 ± 14 MPa after the incubation period. The range of values of the stiffness of PCL scaffolds is in agreement with a study of Domingos et al [294] reporting a compressive modulus of 52.5 ± 4.5 MPa for scaffolds with a comparable lay down pattern (0°/90°) and a strut distance of 550 µm. Hutmacher et al [69] measured a compressive modulus of 41.9 ± 3.5 MPa for PCL scaffolds. This slightly decreased value could be caused by a varied lay down pattern of 0°/60°/120°, as also reported by Domingos et al [294], who
reported decreased stiffness in comparison to a 0°/90° pattern, which was used in our study. No differences in the mechanical properties of pre-conditioned PCL scaffolds have been also reported by Domingos et al [294]. This issue is discussed controversially in the literature as Hutmacher et al [69] showed an influence of the pre-treatment of the scaffolds on their mechanical properties. PCL scaffolds were not stiffer than PCL-PEG 8020 scaffolds before incubation, but afterwards as the stiffness of the blend dropped from 46 ± 15 MPa to 29 ± 6 MPa, the difference was significant. Moreover, PCL scaffolds were significantly stiffer than PCL-PEG 7030 scaffolds before (21 ± 5 MPa) as well as after (11 ± 5 MPa) the incubation. There was also a significant difference between PCL-PEG 8020 and PCL-PEG 7030 scaffolds before incubation. In Figure 64 b) the stiffness of scaffolds with 14 struts or 10 struts per layer is shown. In general, the scaffolds with a higher number of struts show a higher stiffness, but this difference is only significant for the PCL scaffolds. This result can be explained by the fact that the change in the porosity caused by the switch from the 14 strut to the 10 strut design is the highest for PCL. Thus, a critical increase of the porosity leads to a decrease of the scaffolds stiffness [294]. Scaffolds with a higher number of struts have a higher number of junctions between adjacent struts, which define the resistance against the compression force in the beginning of the test [76],[294]. An increasing amount of PEG reduces the stiffness of the scaffolds. A possible reason could be a change in the crystallization behaviour of the PCL phase as the crystallisation and phase separation in PCL-PEG blends is a competitive process [295]. Schuurman et al [50] investigated the mechanical properties of PCL and PCL/alginate scaffolds fabricated via a sequential plotting process showing that the PCL phase dominates the mechanical stability of such scaffolds and that there is no significant effect of the addition of alginate. Only compared to pure alginate bulk material the mechanical stability is increased for both types of scaffolds.

7.4 Hard-soft phase scaffolds: Processing and in-vitro characterisation

7.4.1 Biocompatibility of the sequential biplotting process – heat influence

An elevated plotting temperature of up to 85 °C, which is necessary for processing PCL-PEG blends, could potentially have a negative impact on the immobilised cells in the soft hydrogel phase. Therefore, the percentage of live cells was determined as
shown in Figure 65 a) by using fluorescence microscopy images of a live-dead staining as presented in Figure 65 b), c). A dense film of ADA-GEL with ST2 cells was plotted on a cell-culture plate (= reference) and on a PCL-PEG 8020 layer, processed right before at 85 °C. The set-up should simulate the situation at the interface of the hydrogel- and the thermoplastic phase, which is possibly the region with the harshest conditions for the cells considering the heat influence.

**Figure 65:** a) Percentage of viable cells on total cell number and b) representative images of the live-dead staining of ST2 cells in ADA-GEL plotted into well plate (= reference) and c) plotted on previously deposited PCL-PEG 8020 blend to evaluate influence of the processing temperature of 85 °C of the blend on cell viability. ©2016 by [34]; licensee MDPI, Basel, Switzerland. [http://www.mdpi.com/1996-1944/9/11/887](http://www.mdpi.com/1996-1944/9/11/887)

The results show that there is no significant difference in the percentage of live cells plotted on the two different substrates. This result indicates the biocompatibility of the sequential bioplotting process at 85 °C. Kim et al [296] also reported a high percentage of live cells for a sequential bioplotting process using PCL, processed at 80 °C, and so at a comparable temperature range. Additional investigations replacing PCL with PLGA plotted at 140 °C or PLA plotted at 190 °C showed a reduced percentage of live cells with increasing processing temperature.

A thermogravic camera was used to determine the temperature profile during the processing of the PCL-PEG 8020 blend with a set temperature of 85 °C, which is comparable to the temperature used later for plotting the 7030 composition, and the ADA-GEL with a set temperature of 37 °C. In Figure 66 optical and thermal infrared
images of the head of the plotter machine carrying the cartridges containing the plotting material and the heating jackets are shown.

**Figure 66:** Optical and thermal infrared images of the head of the plotter machine carrying the cartridges filled with the material. Cartridge on the right side filled with PCL-PEG blend (heating jacket at 85 °C) and middle cartridge filled with the ADA-GEL/ST2 cells mixture (heating jacket at 37 °C).

In Figure 67 optical and thermal infrared images are presented, which visualise the temperature of the template and the deposited material during the processing of the PCL-PEG layer (a,b) as well as during the deposition of the ADA-GEL (c,d).

**Figure 67:** Optical and thermal infrared images of the plotting of the PCL-PEG 8020 layer (a,b) at 85 °C and ADA-GEL hydrogel at 37 °C (c,d) on top of the thermoplastic layer.
The results show that besides the elevated temperature, which is necessary to guarantee the flow rate of the PCL-PEG blend, the temperature of the substrate and the blend solution after the deposition is moderate at around 38 °C (Figure 67 a,b). Thus, the cooling rate is sufficiently fast enabling the deposition of the ADA-GEL/ST2 cell mixture on this PCL-PEG layer, as shown in Figure 67 c,d, without a negative effect of the heat of the PCL-PEG layer on the cells. This result agrees with the outcomes presented in Figure 65, showing high cell viability. Izadifar et al [96] also measured fast cooling rates for pure PCL processed at 80 °C.

### 7.4.2 Cell viability during the maturation phase of the hybrid constructs

In Figure 68 the long-term cell viability of the PCL-PEG ADA-GEL scaffolds over an incubation time of 28 days is shown. The cell viability increased constantly from day 3 to day 21 and balanced after day 21. This result corroborates the previous finding that the heat influence during sequential bioplotting is compatible with ST2 cells. Thus, after surviving the processing, the cells proliferated, which was confirmed by the fact that WST-8 assay signal increased during the maturation phase of the constructs.

![Cell viability of a PCL-PEG 7030 ADA-GEL construct with ST2 cells up to 28 days of incubation.](http://www.mdpi.com/1996-1944/9/11/887)

Kim et al [296] also measured an increase of the WST-8 assay signal during the maturation phase of up to 14 days of hybrid constructs consisting of PCL, processed at 80°C, as a hard phase. It becomes apparent that the temperature range of 80 °C to
90 °C avoids cell damage and does not negatively affect the long-term cell development. In their study Kim et al [296] additionally tested pure PLGA, plotted at 140 °C, as a hard phase. Such hybrid constructs showed no increase of the WST-8 assay signal during the maturation, indicating a negative influence of the elevated processing temperature on the long-term cell development. Hybrid constructs fabricated of PCL and cell-loaded alginate also showed an increase in cell number over an incubation period of 14 days [96]. The increase of the cell viability was also detected in pure ADA-GEL/ST2 cell constructs, as it was presented in Chapter 6. Thus, the sequential bioplotting process exhibited no negative influence on the long-term development of the ST2 cells. This result of the cell viability kinetic is consistent with similar studies done with human osteoblast-like MG-63 cells, which were encapsulated in ADA-GEL hydrogel bioplotted structures, as presented in Chapter 5, as well as in microcapsules [31]. Bioplotted ADA-GEL structures loaded with HCT116 cells also showed high cell viabilities after the plotting process [258].

7.4.3 Cell distribution and cell morphology

The samples were observed over the whole incubation time by optical microscopy. In Figure 69 a selection of images showing samples right after the fabrication (upper row), and at day 14 (middle row) and day 28 (bottom row) of the incubation period, is presented.
Immediately after fabrication, single, round shaped cells are visible in the hydrogel phase. With ongoing incubation time, the cells proliferated. Thus, cell agglomerates and cells with spreading morphology became visible. An interaction of the cells with the PCL-PEG phase was also observed, indicating cells migrating from the hydrogel to the support structure. This interaction and adhesion of the cells to the hard phase increased over time and can be confirmed after 28 days of incubation. The proliferation of the cells is possibly the reason for the increase of the cell viability reported in Figure 68.

For imaging the cell adhesion and cell distribution after 28 days of incubation staining of the actin cytoskeleton (red) and the cell nuclei was performed, as shown in Figure 70. In agreement with the results of optical microscopy, fluorescence microscope images confirmed that cells migrated and proliferated on and along the PCL-PEG support structure and completely covered it, as shown in the overview images in Figure 70 a) and b). The detailed spot image in Figure 70 d) shows that cells achieved a spreading morphology on the hard phase. Furthermore, the images indicate that cells proliferated. It is also observed that large cell agglomerates formed in the hydrogel, are seen in Figure 70 a). Moreover, in Figure 70 c) the whole area in between the hard phase is seen to be densely populated with cells. Figure 70 e) shows a cell agglomerate and also single spread cells in the hydrogel phase. The results show that a combination of PCL-PEG 7030 blend and ADA-GEL hydrogel enables migration of the cells from the hydrogel phase to the support structure. Thus, the hard phase is not only essential for the mechanical support, but also it affects cellular response. This result is in contradiction to studies using PCL and cell-loaded pure alginate as in this case no influence or interaction of the immobilised cells with the PCL structure was reported [96]. The present results could be explained by considering the favourable properties of the ADA-GEL in comparison to pure alginate which are related to faster degradation, influencing cell mobility, and cell-material interaction [30],[31].
It was shown that PCL-PEG blends are appropriate for sequential bioplotting applications and scaffolds were produced at relatively reduced temperature in comparison with pure PCL. The wetting and cell behaviour were improved in comparison to pure PCL. A disadvantage of PCL is its long-term stability of several years, which could eventually hinder the ingrowth of tissue [297]. The short-time degradation behaviour was increased by PEG blending in comparison to pure PCL. It remains an interesting task for the future to evaluate the long-time degradation of the scaffolds (several months). The mechanical properties were adjusted by varying the PEG content. The use of ADA-GEL instead of pure alginate enables cells to migrate leading to the effective interaction of cells also with the support PCL-PEG structure. Possibly, this behaviour is beneficial for the establishment of the interface between new developed tissue and the support structure, which will only degrade over a longer time period providing sustained (time-dependent) mechanical support for the newly formed tissue.

This concept of bioplotting hybrid constructs has shown already promising results in-vivo [297]. Thus, it seems to be a successful approach to overcome the often limited mechanical properties of hydrogels, but still to take advantage of the bioplotting concept involving cell and material deposition in one step.
8 Conclusions and Outlook

The results of the presented work contribute to current efforts to establish biofabrication approaches for the investigation of bone disease, bone remodelling mechanisms and bone regeneration. The constituents of the evaluated biofabrication approach were i) materials: ADA-GEL hydrogel in different modifications and PCL-PEG blends, ii) additive manufacturing technologies: bioplotting technique and fused deposition modelling, and iii) different cell types related to bone tissue. Within this work all process steps of a biofabrication approach have been addressed, starting from the materials selection and characterisation, the establishment and assessment of different processing strategies as well as the investigation of the cell development interacting with the hydrogel properties during the maturation phase of the fabricated constructs.

As part of the material characterisation of the ADA-GEL hydrogel system in connection to the application in the extrusion-based bioplotting technique, the rheological properties of the hydrogel were evaluated. In comparison to pure ALG, as a widely used hydrogel system in biofabrication and so an adequate reference system, the ADA was characterised by over a factor 10 lower viscosity values for equal concentrations of the solutions. This outcome could be explained by the reduced molecular weight of ADA, which could be adjusted by varying the periodate equivalent during oxidation of the ALG. It was shown that the thermoreversible gelation mechanism of the GEL component of ADA-GEL could compensate the drop of the viscosity of ADA in comparison to pure ALG, thus enabling the use of ADA-GEL in the bioplotting process. For the first time ADA-GEL was successfully applied in a bioplotting process, gaining constructs with defined geometry. Thus, the GEL, besides its positive aspects regarding improved cell adhesion properties [31], has also an important function in the bioplotting of ADA-GEL considering the shape stability after the extrusion process. Nevertheless, considering the demands of processing and cell response on bioinks, the ADA-GEL system most significant feature is the positive development of different cell types in this 3D matrix. The processing properties of the ADA-GEL, like the shape fidelity, could still be improved. These limitations could be compensated using the hard-soft plotting approach. Here the hard phase structures also act as a kind of mould or skeleton, when the ADA-GEL loaded with cells is
deposited in the free space between them. An additional future attempt to improve the shape fidelity after the ADA-GEL deposition could be the use of a cooled template to enhance the thermal gelation mechanism of the GEL component of ADA-GEL and so to give the constructs an improved temporary stability before the ionic gelation step [116]. Nevertheless, the condensation of humidity would be a risk of contamination for the process. Methacrylation enables the photocrosslinking of ADA [165] as well as GEL [118]. This could be possibly a third crosslinking mechanism besides the ADA and GEL crosslinking over the Schiff’s base formation and the ionic gelation based on the ADA component, which could be introduced to the ADA-GEL hydrogel system. This would provide the possibility of pre-crosslinking right after the hydrogel deposition being thus possible to improve the shape stability.

Further studies were done evaluating the mechanical properties of the ADA-GEL system regarding its single components (ADA and GEL) as well as assessing the influence of different crosslinking mechanisms. It was found that the mechanical performance of the ADA-GEL is mainly based on the ionic gelation mechanism of the ADA component. This gives the possibility for modification of the ADA-GEL system using other gelation ions than Ca$^{2+}$, e.g. Ba$^{2+}$, Cu$^{2+}$ or Zn$^{2+}$. Besides the adaptation of the mechanical properties and the degradation behaviour of the ADA-GEL matrix, these ions could also influence the cell response. The effect of released therapeutic ions has been discussed in the context of bioactive glasses [298], which have been also added to ADA-GEL to generate composite materials [241],[263] and so represent a very versatile alternative for modifying the ADA-GEL system. Moreover, it could be demonstrated that the mechanical properties of ADA-GEL could be tailored depending on the periodate equivalent used for ADA preparation. Decreased periodate equivalents resulted in ADA-GEL with increased rYm values. The degradation mechanisms of ADA-GEL, namely: i) GEL release and ii) hydrolytic degradation, were investigated for different ADA-GEL compositions by compiling degradation kinetics data. ADA-GEL with increased stock solution concentration of ADA and GEL showed a percentage reduction of released GEL and enhanced long term stability. The already mentioned possibilities of introducing a photocrosslinking mechanism to the ADA-GEL would be also interesting for adjusting the mechanical properties and the degradation behaviour of the hydrogels. Regarding the
degradation of the ADA-GEL/cell system, it would be also interesting to evaluate the influence of cells on degradation behaviour.

The biocompatibility of the bioplotting process was demonstrated, showing a high percentage of live cells of different origin. Interestingly, it was shown that harsh processing parameters like enhanced plotting pressures could be compensated by ST2 cells in dependence of the ADA-GEL composition surrounding them and so acting as a protective shield. In general, the processing step seems not to be the major obstacle regarding the generation of hydrogel/cell constructs. Nevertheless, the long term influence of this mechanical stimulation during processing on cell behaviour is not yet extensively in the focus of research [265].

The most important challenge seems to be the adjustment of the hydrogel properties in the maturation phase. Here tailoring of the hydrogel degradation kinetic and cell development as well as ECM secretion must be synchronised. MG-63 osteoblast like cells, an osteosarcoma cell line, was applied in the developed bioplotting approach. It was shown that the cells proliferate and show good cell-material interaction in the degrading 3D ADA-GEL matrix. MG-63 cells with spread morphology could be found throughout the 3D construct. Furthermore, the release of VEGF from MG-63 cells could be detected. This is an interesting aspect as angiogenesis is an issue in bone TE as well as cancer research, and increased levels of VEGF are relevant in this regard.

ST2 cells were biopotted employing different compositions of the ADA-GEL regarding stock solution concentrations and amount of periodate used for ADA preparation. Cell proliferation and cell morphology were investigated over incubation times up to 42 days. Higher values of the rYm and reduced degradation kinetics slowed down cell proliferation and partially hampered cell spreading. Nevertheless, a composition with an adjusted molar mass and concentration could be found for ST2 cells enabling enhanced mechanical properties, handability and proliferation of ST2 cells. In this context, advanced studies regarding the pore size distribution in the different ADA-GEL modifications would be interesting, as this is an essential parameter regarding cell migration. These possibilities to adjust the properties of the ADA-GEL and so the cell response will be relevant for fabricating constructs out of different ADA-GEL modifications, whereas certain areas are optimised for a specific cell type on a very
local scale, which is highly interesting for co-culture set-ups [37]. In this context the concept of plotting pre-fabricated microcapsules with encapsulated cells [257] could be used to reach this local compartmentalisation of the constructs with different ADA-GEL modifications, creating in this way optimised cell niches on a very local scale. Besides the mentioned possibilities for the modification of the ADA component of the ADA-GEL, also the GEL could be partially replaced with fibrous proteins like keratin, silk fibroin or elastin [133] for creating more complex cellular niches thus enhancing the versatility of the plotting process. Also the modification with peptide sequences is a choice [232]. Silk fibroin-gelatin hydrogels have already been successfully adapted for bioprinting approaches [143].

The ADA-GEL system has been mechanically adjusted to fit with the osteoid matrix and a ST2/RAW cell co-culture was biofabricated within this osteoid-like ADA-GEL hydrogel. It was shown that the co-culture of these two cell types has beneficial aspects considering the cell response. Especially, the synthesis of OPN and VEGF was increased in the co-culture set-up. The replacement of the used cell lines of this system with a primary cell model as well as the evaluation of osteogenic and osteoclastogenic differentiation factors will be a next step. Furthermore, this osteoid-like model could be used for investigations with expanded incubation times to evaluate the mineralization process induced by the osteoblast and osteoclast crosstalk. Another task will be to adapt the fabricated constructs for dynamic cultivation using bioreactors. This will be especially necessary when the construct dimensions will be increased to fit with required clinical dimensions of several cm³ to guarantee the transport of oxygen and nutrients for the immobilised cells. The implementation of a vascular system in the constructs during the bioplotting process is a further, necessary step [299] and therefore the introduction of endothelial cells will be necessary.

Considering the aspect of shape fidelity, the sequential bioplotting process using PCL-PEG blends has been already mentioned. PCL-PEG blends are appropriate for sequential bioplotting applications. The advantages in comparison to pure PCL were the improved wetting behaviour and cell behaviour. Considering that PCL is stable for several years, the ingrowth of tissue may be hindered [297]. While the PCL-PEG blends showed increased short-time degradation behaviour in comparison to pure
PCL, the long-term (in vitro / in vivo) behaviour should be investigated in future studies. The mechanical performance of this hybrid constructs is enhanced in comparison to the pure hydrogel constructs. Interestingly, an effective interaction of cells also with the PCL-PEG support structure was detected. Possibly, this behaviour is beneficial for the establishment of the interface between new developed tissue and the support structure. Moreover, this shows the importance of optimizing the support structure not only in terms of mechanical stability, but also regarding cell interactions. Other attempts have been reported to modify the hard phase in such constructs. PCL protective layers were plotted to protect the cells of the negative heat influence, while plotting PLA (190°C) and PLGA (140°C) layers in the constructs [296]. The addition of bioactive glass inclusions into PCL-PEG struts is another approach leading to improved bioactivity of the constructs intended for bone tissue engineering.

As presented, here, the application of the ADA-GEL hydrogel system in combination with the AM technique of bioplotting for biofabrication offers a huge field of possibilities in designing complex 3D tissue structures. Especially the combination of different AM techniques in one processing platform, being increasingly provided by the producers of commercial printer devices, could be an essential step forward. With the help of combining different techniques the hierarchical resolution of hybrid constructs could be increased. Especially, the addition of electrospinning to the techniques of bioplotting and fused deposition modelling has shown promising results regarding the cell behaviour in such structures [105]. Considering the very high resolution of melt electrospinning writing (MEW) in the submicron range [300] combined with an ordered filament deposition in comparison to the more random deposition provided by solution electrospinning, the integration of MEW with bioplotting on a common platform would be also beneficial.

In general, the evaluation of different hydrogels and the determination of the appropriate processing parameters are very time-consuming tasks and this represents a “bottle neck” delaying rapid progress in the field. The application of mathematical modelling and computer simulations could possibly reduce development time and gain a higher throughput [6]. This is an example for the interdisciplinary nature of the biofabrication field encompassing the expertise of cell biologists, material engineers, chemists and mechanical engineers. To reach the ambitious future goal of generating
functioning organs a further and more effective convergence and interaction of the different disciplines will be necessary.
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Appendix

Figure A 1: Reduced and inherent viscosities for a) ALG and b) ADA with high oxidation c) ADA with intermediate oxidation d) ADA with low oxidation of various concentrations.
Figure A 2: Protein concentration of different cell type set-ups after 21 days of incubation measured by Bradford test [260].

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Figure A 3: LSCM images of the actin cytoskeleton (red) and the cell nuclei (green) of the RAW single culture (a, b) and the ST2/RAW cells co-culture (c, d) after 21 days of incubation [260].

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Figure A 4: Fluorescence microscope images of the actin cytoskeleton (red) and the cell nuclei (green) of the ST2/RAW cells co-culture after 21 days of incubation [260].

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