Towards regeneration of articular cartilage

Construction and evaluation of unidirectional collagen scaffolds

Michiel W. Pot
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Towards regeneration of articular cartilage

Construction and evaluation of unidirectional collagen scaffolds

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Chapter 1

General introduction
and outline of the thesis
Articular cartilage

The human knee joint is a complex synovial joint, consisting of a joint capsule, which is stabilized by ligaments and muscles, and articular cartilage, which is a hyaline tissue covering the ends of the long bones [1-4]. Articular cartilage provides a low-friction and load-bearing surface for efficient and flexible motion of joints [2, 5, 6] with an average thickness of 2.4 mm in load bearing areas in adults [5, 7]. Cartilage consists of chondrocytes embedded in a complex extracellular matrix (ECM, Fig. 1) [8, 9]. The cells are dispersed in the ECM at a low cell density of only 1-5% of the total volume [5-7]. The matrix components are synthesized and secreted by the chondrocytes, which also regulate matrix metabolism [4, 8]. The ECM consists mainly of water (about 80%) and solid materials (about 20%), where the “solid materials” contain collagen (60%), proteoglycans (30%) and other (glyco)proteins (10%) [7]. Collagen fibers and proteoglycans have important functions in resisting shear stresses and shock absorption [8]. The proteoglycans consist of a core protein with negatively charged glycosaminoglycans (GAGs), such as chondroitin sulfate and keratan sulfate [2]. GAGs are highly negatively charged and thereby attract small cations and water. This results in high water content and load-dependent deformation of the tissue. The ECM also functions as an environment to transduce signals to cells, store growth factors and cytokines, protect chondrocytes from mechanical stress, and

Figure 1  Cross-section of healthy articular cartilage and the underlying subchondral bone (Safranin-O staining). Cells (blue) are dispersed in an extracellular matrix, consisting of water, collagen, proteoglycans and glycosaminoglycans (red). The subchondral bone (green) is found below the cartilage layer.
help cells to maintain their phenotype [8]. Although the tissue appears biologically simple due to its avascular, aneural, alymphatic nature, and low metabolic activity, articular cartilage has a complex ECM organization that is important for its biomechanical properties [4, 5, 10].

Treatment of cartilage defects and the clinical problem

Traumatic injuries and osteoarthritis can result in disruption or loss of structure and function of cartilage, and patients with such defects often suffer from progressive joint dysfunction and pain [2, 4]. These defects do not heal spontaneously due to the avascular nature of the tissue and it remains a challenge to restore articular cartilage. Knee arthroplasty is a surgical procedure that may be applied to replace the articular surface by a prosthesis. Limitations of this surgical treatment are the costs and invasiveness, and revisions may be needed in time. Treatments such as microfracture surgery [11], mosaicplasty [12] and autologous chondrocyte implantation [13] are applied to regenerate cartilage tissue. Microfracture surgery, also known as bone marrow stimulation, aims to induce a reparative response by damaging the subchondral bone plate below the cartilage lesion to initiate bleeding and releasing mesenchymal stem cells, which form neotissue [14]. This procedure may be advantageous over other regenerative approaches because patients can be treated with one-stage minimally invasive surgery and patients will therefore experience less pain, have a shorter recovery period and require shorter hospitalization time. Moreover, harvesting of autologous cells and tissues is not needed, which avoids problems like donor-site morbidity, cell culture costs, off-the-shelf availability, regulatory issues, and multiple-stage surgical procedures [15]. The clinical outcome mainly depends on patient age and defect size, and the treatment results in long-term pain relief and restored knee function in 75% of the patients with subchondral defects [4]. Despite therapy, the formed neotissue generally consists of fibrocartilage, which has inferior mechanical and biological properties compared to native cartilage [15]. The difficulty for the regeneration of hyaline cartilage by bone marrow stimulation may be explained by the lack of direct control of the chondrogenic process, and the need to regenerate more durable cartilage tissue persists. A template to guide and stimulate cartilage regeneration as addressed in this study may be the solution [16].
Cartilage regeneration using regenerative medicine and tissue engineering

Regenerative medicine and tissue engineering may offer promising alternatives and/or additions to clinical strategies that aim to restore damaged cartilage tissue. Regenerative medicine strives to restore normal body functions by repairing or replacing tissues and organs, and it encompasses various disciplines such as drug delivery, nanotechnology, proteomics, genomics, biochemistry, chemical engineering and tissue engineering. The field of tissue engineering focuses on applying scaffolds, cells and biologics to create functional tissues with the goal to restore, maintain, or improve tissue and organ function (Fig. 2) [17].

Regenerative medicine may offer a promising addition to bone marrow stimulation by the implantation of scaffolds. The general efficacy of implanting acellular scaffolds after applying bone marrow stimulation (microfracture and

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**Figure 2**  Regenerative medicine and tissue engineering aim to regenerate tissues and organs. Regenerative medicine strives to restore normal body functions by repairing or replacing tissues and organs. Tissue engineering approaches can be applied to achieve this goal, which generally encompasses the construction of scaffolds and the incorporation of cells and biologics to create functional tissues.
subchondral drilling) was demonstrated in a systematic review of literature and meta-analysis [18]. Results indicated superior cartilage regeneration after implantation of acellular scaffolds, which further improved by incorporation of biologics, compared to bone marrow stimulation alone. Although these results indicated that bone marrow stimulation in combination with the implantation of scaffolds is a promising strategy to restore cartilage tissue, the quality of neotissue does not yet resemble the tissue quality of native cartilage. Also regenerated cartilage using commercial products such as TruFit™ Bone Graft Substitute [19], ChondroMimetic™ [19] and Fin-Ceremica’s osteochondral biomimetic scaffold [20, 21] lacks the tissue quality of native articular cartilage [18]. Therefore, the need to improve implants for cartilage regeneration remains.

Unidirectional collagen scaffolds

In this thesis, the development of unidirectional collagen scaffolds and modifications for improvements related to mechanical strength, instructive cues and bioactivity were investigated. Unidirectional scaffolds are favorable to reconstruct tissues displaying an anisotropic extracellular matrix (ECM) as they provide a template for aligned ECM deposition to structurally and mechanically integrate with surrounding tissue [22, 23]. The newly formed ECM is deposited according to the scaffold structure [23, 24], which mimics the orientation of unidirectional collagen fibrils and steers the arrangement of chondrocytes in vertical columns, while joint loading should ultimately remodel the tissue to resemble the complex organization of native cartilage [5, 6]. Type I collagen is the most abundant scaffolding material in the body, and can be used to construct biocompatible, biodegradable and bioactive collagen scaffolds by lyophilization [25]. However, the use of type II collagen seems a logical choice for scaffold construction since type II collagen is the structural component in cartilage used by nature. Comparing type I and type II collagen scaffolds indicated that type I collagen scaffolds are also suitable for cartilage regeneration since dedifferentiated chondrocytes are able to redifferentiate in PLGA-type I collagen hybrid meshes [26] and gene expressions for chondrocytes were similar when cultured in type I and type II collagen scaffolds [27]. Additionally, the use type I over type II collagen to construct collagen scaffolds may be favorable since less cell migration was found in type II collagen scaffolds in vivo because cells were directed into a chondrogenic phenotype upon arrival in the scaffolds, while type I collagen scaffolds facilitated the influx of progenitor cells from the subchondral bone towards the defect site and throughout the implanted scaffolds [28]. Type I unidirectional collagen scaffolds have been implanted in osteochondral defects
where they facilitated the migration of bone marrow-derived mesenchymal stem cells (MSCs) [29].

**Improving unidirectional collagen scaffolds**

Cartilage regeneration may be improved by applying unidirectional collagen scaffolds with improved bioactivity. Regeneration guided by the unidirectional scaffold as a template and activated by incorporated biological stimuli has not been investigated [29].

MSCs sense the scaffold substrate upon arrival in the scaffold. Intrinsic structural properties of scaffolds have been shown to regulate cellular migration, attachment, viability and differentiation [30, 31]. For example, it has been shown that MSCs preferentially adhere to ridged surfaces [32] and that micro- and nanoscale features can enhance chondrogenesis [33-35]. Incorporation of 3D topographical features in unidirectional collagen scaffolds may enhance chondrogenesis of MSCs.

Bone and cartilage development is guided by various growth factors, of which the spatiotemporal presence of these biologics guides osteochondral maturation [36]. Of these biologics, incorporation of stromal cell-derived factor-1 to the scaffold may results in the attraction of MSCs from the bone marrow towards the implant [37]. Growth factors such as bone morphogenetic protein (BMP), transforming growth factor beta (TGF-β) and fibroblast growth factor (FGF) have been incorporated in scaffolds to stimulate cartilage regeneration [38]. Growth factors can be loaded to collagen scaffolds by their non-covalent interaction with glycosaminoglycans (GAGs) covalently immobilized to collagen scaffolds [39-45]. The non-covalent immobilization of growth factors to GAGs is important for growth factor signaling, controlling the dose and bioactivity, and to protect growth factors from degradation [46, 47]. Unidirectional collagen scaffolds with enhanced bioactivity may be prepared by loading of growth factors stimulating cartilage regeneration.

Various strategies to improve unidirectional collagen scaffolds were developed, as described in this thesis, and were related to (Fig. 3):

1. **incorporation of topographical features to steer chondrogenic differentiation of MSCs**
2. **enhancement of bioactivity to stimulate osteochondral regeneration**
Aim and outline of this thesis

Central concept and clinical considerations: development of clinically applicable instructive implants to regenerate cartilage

This research focuses on the construction and evaluation of instructive unidirectional collagen scaffolds. The vision of the research strategy encompasses the implementation of different components in scaffolds, which are considered important features for implants to regenerate articular cartilage. Overall, increased bioactivity in an unidirectional collagen scaffold, which facilitates infiltration of bone marrow-derived MSCs [29] and steers deposition of ECM...
according to the architecture of the template [23, 24], may result in a medical device inducing cartilage regeneration.

Unidirectional collagen scaffolds are acellular devices and can be prepared from medical-grade collagen in GMP facilities. As a new technology in clinical practice, the consideration between acellular and cell-laden scaffolds is important. By applying acellular scaffolds, the advantages of bone marrow stimulation are maintained, including off-the-shelf availability and the absence of cell culture costs [48, 49].

The overall goal of this study was to develop an off-the-shelf construct to be used in combination with bone marrow stimulation for the regeneration of articular cartilage.

In chapter 2, a systematic review and meta-analysis was performed to assess all current evidence for the efficacy of articular cartilage regeneration using acellular biomaterials implanted in the knee and ankle joint after bone marrow stimulation in animal models. This systematic review aimed to provide an overview of currently existing knowledge and identify knowledge gaps, to provide transparency on the quality of performed in vivo studies, and to aid the design of future animal studies and clinical trials.

In chapter 3, a systematic search of literature including a meta-analysis was conducted to assess the efficacy of implanting cell-seeded versus cell-free implants on cartilage regeneration.

In chapter 4, an adjustable directional freezing method to develop porous collagen scaffolds with aligned unidirectional pores is described.

In chapter 5, the effect of pore orientation in collagen scaffolds on cartilage regeneration in vitro is described.

In chapter 6, 3D topographical features were incorporated in collagen scaffolds by lyophilization, and the effect on cell differentiation is described.

In chapter 7, the bioactivity of unidirectional collagen scaffolds was improved by the incorporation of BMP2.

In chapter 8, we changed gears, and focused on another organ, the bladder, where we introduced unidirectional pores using a different methodology, allowing the construction of a large acellular spherical hollow construct.

In chapter 9, the findings of this thesis are summarized and future perspectives are indicated.
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Chapter 2

Improved cartilage regeneration by implantation of acellular biomaterials after bone marrow stimulation: a systematic review and meta-analysis of animal studies

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Abstract

Microfracture surgery may be applied to treat cartilage defects. During the procedure the subchondral bone is penetrated, allowing bone marrow-derived mesenchymal stem cells to migrate towards the defect site and form new cartilage tissue. Microfracture surgery generally results in the formation of mechanically inferior fibrocartilage. As a result, this technique offers only temporary clinical improvement. Tissue engineering and regenerative medicine may improve the outcome of microfracture surgery. Filling the subchondral defect with a biomaterial may provide a template for the formation of new hyaline cartilage tissue. In this study, a systematic review and meta-analysis were performed to assess the current evidence for the efficacy of cartilage regeneration in preclinical models using acellular biomaterials implanted after marrow stimulating techniques (microfracturing and subchondral drilling) compared to the natural healing response of defects. The review aims to provide new insights into the most effective biomaterials, to provide an overview of currently existing knowledge, and to identify potential lacunae in current studies to direct future research. A comprehensive search was systematically performed in PubMed and EMBASE (via OvidSP) using search terms related to tissue engineering, cartilage and animals. Primary studies in which acellular biomaterials were implanted in osteochondral defects in the knee or ankle joint in healthy animals were included and study characteristics tabulated (283 studies out of 6688 studies found). For studies comparing non-treated empty defects to defects containing implanted biomaterials and using semi-quantitative histology as outcome measure, the risk of bias (135 studies) was assessed and outcome data were collected for meta-analysis (151 studies). Random-effects meta-analyses were performed, using cartilage regeneration as outcome measure on an absolute 0-100% scale. Implantation of acellular biomaterials significantly improved cartilage regeneration by 15.6% compared to non-treated empty defect controls. The addition of biologics to biomaterials significantly improved cartilage regeneration by 7.6% compared to control biomaterials. No significant differences were found between biomaterials from natural or synthetic origin or between scaffolds, hydrogels and blends. No noticeable differences were found in outcome between animal models. The risk of bias assessment indicated poor reporting for the majority of studies, impeding an assessment of the actual risk of bias. In conclusion, implantation of biomaterials in osteochondral defects improves cartilage regeneration compared to natural healing, which is further improved by the incorporation of biologics.
Introduction

Articular cartilage is a specialized tissue that covers joint surfaces and provides a low-friction and load-bearing surface for a smooth motion of joints. The structure and function of the tissue can be compromised by traumatic injuries and degenerative joint diseases. Due to its avascular nature, damaged cartilage tissue does not heal spontaneously and it remains a challenge to fully restore tissue function [1, 2].

The surgical options to treat patients with a localized cartilage defect are limited to cartilage regeneration approaches such as autologous chondrocyte implantation and microfracture surgery [3, 4]. The latter strategy, also known as bone marrow stimulation, is relatively simple, minimally invasive and inexpensive. During this procedure the subchondral bone plate below the cartilage lesion is perforated to initiate bleeding and induce a reparative response. The principle behind this regenerative resurfacing strategy is the migration of non-differentiated bone marrow-derived multipotent stem cells from the subchondral bone into the defect site leading to the formation of new cartilage tissue [5-7]. Patients treated with bone marrow stimulation generally show clinical improvements up to 1.5 - 3 years after surgery. However, five years after surgery higher incidences of clinical failures are observed [8, 9]. The newly formed tissue generally consists of fibrocartilage repair tissue rather than hyaline cartilage, has limited filling of the defect, integrates poorly with the surrounding tissue and has inferior mechanical properties compared to hyaline cartilage [10]. Therefore, the need for regeneration of more durable cartilage tissue persists.

Regenerative medicine and tissue engineering may offer promising alternatives and/or additions to clinical strategies that aim to restore damaged cartilage tissue. The construction of biomaterials and the incorporation of cells and biologics in these implants have been widely investigated for this purpose. Biomaterials can be implanted in osteochondral defects created by applying marrow stimulating techniques (microfracture and subchondral drilling [11]) to guide and stimulate the formation of cartilage tissue [12]. During microfracture surgery an arthroscopic awl is used to penetrate the subchondral bone, while with subchondral drilling a high speed drill is applied to penetrate the trabecular bone. Different strategies have been applied including the implantation of biomaterials with and without cells. Acellular biomaterials offer various advantageous properties such as lack of donor-site morbidity, absence of cell culture costs, off the shelf availability, fewer regulatory issues, and application of one-stage surgical procedures [13, 14]. Many researchers have explored the approach of implanting acellular biomaterials and investigated the use of various biomaterials in vivo, such as natural (e.g. collagen [6, 15-17], chitosan [18-21],
alginate [22-24] and hyaluronic acid [4, 25-27]) and synthetic polymers (e.g. poly-caprolactone [28-30], polyvinyl alcohol [31-33] and poly(lactic-co-glycolic acid) [34-37]). To combine the advantageous properties of these materials, multilayered biomaterials (e.g. β-tricalcium phosphate-hydroxyapatite/hyaluronate-atelocollagen [2], ceramic bovine bone-gelatin/gelatin-chondroitin sulfate-sodium hyaluronate [38]), blends (e.g. poly(glycolic acid)-hyaluronic acid [7] and type I collagen-hyaluronic acid-fibrinogen hydrogel [39]) have been constructed. Biologics are natural factors that can be used to stimulate tissue regeneration, e.g. by inducing proliferation and differentiation of cells. Biologics such as growth factors of the transforming growth factor β (TGF-β) superfamily and others have been incorporated in biomaterials to guide and stimulate the formation of hyaline cartilage tissue [40]. Moreover, it has been reported that the animal model of choice may have a significant impact on study outcome of articular cartilage regeneration [41]. Currently, there is no systematic overview of the current

**Figure 1** Illustration of cartilage regeneration by implantation of biomaterials after bone marrow stimulation. The implanted biomaterials provide a template to guide cartilage regeneration by bone marrow derived mesenchymal stem cells.
literature assessing the effect of various parameters (e.g. applied biomaterials, incorporated biologics and animal models) on cartilage regeneration.

The aim of this systematic review and meta-analysis is to assess all current evidence for the efficacy of articular cartilage regeneration using acellular biomaterials implanted in the knee and ankle joint after microfracture and subchondral drilling in animal models. Additionally, we strive to provide transparency on the quality of performed in vivo studies, in order to aid the design of future animal experiments and clinical trials. We provide a systematic and unbiased overview of the current literature addressing regeneration of articular cartilage using a wide range of acellular biomaterials containing various biological cues (as illustrated in Fig. 1). Results of semi-quantitative histological scoring systems are used as a quantitative outcome parameter for outcome assessment of cartilage regeneration. Although microfracture surgery and subchondral drilling strive to stimulate cartilage and osteochondral regeneration, respectively, both are generalized in this study as cartilage regeneration. Moreover, the evaluation of different subgroups (natural and synthetic origin of the biomaterials, structure of the materials (scaffolds vs. hydrogels), incorporated biological cues, and animal models) was included to gain insights in which parameters affect cartilage regeneration and to what extent.

**Materials and Methods**

**Search strategy**
To identify relevant peer-reviewed articles, a comprehensive search of the literature using PubMed and EMBASE (via OvidSP) was conducted, using the methods defined by de Vries et al. [42] and Leenaars et al. [43]. The last search date was April 3rd 2015. In both databases, a tissue engineering search component developed by Sloff et al. [44], consisting of equivalents for tissue engineering (e.g. tissue regeneration, regenerative medicine, bio-engineering or biomatrices), was combined with a cartilage search component, consisting of equivalents for cartilage and cartilage-related surgeries (e.g. chondral, chondrogenic, surgery, microfracturing or implants). The search components were constructed using MeSH terms (PubMed) and EMTREE terms (EMBASE) and additional free-text words from titles or abstracts ([tiab] or ti,ab). The obtained tissue engineering-related and cartilage-related results were filtered for animal studies using previously described animal search filters [45, 46]. The complete search strategy is attached in Supplementary Information 1. No language restrictions were used.
Study selection
References from the PubMed and EMBASE search strategies were combined and duplicates were manually removed from EndNote, with the preference of PubMed over EMBASE. All screening phases were performed by two independent reviewers (MP and VG) and reported according to the “Preferred Reporting Items for Systematic Reviews and Meta-Analysis” (PRISMA) guidelines [47]. References were first screened based on title and were excluded based on the following criteria: 1) titles showed no relevance to regeneration of articular (hyaline) cartilage, 2) it was specifically stated in the title that the conducted experiment was an in vitro study only, 3) osteoarthritis animal models were used, 4) only ex vivo studies were performed, and 5) deceased animals were used. In case of doubt or disagreement, references were included for further screening. The second screening phase consisted of a title/abstract screening in Early Review Organizing Software (EROS, Institute of Clinical Effectiveness and Health Policy, Buenos Aires, Argentina, www.eros-systematic-review.org). References were included based on the following inclusion criteria: 1) primary study, 2) animal model, 3) bone marrow stimulation by microfracturing or creation of an osteochondral defect, and 4) biomaterial implantation. Articles were only excluded when it was specifically stated in the abstract that the study was performed without healthy animals or acellular biomaterials, or if biomaterials were not implanted in the knee or ankle joint. Articles were not excluded in case important information in the abstract was missing. These articles were assessed in the full-text screening phase. For the full-text screening, articles were included if they met all of the following inclusion criteria: 1) primary study, 2) animal model, 3) healthy animals, 4) articular cartilage regeneration, 5) knee or ankle joint, 6) bone marrow stimulation by microfracturing or creation of an osteochondral defect, and 7) implantation of an acellular biomaterial. In general, if results of the two reviewers were different, articles were discussed until consensus was reached. In case of double publication, one of the studies was removed. During the screening phase, no selection was made based on publication language. The risk of bias assessment and meta-analysis was applied to studies with a comparison between a non-treated empty defect control and biomaterial implantation, and with semi-quantitative histological scoring system results as outcome data.

Study characteristics
From the studies included after the full-text screening, the following details were obtained: general information (author and year of publication), animal characteristics (species, strain, sex, age, weight and the number of animals), information related to the surgical defect (size, depth and location), experimental conditions, biomaterial, biologics, evaluation time points and all outcome measures used, i.e.
macroscopic evaluation, semi-quantitative macroscopic evaluation, histology, immunohistochemistry, semi-quantitative histological scoring, and biomechanical tests. Data from semi-quantitative histological scorings were used in the meta-analysis (described in section 2.5). Histological scoring systems applied in different studies consisted of scoring parameters like cell morphology, Safranin-O staining, integrity of surface, thickness, surface of area filled with cells, chondrocyte clustering, degenerative changes, restoration of the subchondral bone and integrity.

**Risk of bias assessment**

A risk of bias analysis was performed to assess the methodological quality of the studies included in the meta-analysis, using an adapted version of the risk of bias tool described by Hooijmans et al. [48] (for all included studies containing a ‘non-treated empty defect’ as control group and studies using semi-quantitative histological scoring systems as outcome measure). A flowchart was constructed (Supplementary Information 2) to score for selection, performance, detection and attrition bias, where the scores ‘−’, ‘?’ and ‘+’ indicate a low, unknown and high risk of bias, respectively. The questions addressed are specified in the Supplementary Information 2. Articles were scored independently by MP and VG, and if the results of the two reviewers were different, results were discussed until consensus was reached. All articles written in Chinese (16 studies) were excluded from the risk of bias assessment only, due to limited resources to independently translate these articles by two native Chinese speakers. However, the data of these studies were extracted and used in the meta-analysis.

**Analysis preparations and meta-analysis**

**Analysis preparations**

The statistical analyses were restricted to those studies containing the outcome measure semi-quantitative histology, making a comparison between a ‘non-treated empty defect’ as control group and implanted biomaterials as experimental group. Data (mean, standard deviation (SD) and number of animals) of the control and experimental group were extracted from the studies, for all available time points. When results were not given numerically, but depicted graphically, the mean and SD were measured using ImageJ (1.46r, National Institutes of Health USA). For studies presenting results in boxplots, the mean and standard deviation were recalculated from the median, range and the sample size according the method described by Hozo et al. [49]. When data were described by a mean and confidence interval (CI), the CI was recalculated to a standard deviation by the following equation for a 95% CI [47]:

$\text{standard deviation} = \sqrt{N} \times \frac{\text{upper limit} - \text{lower limit}}{3.92}$
For some studies, data were unclear and assumptions were made, which are listed in Supplementary Information 3. To compare studies with different histological score system scales, means and standard deviations were converted to a 100% scale by dividing the result by the maximum achievable histological score and multiplying by 100%. In case of missing or unclear data, authors were e-mailed to retrieve the data. When data could not be obtained, these studies were excluded from the meta-analysis (reasons for exclusion are also given in Supplementary Information 3). Results of studies with several experimental groups were combined, following the approach described in the Cochrane Handbook, table 7.7 [47]. The same approach was followed to combine results of different animals on several time points in the same group in the same study. One study (Hamanishi, 2013 [50]) had an SD of zero, which caused problems in the analyses. Therefore, the SD was changed to 4.29, equal to the SD of the experimental group of the same study at the same time point. The resulting data were used to calculate the treatment effect and corresponding standard error (SE) per study.

Meta-analysis
The following main research question was assessed: Does an overall beneficial effect exist of implanting acellular biomaterials in osteochondral defects compared to non-treated empty defects?

First, in order to select the appropriate statistical random-effects meta-analysis model, we compared a univariate approach to the bivariate approach. In the bivariate approach, separate outcomes for control and experimental group were used with their respective SEs. The correlation between these two outcomes was modeled with a compound symmetry covariance matrix, as this resulted in a much lower Akaike Information Criterion value than the use of an unstructured covariance matrix. Results were compared with those of the univariate approach, based on the treatment effect and SE per study. Results of the univariate and bivariate approaches were very similar and we therefore proceeded with the univariate approach, when applicable in combination with likelihood ratio tests.

Restricted to the experimental groups, the following sub-questions were addressed to evaluate whether the treatment effect depended on specific variables: 1) Is there a difference between the use of natural and synthetic biomaterials?; 2) Does the structure of the biomaterials affect cartilage regeneration?; 3) Do differences among various material subgroups exist?; 4) Does incorporation of biologics have a beneficial effect on cartilage regeneration compared to control biomaterials?; 5) Do differences among subgroups of biologics exist?; 6) Do different animal models result in variations in cartilage regeneration? Results are shown as % cartilage regeneration (95% CI: [lower CI, upper CI]. Some studies have more than one experimental group. Therefore, the total number of
studies and number of experimental groups (no. of studies/groups) are provided. Sensitivity analyses were performed to evaluate the effect of time (e.g. all time points, short (≤ 8 weeks), long time points (> 8 weeks), or the maximum time point), outliers (excluding consecutively the studies with the 10% highest / lowest pooled SD, and studies with the 10% highest / lowest SE), implant location, bone marrow stimulating technique applied (microfracturing vs. subchondral drilling), language (excluding studies reported in Chinese as the risk of bias of these studies was not assessed), and excluding studies where assumptions had to be made. Based on a pilot analysis, data of all time points were used for subgroup analyses. Subgroup analyses were only performed for subgroups consisting of more than two groups.

The statistical analyses were performed with SAS/STAT® software version 9.2 for Windows, copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA. The funnel plot shows the overall outcome of the pooled effect size of each study. I² was used as a measure of heterogeneity. The forest plot was created with ReviewManager (RevMan, Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014).

Results

Search and study inclusion
The searches conducted in PubMed and EMBASE (Supplementary Information 1) resulted in 4401 and 5986 studies, respectively, leaving 6688 studies after removal of duplicates. These studies were screened by title and title/abstract, which resulted in 1088 included studies after the title screening and 517 included studies after the title/abstract screening. Screening articles by full-text and subsequently selection for studies with empty defect controls as well as semi-quantitative histology as outcome measure resulted in 283 included studies after full-text assessment, of which 151 and 135 articles could be used for the meta-analysis and risk of bias assessment, respectively (Fig. 2). The studies from Xie et al. [51], Yao et al. [52] and Zhou et al. [53] could not be retrieved as a full text and these studies were therefore excluded. An overview of all included studies after full-text assessment as well as studies included for the risk of bias assessment and meta-analysis is provided in Supplementary Information 3. All references and abbreviations can be found in Supplementary Information 4. In this table, remarks are provided related to exclusion reasons for risk of bias assessment and meta-analysis (e.g. duplicate publication and incomplete data). Assumptions made for certain studies are also stated in this table.
**Study characteristics**

The study characteristics (Supplementary Information 3) clearly show substantial variation among studies. A wide range of animal species was used, from small (rat and rabbit) to larger animal models (dog, minipig, goat, pig, sheep and horse). A large variation was observed between the ages of animals (e.g. the age of rabbits ranged from 6 weeks to >2 years). Often ages were not described or specified specifically (e.g. as adult or mature). Generally, the animals were older (range of years) in large animal models compared to animals used in small animal models (range of months). The defects were created at different locations in the knee joint, such as the trochlea, condyle (medial and lateral), femur and intercondylar fossa. In addition, a large variation was found in the dimensions of the prepared defects, e.g. the dimensions of the defects created in rabbits ranged from 4-7 mm in diameter and 0.8-9 mm in depth. Microfracture surgery and subchondral drilling

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**Figure 2** PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analysis) flowchart of the systematic search of literature.
was performed in 25 and 258 studies, respectively. The implanted biomaterials were of natural or synthetic origin or combinations thereof, and consisted of single-layered or multilayered implants or blends thereof. Implants were constructed from a wide range of materials or combinations thereof, such as collagen, chitosan, hyaluronic acid, alginate, fibrin, hydroxyapatite, poly(lactic-co-glycolic acid), polycaprolactone, poly(glycolic acid) and poly(ethylene glycol), and used in different states: scaffolds, hydrogels, or hybrid mixtures of both. Various biological cues were incorporated in the biomaterials prior to implantation or administered afterwards by injection into the knee joint, mostly growth factors of the TGF-β superfamily such as bone morphogenetic protein 2 (BMP-2) and TGF-β1, but also fibroblast growth factor (FGF) and platelet-rich plasma (PRP). The maximum follow-up time was 1 year, but studies mainly investigated relatively short-term effects of implanted biomaterials on cartilage regeneration (up to 6 months).

Risk of bias assessment

A risk of bias assessment was performed to assess risks of bias (selection, performance bias, detection and attrition bias) in studies included for the meta-analysis (Fig. 3). An overview of all scores per individual study is provided in Supplementary Information 6.

Figure 3 Risk of bias of all included studies in the meta-analysis. The green, orange and red colors depict the percentages of studies with low, unknown or high risk of bias of the total number of assessed studies. The risk of bias assessment indicated a general lack of details regarding the experimental setup, as indicated by the orange bars. The green bars represent a low risk of bias, mainly for the difference between groups at the moment of surgical intervention and addressing incomplete outcome data. High risk of bias was infrequently scored, as indicated by the red bars. Q4-Q6 are not depicted in the graph, but are described in Supplementary Information 6.
The risk of bias assessment showed that details with respect to the randomization method were not provided (Q1). It was often described that animals were randomized across different groups without describing the method of randomization, thereby limiting assessment of the adequacy of randomization and therefore the actual risk of selection bias. Another notable observation from the experimental designs studied was that only in a limited number of studies it was described that power calculations were performed, whereas sufficient power in animal experiments is a requirement for performing adequate studies. The actual power analyses were never provided in the studies. Due to a lack of information, it was also difficult to assess possible bias by differences in implantation sites (with differences in load-bearing conditions, Q2.1) and differences between groups related to the age, sex and weight of the animals at the start of the experiment (Q2.2). Generally, baseline characteristics of animals prior to implantation of biomaterials (e.g. some animals received additional surgery related to harvesting of cells for biomaterials combined with cells, Q2.3) were similar. When implanting biomaterials, no details were described on blinding different biomaterials (Q3).

Blinding of the empty defect and biomaterial conditions should be performed to limit bias. However, blinding between the empty defect and biomaterial group is impossible in case only one biomaterial is implanted. More than half of the studies conducted blinded outcome assessment while performing the histological scoring, resulting in low risk of detection bias, whereas the other studies had an unknown risk (Q7). For most studies, no incomplete outcome data were described/ found, resulting in low risk of attrition bias. For some studies, dropouts were described/ found, resulting in differences between groups and high risk of bias (Q8). Overall, the risk of bias analysis generally revealed poor reporting of the experimental design for the majority of the studies, impeding an assessment of the actual risk of bias.

Data synthesis
For an overview of the meta-analysis and results obtained, see Table 1. The histological scores of defects implanted with biomaterials and non-treated empty defects are presented as a percentage on a 100% scale, where 0% and 100% indicate poor and perfect cartilage regeneration, respectively. Data are presented as the effect (%) with 95% CI.

Overall effect biomaterial implantation
The meta-analysis indicates a significant improvement of cartilage regeneration using acellular biomaterials implanted after applying marrow stimulating techniques compared to non-treated empty defects (15.6% (95% CI: [12.6, 18.6],
p < 0.0001). The forest plot (Supplementary Information 7) depicts the outcome effect of each individual study. In 73 studies cartilage regeneration significantly improved by the incorporation of biomaterials. In 48 studies no effect was found, whereas in only 6 studies a negative effect on cartilage regeneration was observed. A similar significant effect was observed taking into account the maximum follow-up only (16.3% [13.1, 19.6], p < 0.0001). Also for short and long term follow-up cartilage regeneration was significantly improved (≤ 8 weeks: 12.5% [9.3, 15.7], > 8 weeks: 17.1% [13.9, 20.2]). No notable differences in cartilage regeneration were found between the results based on the maximum follow-up time per study versus those based on all time points per study. Therefore, further subgroup analyses were made using results from all time points together.

**Natural and synthetic materials**
The subgroup analysis assessing cartilage regeneration using materials of different origin, natural and synthetic, indicated no significant differences (p = 0.887) between natural (53.0% [49.31, 56.63]) and synthetic materials (53.7% [48.75, 58.65]).

Dividing the group of materials into subgroups allows comparison of cartilage regeneration using different biomaterials. The following subgroups were studied: 1) collagen, 2) chitosan, 3) hyaluronic acid-based biomaterials, 4) alginate, 5) fibrin, 6) bone material-based, 7) PLGA, and 8) PAMPS-PDMAAm DN hydrogel. No significant differences between the biomaterial subgroups were found (Table 1).

**Material structure**
Materials were divided in three groups based on their structure: 1) scaffolds, 2) hydrogels, and 3) blends. Cartilage regeneration was similar after use of scaffolds (53.1% [49.53, 56.74]), hydrogels (54.2% [49.39, 59.07]) and blends (55.7% [42.0, 69.3], p = 0.973.

**Biologics**
Incorporation of biologics in the biomaterials resulted in a statistically significant improvement in cartilage regeneration of 7.6% [2.1, 13.0], p = 0.007, compared to the implantation of control biomaterials. Including only those studies with a direct comparison between control biomaterials and biomaterials loaded with biologics resulted in an improved cartilage regeneration of 14.6% [5.9, 23.4], p = 0.003. Comparing various biological cues including BMP, FGF, PRP and TGF indicated no significant differences in improvement of cartilage regeneration between these biologics.
Table 1 Overview of the meta-analysis results for the main research question assessing the overall beneficial effect of implanting acellular biomaterials in osteochondral defects compared to non-treated empty defects and sub-questions evaluating the effect of specific variables on the treatment effect.

<table>
<thead>
<tr>
<th>Meta-analysis</th>
<th>No. of studies/groups</th>
<th>Subgroups</th>
<th>Cartilage regeneration (% [95% CI])</th>
<th>Mean difference (% [95% CI])</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Overall effect</td>
<td>127/400</td>
<td>Biomaterial</td>
<td>53.6 [50.7, 56.6]</td>
<td>15.6 [12.6, 18.6]</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>127/247</td>
<td>Empty defect</td>
<td>38.1 [35.1, 41.0]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Origin materials</td>
<td>76/222</td>
<td>Natural</td>
<td>53.0 [49.3, 56.6]</td>
<td>-0.73 [-6.5, 5.0]</td>
<td></td>
</tr>
<tr>
<td>39/137</td>
<td>Synthetic</td>
<td>53.7 [48.8, 58.7]</td>
<td></td>
<td></td>
<td>p = 0.887</td>
</tr>
<tr>
<td>3. Material subgroups</td>
<td>20/68</td>
<td>Collagen</td>
<td>49.5 [41.1, 57.8]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/17</td>
<td>Chitosan</td>
<td>57.5 [40.8, 74.2]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5/11</td>
<td>Hyaluronic acid</td>
<td>47.9 [31.7, 64.1]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5/16</td>
<td>Alginate</td>
<td>63.0 [46.9, 79.0]</td>
<td></td>
<td></td>
<td>p = 0.804</td>
</tr>
<tr>
<td>3/10</td>
<td>Fibrin</td>
<td>55.3 [34.4, 76.3]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5/11</td>
<td>Bone</td>
<td>51.2 [35.2, 67.2]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15/52</td>
<td>PLGA</td>
<td>58.5 [49.0, 68.0]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/21</td>
<td>PAMPS-PDMAAm DN</td>
<td>47.9 [31.7, 64.1]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Scaffold structure</td>
<td>78/258</td>
<td>Scaffolds</td>
<td>53.1 [49.5, 56.7]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41/127</td>
<td>Hydrogels</td>
<td>54.2 [49.4, 59.1]</td>
<td></td>
<td></td>
<td>p = 0.973</td>
</tr>
<tr>
<td>7/17</td>
<td>Blends</td>
<td>55.7 [42.0, 69.3]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Biologicals</td>
<td>113/291</td>
<td>No biologicals</td>
<td>51.7 [48.6, 54.9]</td>
<td>7.56 [2.1, 13.0]</td>
<td></td>
</tr>
<tr>
<td>35/109</td>
<td>Biologicals</td>
<td>59.3 [54.0, 64.6]</td>
<td></td>
<td></td>
<td>p = 0.007</td>
</tr>
</tbody>
</table>

The total number of studies and number of experimental groups included in the meta-analysis are shown (some studies have >1 experimental group, no. of studies/groups). The quality of cartilage regeneration is presented on a 100% scale, where 100% represents the maximum achievable regeneration. No significant differences were found between natural and synthetic materials, between the various material subgroups, and between the biomaterial structures (hydrogels versus scaffolds versus blends), and between animal species. ED: empty defect, B: biomaterials.
Table 1
Overview of the meta-analysis results for the main research question assessing the overall beneficial effect of implanting acellular biomaterials in osteochondral defects compared to non-treated empty defects and sub-questions evaluating the effect of specific variables on the treatment effect.

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>No. of studies/groups</th>
<th>Cartilage regeneration (% [95% CI])</th>
<th>Mean difference (% [95% CI])</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall effect</td>
<td>127/400</td>
<td>53.6 [50.7, 56.6]</td>
<td>15.6 [12.6, 18.6]</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Origin materials</td>
<td>76/222</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural</td>
<td>39/137</td>
<td>53.0 [49.3, 56.6]</td>
<td>-0.73 [-6.5, 5.0]</td>
<td>p = 0.887</td>
</tr>
<tr>
<td>Synthetic</td>
<td>37/85</td>
<td>53.7 [48.8, 58.7]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Material subgroups</td>
<td>20/68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>6/17</td>
<td>49.5 [41.1, 57.8]</td>
<td></td>
<td>p = 0.804</td>
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<tr>
<td>Chitosan</td>
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</tr>
<tr>
<td>Hyaluronic acid</td>
<td>5/11</td>
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<td></td>
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</tr>
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</tr>
<tr>
<td>Fibrin</td>
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</tr>
<tr>
<td>Bone</td>
<td>5/11</td>
<td>51.2 [35.2, 67.2]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLGA</td>
<td>15/52</td>
<td>58.5 [49.0, 68.0]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAMPS-PDMAAm DN</td>
<td>6/21</td>
<td>47.9 [31.7, 64.1]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scaffold structure</td>
<td>78/258</td>
<td>53.1 [49.5, 56.7]</td>
<td></td>
<td>p = 0.9734</td>
</tr>
<tr>
<td>Hydrogels</td>
<td>1/127</td>
<td>54.2 [49.4, 59.1]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blends</td>
<td>7/17</td>
<td>55.7 [42.0, 69.3]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biologicals</td>
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</tr>
<tr>
<td>Biologicals</td>
<td>78/222</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP</td>
<td>9/35</td>
<td>56.6 [-6.3, 119.6]</td>
<td>18.7 [-0.0, 37.3]</td>
<td>p = 0.780</td>
</tr>
<tr>
<td>FGF</td>
<td>5/20</td>
<td>51.8 [-43.9, 147.4]</td>
<td>3.1 [-13.2, 19.4]</td>
<td></td>
</tr>
<tr>
<td>PRP</td>
<td>8/14</td>
<td>55.9 [-20.9, 132.8]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF</td>
<td>6/16</td>
<td>60.2 [-7.5, 128.0]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs</td>
<td>3/5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goats</td>
<td>5/13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macaques</td>
<td>1/3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minipigs</td>
<td>10/20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbits</td>
<td>94/333</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>13/23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The total number of studies and number of experimental groups included in the meta-analysis are shown (some studies have >1 experimental group, no. of studies/groups). The quality of cartilage regeneration is presented on a 100% scale, where 100% represents the maximum achievable histological score and thus the best cartilage regeneration. Implantation of biomaterials significantly improved cartilage regeneration compared to non-treated empty defects, which was further improved by the incorporation of biologics. No significant differences were found between natural and synthetic materials, between the various material subgroups, and between the biomaterial structures (hydrogels versus scaffolds versus blends), and between animal species. ED: empty defect, B: biomaterials.
Animal models
Evaluation of the animal models used showed no significant differences ($p = 0.348$) between the effects of biomaterials implanted in dogs, goats, macaques, minipigs, pigs, rabbits, rats or sheep (Table 1).

Sensitivity analyses
Sensitivity analyses were performed to assess the robustness of the meta-analysis with respect to the overall effect. The sensitivity analyses indicated that exclusion of studies with assumptions and studies written in Chinese (no risk of bias assessment analyzed) had no effect on the estimated difference in biomaterial regeneration. Moreover, including only studies with SDs or SEs in the 10-90% range did not notably change the overall outcome effect. In a post-hoc analysis, we investigated cartilage regeneration using biomaterials implanted at different locations including condyles, femur, intercondylar fossa and the trochlea. No differences were found comparing these implant sites ($p = 0.143$). In another post-hoc analysis, we compared cartilage regeneration of empty defects or defects filled with biomaterials after applying microfracturing or subchondral drilling. For empty defects ($p = 0.152$) and biomaterial implants ($p = 0.063$) no significant differences between the two bone marrow stimulating techniques were found.

Figure 4 Funnel plot of included studies to assess the overall effect of the implantation of acellular biomaterials compared to non-treated empty defect controls. The figure indicates no substantial asymmetry.
Publication bias
A funnel plot (Fig. 4) was prepared for all included studies to analyze the overall comparison between acellular biomaterials and non-treated empty defect controls. No extensive asymmetry was observed, indicating an absence of considerable publication bias.

Discussion
The regeneration of damaged cartilage has been widely investigated using preclinical models. However, the efficacy of cartilage regeneration using implantation of acellular biomaterials has never been assessed using a systematic review and meta-analysis. This systematic review aimed (a) to provide an overview of currently existing knowledge and identify knowledge gaps, (b) to provide transparency on the quality of performed in vivo studies, and (c) to aid the design of future animal studies and clinical trials. The results could provide insight in strategies for future (pre) clinical research related to biomaterial properties, incorporation of biologics, choice of a suitable animal model, and their effects on cartilage regeneration.

The general findings of this systematic review and meta-analysis are that the implantation of biomaterials improves cartilage regeneration compared to non-treated osteochondral defects by 16% (95% CI). There were only six out of 151 studies that showed a negative effect of biomaterial implantation on cartilage regeneration. In 48 studies no significant effect on cartilage regeneration was found. For those studies with improved cartilage regeneration (73 studies), clinical studies will have to confirm the beneficial effect of implantation of biomaterials on cartilage regeneration in human patients. Filardo et al. described the implantation of an osteochondral biomimetic scaffold consisting of a type I collagen cartilage-like layer, a type I collagen/hydroxyapatite intermediate layer, and a mineralized blend of type I collagen and hydroxyapatite as a subchondral bone compartment, to treat patients with osteochondritis dissecans. For these patients, clinical scores improved significantly after the first two years and evaluation by MRI indicated good defect filling and implant integration, but also heterogeneous tissue regeneration and changes of the subchondral bone [54]. In two studies included in this systematic review and meta-analysis, this osteochondral biomimetic scaffold was also implanted in sheep. Cartilage regeneration after six months was 81.8% ± 8.9% (empty defect: 23.2% ± 20.7%) and 81.2% ± 5.1% (empty defect: 23.4% ± 6.7%). A direct comparison between the degree of cartilage regeneration described in the preclinical studies and clinical study is not possible since no histological results were described in the clinical study. In
addition, outcome measures used in preclinical studies may not predict the clinical outcome. For example, a randomized controlled clinical trial with BST-CarGel, a chitosan-based medical device, showed greater lesion filling and superior repair tissue quality compared to bone marrow stimulation after twelve months implantation, but without notable clinical differences related to pain, stiffness and physical function between both groups [55]. A remarkable observation is the difference in follow-up between the studies, which may explain the good histological scores in the preclinical studies after six months and heterogeneous tissue regeneration and changes of the subchondral bone after two years in human patients. In general, clinical studies demonstrated improved cartilage regeneration by the implantation of biomaterials after bone marrow stimulation, but there is still room for improvement regarding clinical outcome and tissue quality.

The only subgroup analysis that showed a statistically significant result between the groups was between control biomaterials and biomaterials loaded with biologics. In future clinical studies assessment of the beneficial properties of implanting biomaterials loaded with biologics is of interest, since a significant improvement of 8% (95% CI) compared to control biomaterials was found and even 14.6% when using studies that directly compared biomaterials with and without biologics. We were not able to perform analyses for the effect of the concentration or subtype of the growth factors due to the small size of these subgroups, although these factors may have a large effect on the outcome. In the study by Ishii et al. a positive effect of FGF-2 was observed by the addition of at least 183 ng to the biomaterials [56], while Maehara et al. showed significant improvements of impregnating biomaterials in 10 µg/ml and not for 100 µg/ml FGF-2 [57]. Loading biomaterials with different BMPs including BMP-2 [4, 58-61] and BMP-7 [62], or TGF subtypes including TGF-β [22] and TGF-β1 [58, 59], resulted in significantly improved cartilage regeneration. However, for clinical application of these medical devices, one should take safety of the products into account as side effects of TGF-β in a joint environment, including fibrosis and osteophyte formation, have been described [63] and patients suffered from major complications after spinal surgery and implantation of high concentrations of BMP/INFUSE [64].

The study characteristics of all included studies were tabulated to provide an extensive overview of the available literature. Besides the internal validity of the studies, the generalizability (external validity) of the study results is of great importance. The latter is affected by factors related to the animal model (species, strain, weight, age, and sex), surgery (location and size of the defect) and follow-up, resulting in heterogeneity between studies. This was also indicated by the relatively high level of heterogeneity (I²) for the main meta-analysis (99.4% [99.4,
99.4%), and the heterogeneity was almost similar for subgroup analyses. We chose to include only healthy animals receiving biomaterials. The screened studies also contained osteoarthritis models that were not included, which may be relevant for future applications to treat patients with osteoarthritis. Therefore, results from this systematic review and meta-analysis may be different compared to results found for osteoarthritis models and future clinical studies with osteoarthritis patients. We assumed that in order to assess the effect of implanted biomaterials on cartilage regeneration, reduction of the influence of confounding parameters would aid the validity of the results and conclusions. In this study, the meta-analysis included all available data of the effect of implanting biomaterials after applying bone marrow stimulating techniques (microfracture and subchondral drilling) compared to empty defects on cartilage regeneration. During microfracture surgery the subchondral bone is penetrated using an arthroscopic awl, whereas during subchondral drilling the trabecular bone is penetrated using a high speed drill, which may result in thermal necrosis [11]. Remarkably, more studies applied subchondral drilling (258 studies) compared to microfracture surgery (25 studies), while microfracture surgery was developed to overcome problems associated with thermal necrosis from subchondral drilling in the treatment of human patients [65]. We did perform a post-hoc meta-analysis to investigate differences in cartilage regeneration after applying both marrow stimulating techniques and subsequent implantation of biomaterials, which resulted in no significant differences between microfracturing and subchondral drilling. A reason for the larger number of animal studies performing subchondral drilling compared to microfracture surgery may be the ease to perform subchondral drilling over microfracture surgery in animals. Although in the included studies various implant locations (i.e. trochlea and condyles) were used, we grouped the results in the meta-analysis. A post-hoc subgroup analysis was performed to compare defect locations, but no overall significant differences were found for biomaterials implanted at different implant locations. Our analysis did not confirm a finding of Chen et al. showing improved chondrogenesis in trochlear versus condylar cartilage defects after bone marrow stimulation in rabbits [66]. This may be explained by various parameters affecting the degree of cartilage regeneration at different implant locations, such as the animal model, follow-up period and rehabilitation protocol.

Different outcome measures such as macroscopic and histological evaluation, semi-quantitative macroscopical and histological evaluation using scoring systems, histomorphometry, PCR and biochemical assays were used to assess the regenerative potential of implanting biomaterials. In this systematic review and meta-analysis, only data from semi-quantitative histological scoring systems were used as outcome measure. We chose to use these data as most authors
presented their results by this method and it allows quantitative comparison of different studies in a meta-analysis. Various histological scoring systems have been used by the authors of included studies, such as the O’Driscoll, Pineda, Wakitani and ICRS scoring system, which were also reviewed by Rutgers et al. [67]. Depending on the histological scoring system, parameters such as cell morphology, matrix staining, surface regularity, structural integrity, defect filling and the restoration of the subchondral bone were evaluated. A limitation of this outcome measure is that the specific topics addressed in the scoring systems greatly differ, i.e. some studies focus on the regeneration of cartilage only, cartilage as well as subchondral bone, or include a biomaterial component (e.g. scoring degradation of the implant). Other outcome measures including macroscopic evaluation, biochemical analysis and biomechanical aspects of the tissue may complete the overview of the tissue quality and provide valuable insights in articular cartilage regeneration, but these outcome measures were only used in a limited number of studies, and therefore not assessed in this analysis.

The risk of bias assessment provided insights in the quality of the experimental design of the studies. Most studies scored a low or unknown risk of bias, however, also little high risk of bias was scored. Low methodological quality (internal validity) may result in an overestimation or underestimation of the intervention effect [68]. In general, details regarding the randomization procedure were not described. Moreover, an observation during the risk of bias assessment was that only few studies included in the systematic review described that power calculations were performed, which is a crucial aspect in conducting experimental studies to ensure sufficient power of experimental designs. As a consequence, studies may lack sufficient power and thereby run the risk of false negative results. Due to the poor reporting of the experimental design for the majority of the studies the assessment of the adequacy of randomization and power calculations, and thus the assessment of the actual risk of selection bias, was inadequate. However, it may also hold true that studies were well designed but there was only poor reporting of the experimental designs [69]. Most researchers scoring the histology sections were blinded and sections were randomized. However, when biomaterials are not (completely) degraded, blinding between biomaterials and empty defects is practically impossible. A lack of blinding of outcome assessors implies the risk of detection/observer bias [70]. Bias may have been introduced by the lack of blinding and randomization and detracts from the overall validity of the results [71, 72]. There is a risk that the positive results found are an overestimation of the true effect of using biomaterials. Introducing standardized protocols such as the golden standard publication checklist [73] or the ARRIVE guidelines [74] may improve reporting of animal studies.
Funnel plots represent the precision of the measured effects, which increases by an increase in study size. Therefore, for small and large studies scatter will be relatively large and little, respectively. As a consequence, generally, in the absence of bias the plot resembles a symmetrical pyramid (a funnel) [47]. An important limitation may be publication bias, since multiple studies were included from the same author and negative results may not be published. It was described in a study by ter Riet et al. that researchers themselves estimate that only 50% of the conducted animal experiments are published. This problem may be solved by statistical corrections for publication bias [75]. In our study, the funnel plot did not show asymmetry and therefore did not indicate the presence of publication bias.

The translational value of animal studies depends on the comparability to the clinical situation. One of the limitations of the performed animal experiments is the short follow-up times. The maximum follow-up time was one year, but most studies investigated cartilage regeneration up to 6 months. This limits the translational value since clinical improvements in humans are generally observed up to 15 - 3 years after microfracture surgery [8, 9]. Moreover, many variations were present in the applied animal models, i.e. animal characteristics (species, strain, sex, age, weight), surgical defects (size, depth and location), applied biomaterials, and incorporated biologics. A review by Chu et al. [76] extensively reflects on benefits and limitations of different animal models used in cartilage repair studies. They state that for humans the volume of a cartilage defect is approximately 550 mm³ and treatment is required for defects with a surface larger than 10 mm². Due to the limited joint size of many animals, larger animal models such as minipig, goat and horse therefore offer superior translational value than smaller animals such as rats, rabbits and dogs. However, all studies contained defect volumes smaller than 550 mm³ and only few studies had defects surfaces larger than 10 mm². Additionally, cartilage thickness differs among various species, with goat, rabbit, minipig and dogs having thinner cartilage than humans. Another drawback for some animal models is the large endogenous repair potential. In humans, untreated defects show little to no regeneration while rabbits display a large regenerative potential, limiting clinical translation. Dog, goat, minipig and horse do not have this large endogenous repair and the use of these animals may therefore be favorable. The maturity of the animals is of great importance when designing animal experiments since open growth plates can impede with the applied treatment. Animal species are skeletally mature at different ages; i.e. rabbits at the age of 16-39 weeks, pigs at 42-52 weeks, dogs at 12-24 months, sheep and goat at 24-36 months and horses at 60-72 months [76, 77].

In this study we did not group studies based on animal maturity. In addition to clinical relevance, other reasons to select an animal model are related to logistical, financial, and ethical considerations. A systematic review conducted by Ahern et
al. [77] investigated the strengths and shortcomings of different animal models and compared these with common clinical lesions in clinical studies. They remarked that smaller animal models are often used due to feasibility, while large animal models may more closely resemble humans. However, no differences were found between animal models in this systematic review and meta-analysis, which may be explained by various parameters affecting the degree of cartilage regeneration such as implant location, defect size, follow-up period and rehabilitation protocol.

In this systematic review and meta-analysis the efficacy of cartilage regeneration using acellular biomaterials was compared to the natural healing response of defects treated with microfracture surgery and subchondral drilling. The risk of bias assessment indicated poor reporting in animal studies, which may be improved in future animal studies. Moreover, to improve the translation towards clinical trials animal experiments should be comparable to the clinical situation. As described in this systematic review a relatively high level of heterogeneity exists between studies related to the animal model, surgery and follow-up, with a need to resemble current clinical settings more closely. In this study we only addressed bone marrow stimulating techniques (microfracture and subchondral drilling) and subsequently the incorporation of biomaterials, but also the regeneration of partial thickness cartilage defects may be beneficial to prevent progression to full-thickness cartilage defects, limit the progression towards osteoarthritis and improve quality of life in patients. In many studies also cell-laden biomaterials have been implanted and the beneficial effect of cellular biomaterials versus acellular biomaterials and the natural healing response has been studied. Although acellular biomaterials offer various advantageous properties over cellular biomaterials such as no donor-site, no cell culture, off the shelf availability, less regulatory issues, and application of one-stage surgical procedures [13, 14], studying the additive value of cellular biomaterials may aid further improvement of marrow stimulating techniques.

**Conclusion**
The systematic review and meta-analysis resulted in a structured, thorough and transparent overview of literature related to the current evidence for the efficacy of cartilage regeneration using acellular biomaterials implanted after microfracturing in animal models. Cartilage regeneration is more effective by implantation of acellular biomaterials in microfracture defects compared to microfracturing alone. The efficacy is further improved by the incorporation of biologics.
**Acknowledgements**

We thank Jie An (Department of Biomaterials, Radboud Institute for Molecular Life Sciences, Radboud university medical center) for full-text screening articles written in Chinese. Gerrie Hermkens from the Radboud university medical center medical library is greatly acknowledged for help retrieving full text studies.

**Supplemental Information**

All supplemental Information are available at http://dx.doi.org/10.7717/peerj.2243#supplemental-information.
References


Supplementary Information 1. PubMed and EMBASE search strategies.

### Animal models

**PubMed**


**EMBASE**

Supplementary Information 1. PubMed and EMBASE search strategies.

Animal models

EMBASE

OR bombina OR epidalea OR calamita OR salamander OR salamanders OR newt OR newts OR triturus OR reptilia OR reptile OR
reptiles OR bearded dragon OR pogona OR vitticeps OR iguana OR iguanas OR lizard OR lizards OR anguis fragilis OR turtle OR
turtles OR snakes OR snake OR aves OR bird OR birds OR quail OR quails OR cotumix OR bobwhite OR colinus OR virginianus OR
poultry OR poultries OR fowl OR fowls OR chicken OR chickens OR galus OR zebra finch OR taeniopygia OR guttata OR canary
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OR owl monkeys OR dourocoulis OR aotus OR spider monkey OR spider monkeys OR atele OR baboon OR baboons OR papio
Cartilage regeneration using acellular implants

### Supplementary Information 1. PubMed and EMBASE search strategies.

#### Animal models

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#### Tissue Engineering

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#### Cartilage

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Supplementary Information 2.
Methodological quality assessment: questions and scoring flowchart.

Questions
1) Randomization: Was the allocation sequence adequately generated and applied (selection bias)?
2) Baseline characteristics: Were the groups similar at baseline (selection bias)?
   Were all groups implanted in one animal and where there differences in load bearing between implantation sites (2.1), and if not all groups were in one animal, was randomization adequately performed?
   How were animal characteristics divided over the groups (2.2)?
   Where there differences between groups at the moment of surgical intervention (2.3)?
3) Implantation concealment: Was the allocation adequately concealed (selection bias)?
4) Random housing: Were the animals randomly housed during the experiment (performance bias)?
5) Blinding: Where the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiments (performance bias)?
6) Random outcome assessment: Were the animals selected at random for outcome assessment (detection bias)?
7) Analysis blinding: Was the outcome assessor blinded (detection bias)?
8) Incomplete outcome data: Were incomplete outcome data adequately addressed (attrition bias)?
Flowchart

1) Was the allocation sequence adequately generated and applied?
   - Allocation sequence specified?
     - Yes
     - No
       - But not random
       - Details?
         - Yes
         - No
           - Partial
           - Complete

2) Were the groups similar at baseline or were they adjusted for confounders in the analysis?
   - Part 1: # conditions per animal?
     - Not all conditions in one animal?
       - One condition per animal?
         - Answer question 1 (allocation)?
           - Yes
           - No
             - Difference in load-bearing between conditions?
               - Yes
               - No
                 - Randomization adequately performed?
                   - Yes
                   - No
                     - Drop out mentioned?
                       - Yes
                       - Not sure
                       - No
                     - Intensive information (%) scaffolds implanted blinded?)
                       - Yes
                       - Not sure
                       - No
   - Part 2: Dividing of characteristics over groups (sex, age, weight)?
     - Not all conditions in one animal?
       - One condition per animal?
         - Yes
         - No
           - Extensive information
             - Yes
             - Not sure
             - No
   - Part 3: Difference between groups at moment of surgical intervention?
     - Yes
     - No

3) Was the allocation adequately concealed?
   - Is there more than 1 scaffold condition and an empty defect?
     - Empty defect and 1 scaffold condition?
       - Empty defect and multiple scaffold conditions?
         - Not enough information
         - Extensive information (e.g., scaffolds implanted blinded)

4) Were the animals randomly housed during the experiment?

5) Were the caregivers and/or investigators blinded from knowledge which intervention each animal received during the experiment?

6) Were the animals selected at random for outcome assessment?
   - In general, for this type of in vivo studies these domains are not essential to be assessed. Therefore, only specific examples were reported in footnotes.

7) Was the outcome assessor blinded?
   - Reviewer(s) blinded?
     - Yes
     - Not sure
     - No

8) Were incomplete outcome data adequately addressed?
   - Dropout mentioned?
     - Yes
     - Not sure
     - No
   - Difference between groups?
     - Intervention related?
       - Yes
       - No
         - Drop out mentioned?
           - Yes
           - Not sure
           - No

In general, for this type of in vivo studies these domains are not essential to be assessed. Therefore, only specific examples were reported in footnotes.
### Supplementary Information 7. Forest plot

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<th>Mean Difference IV, Random, 95% CI</th>
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Cartilage regeneration using acellular implants

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Kawaguchi et al. 2011 22.59 [12.56, 32.62]  
Makina et al. 2013 23.61 [14.53, 32.20]  
Schragermenn et al. 2009 23.03 [12.79, 34.47]  
Ban et al. 2010 24.51 [17.24, 31.78]  
Igarashi et al. 2012 24.64 [18.00, 30.46]  
Jagdizinski et al. 2014 25.00 [17.69, 32.31]  
Li et al. 2015 25.62 [21.81, 29.43]  
Fukui et al. 2014 26.01 [18.80, 43.16]  
Tarnai et al. 2005 26.11 [18.33, 33.80]  
Forhoeve et al. 2014 26.42 [5.13, 57.97]  
Boulegava et al. 2012 27.48 [19.21, 35.76]  
Nagura et al. 2007 27.66 [17.80, 37.48]  
Zha et al. 2008 27.73 [23.20, 32.16]  
Tomkova et al. 2010 27.81 [17.40, 38.22]  
Zhang et al. 2010 27.81 [23.16, 32.46]  
Eggenout et al. 2009 27.96 [8.39, 47.53]  
Ichii et al. 2007 28.57 [13.59, 43.56]  
Ogasawara et al. 2012 29.47 [10.86, 48.06]  
Huth et al. 2014 30.09 [17.66, 42.31]  
Gupta et al. 2014 30.60 [19.28, 42.04]  
Mori et al. 2013 31.32 [20.21, 42.43]  
Kasimura et al. 2011 31.66 [24.85, 38.47]  
Dai et al. 2014 32.04 [27.84, 39.57]  
Giorlando et al. 2011 32.78 [22.98, 42.56]  
Reyes et al. 2013 33.66 [17.79, 49.51]  
Luchowskii et al. 2006 34.39 [28.80, 41.70]  
Reyes et al. 2010 36.04 [25.97, 46.11]  
Reyes et al. 2012 36.22 [28.34, 46.16]  
Dai et al. 2016 40.43 [35.34, 45.52]  
Jiang et al. 2013 42.20 [25.49, 58.91]  
Sakata et al. 2014 42.98 [33.37, 51.90]  
Ko (2) et al. 2010 46.61 [39.63, 54.15]  
Woeber et al. 2009 46.66 [33.67, 59.65]  
Cui et al. 2000 47.62 [44.91, 50.33]  
Zhang et al. 2013 48.28 [38.87, 57.69]  
Ghosn et al. 2014 48.69 [39.02, 57.36]  
Cui et al. 2013 49.09 [41.21, 56.96]  
Ko et al. 2010 58.58 [42.95, 74.21]  
Zhang et al. 2014 64.31 [53.97, 74.65]  

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Chapter 3

Augmented cartilage regeneration by implantation of cellular versus acellular implants after bone marrow stimulation: a systematic review and meta-analysis of animal studies

Michiel W. Pot | Toin H. van Kuppevelt | Veronica K. Gonzales | Pieter Buma
Joanna in ’t Hout | Rob B.M. de Vries | Willeke F. Daamen
Abstract

Bone marrow stimulation may be applied to regenerate focal cartilage defects, but generally results in transient clinical improvement and formation of fibrocartilage rather than hyaline cartilage. Tissue engineering and regenerative medicine strive to develop new solutions to regenerate hyaline cartilage tissue. In this systematic review and meta-analysis, we provide a comprehensive overview of current literature and assess the efficacy of articular cartilage regeneration by implantation of cell-laden versus acellular biomaterials in the knee and ankle joint after bone marrow stimulation in animal models. PubMed and EMBASE (via OvidSP) were systematically searched using tissue engineering, cartilage and animals search strategies. Included were primary studies in which cellular and acellular biomaterials were implanted after applying bone marrow stimulation in the knee or ankle joint in healthy animals. Study characteristics of all included studies (146 studies out of 7354 studies found) were tabulated and for those studies applying semi-quantitative histology as outcome measure, outcome data were collected for meta-analysis (117 studies). Cartilage regeneration was expressed on an absolute 0-100% scale and random effects meta-analyses were performed. Implantation of cellular biomaterials significantly improved cartilage regeneration by 18.6% compared to acellular biomaterials. No significant differences were found between biomaterials loaded with stem cells and those loaded with somatic cells. Culture conditions of cells did not affect cartilage regeneration. Cartilage formation was reduced with adipose-derived stem cells compared to other cells, but still improved compared to acellular scaffolds. Assessment of the risk of bias was impaired due to incomplete reporting for most studies. Implantation of cellular biomaterials improves cartilage regeneration compared to acellular biomaterials.
Introduction

Articular cartilage facilitates joint loading and movement by resisting compressive and shear forces [1]. For patients, localized cartilage defects can have detrimental long term effects such as joint dysfunction, pain, and degenerative osteoarthritis. Once cartilage is damaged, the tissue does not heal spontaneously due to its avascular nature [2]. Clinical treatments to heal full-thickness cartilage defects and osteochondral lesions include bone marrow stimulation techniques, such as microfracture and subchondral drilling, and autologous chondrocyte implantation. The defect size generally determines the treatment applied, where microfracture and autologous chondrocyte implantation can be used to treat small (< 2.5 cm²) and large lesions (> 2.5 cm²), respectively [3]. Microfracture surgery is an attractive procedure as it is a relatively simple, minimally invasive and inexpensive one-step approach. During the procedure multiple perforations, so-called microfractures, are made in the subchondral bone plate to induce bleeding and provoke a reparative response in the cartilage defect. The formed blood clot consists of bone marrow-derived mesenchymal stem cells (BM-MSCs), growth factors and other proteins, providing an environment supportive of cartilage formation [4]. The repaired tissue, however, generally consists of fibrous cartilage, which lacks the mechanical properties of native hyaline cartilage [5]. Microfracture only results in temporary clinical improvement [6], and the demand for improved cartilage regeneration persists.

Cartilage regeneration may be improved by applying tissue engineering and regenerative medicine (TERM) approaches in addition to bone marrow stimulating techniques. TERM encompasses the development of biomaterials, which can be loaded with cells and biologics [7]. Upon implantation of these biomaterials and infiltration of BM-MSCs, the biomaterial may act as a template to guide and stimulate cartilage regeneration [3]. In a previous systematic review and meta-analysis, we showed that in animal models the implantation of acellular biomaterials in addition to bone marrow stimulation was more effective in regenerating cartilage in vivo than bone marrow stimulation alone, and the efficacy was further improved by the incorporation of biologics [8].

Bone marrow stimulation may be even more effective in case the biomaterials are loaded with cells. Implantation of biomaterials loaded with cells after applying bone marrow stimulation has been widely investigated in vivo, and included loading of chondrocytes [9-11], BM-MSCs [12-14], synovium-derived mesenchymal stem cells (SD-MSCs) [15-17], adipose-derived stem cells (ADSCs) [18-20], periosteal cells [21, 22], fibroblasts [23], umbilical cord stem cells (UCSC) [23, 24] and embryonic stem cells (ESC) [25]. Cells are either used directly after harvesting [26, 27] or after an additional in vitro step to expand [28, 29] and/or differentiate the cells [30, 31].
In this systematic review and meta-analysis, we present a comprehensive, unbiased overview of all current literature regarding the regeneration of articular cartilage by implantation of cell-laden versus cell-free biomaterials in the knee and ankle joint after bone marrow stimulation in animal models (as illustrated in Fig. 1). At present, a complete overview is lacking and the efficacy of cartilage regeneration by these approaches has not been investigated using a systematic review and meta-analysis. Since strategies to heal cartilage defects are in theory also applicable for osteochondral lesions [3], microfracture and subchondral drilling were in the context of this SR both regarded as cartilage regeneration.

**Figure 1** Illustration of articular cartilage regeneration by implantation of cellular and acellular biomaterials after applying bone marrow stimulation. The figure was adapted from Pot et al. [8].
We further investigated the effect of loading biomaterials with (1) stem cells versus somatic (differentiated) cells, (2) different cell types (e.g. chondrocytes, MSCs and ADSCs), and (3) culture conditions of cells (e.g. direct use after harvesting, in vitro expansion and/or differentiation). In the meta-analysis, histological scores from semi-quantitative histological scoring systems were used as outcome data to assess the effect on cartilage regeneration. Moreover, we aimed to provide transparency on the quality of included in vivo studies, to make suggestions on how to improve the design of future animal studies and eventually clinical trials, and to provide insights in strategies for future (pre) clinical research related to cellular or acellular approaches.

**Materials and Methods**

**Search strategy**

An extensive literature search was performed in PubMed and EMBASE (via OvidSP) to identify relevant peer-reviewed articles, according to the methods described by de Vries et al. [32] and Leenaars et al. [33]. Articles were searched until June 29, 2016. The search strategy consisted of search components for tissue engineering [34] and cartilage [8]. The search components were developed using synonyms for tissue engineering and cartilage, and were constructed using MeSH terms (PubMed) and EMTREE terms (EMBASE), as well as free-text words from titles or abstracts ([tiab] or /ti,ab.) [8]. Subsequently, results were refined for animal studies by applying animal search filters [35, 36]. No language restrictions were applied.

**Study selection**

After obtaining all references through searches in PubMed and EMBASE, duplicates were manually removed in EndNote X7 (Thomson Reuters, Philadelphia, Pennsylvania) by one review author (MP). Resulting references were screened for their relevance by two independent reviewers (MP and VG/WD) based on (1) title, (2) title/abstract and (3) full-text using Early Review Organizing Software (EROS, Institute of Clinical Effectiveness and Health Policy, Buenos Aires, Argentina, www.eros-systematic-review.org). In case of disagreement between both reviewers or any doubt, references were included for further screening. An overview of all exclusion criteria applied per screening phase is provided in Supplementary Information 1.

Studies were included for risk of bias assessment and meta-analysis when semi-quantitative histological scoring was used as outcome measure.
Study characteristics
Study characteristics were extracted from the studies by MP. Basic information (author and year of publication), animal model characteristics (species, strain, sex, etc.), experimental characteristics (surgery, biomaterial, follow-up, etc.), cell characteristics (cell type, culture conditions, etc.) and outcome characteristics (macroscopic evaluation, histology and semi-quantitative histological scoring, etc.) were obtained.

Risk of bias assessment
The methodological quality was assessed for all studies included in the meta-analysis (studies that evaluated cartilage regeneration using semi-quantitative histological scoring as outcome data). A risk of bias analysis was performed according to an adapted version [8] of the tool described by Hooijmans et al. [37]. Selection, performance, detection and attrition bias were scored independently by MP and VG/WD using questions and a flowchart [8], with ‘-’, ‘?’ and ‘+’, indicating low, unknown and high risk of bias, respectively. In case of differences between both reviewers, results were discussed until consensus was reached. Unfortunately, 16 articles were published in Chinese and we did not have the resources to obtain certified translations of these articles. We were, however, able to successfully extract the data of these studies using Google Translate and used the data in the meta-analysis. A sensitivity analysis was performed to evaluate the effect of language (exclusion of articles published in Chinese, see section 2.5.2).

Analysis preparations and meta-analysis
Analysis preparations
Meta-analyses were performed for the outcome measure semi-quantitative histology; data were used from studies that compared biomaterials with (experimental group) and without cells (control group). In general, these histological scoring systems evaluate the degree of cartilage regeneration by scoring parameters including e.g. Safranin-O staining, surface integrity and cartilage thickness [38].

Outcome data (mean, standard deviation (SD) and number of animals) were extracted from the studies for all time points as follows: 1) numerical data were obtained from the text or tables, 2) results depicted graphically were obtained by measuring the mean and SD using ImageJ (1.46r, National Institutes of Health USA), 3), results presented in boxplots were recalculated from the median, range and the sample size to the mean and SD [39], and 4) in case results were presented as mean and confidence interval (CI) per group, the following equation was used to recalculate the CI to a standard deviation for a 95% CI [40]:

$$SD = \sqrt{N} \times \frac{upper \ limit - lower \ limit}{3.92}$$
If data were missing or unclear, authors were contacted to provide the data. Studies were excluded from the meta-analysis in case data could not be retrieved or remained unclear (i.e. missing SD, all SD similar to corresponding mean, and histological scores exceeding maximum), unless data were sufficiently clear to make assumptions (i.e. group size and number of animals per time point and analyses, see Supplementary Information 2). A sensitivity analysis was performed to evaluate the effect of assumptions (exclusion of articles where assumptions were made, see section 2.5.2). Histological scoring systems describe the degree of cartilage regeneration, however with different scoring scales. To compare data from different studies, all data was converted to a 100% cartilage regeneration scale by dividing both the mean and SD by the maximum score of the scoring system applied and subsequently multiplying the outcome by 100%. In our SR, healthy tissue is represented as 100% cartilage regeneration (highest score). Lower percentages indicate less regenerated cartilage tissue. When results of experimental groups could be combined per study (i.e. outcome of various biomaterials seeded with one cell type), we followed the approach described in the Cochrane Handbook, table 7.7 [40]. Time points of treatment groups were combined using the same approach. Subsequently, per study the mean and corresponding standard error (SE) per treatment group were calculated.

**Meta-analysis**

The following main research question was assessed: Is there an overall beneficial effect on cartilage regeneration (expressed as histological score) of implanting biomaterials loaded with cells compared to acellular biomaterials?

We used a bivariate approach to model a random effects meta-analysis, i.e. separate outcomes for the control and experimental group were used with their respective SEs. The correlation between these two outcomes was modeled with a compound symmetry covariance matrix, as this resulted in a the lowest Akaike Information Criterion value.

To evaluate the effect of specific variables on the treatment outcome for the experimental group (biomaterials loaded with cells), the following sub-questions were addressed: 1) Is there a difference between the use of stem cells and somatic (differentiated) cells (stem cells vs. somatic cells)?: 2) Do differences among various cell subgroups exist (e.g. chondrocytes vs. other cells)?: 3) Is there a difference between biomaterials loaded with cells which were not cultured *in vitro*, were expanded *in vitro* or were differentiated *in vitro* (during surgery vs. expansion, surgery vs. differentiation, and expansion vs. differentiation)? Results are depicted as % cartilage regeneration (95% CI: [lower CI, upper CI]). The mean difference (%) [95% CI]) is presented as condition A – condition B (e.g. ‘stem cells - somatic cells’ or ‘surgery – differentiation’). Based on a previous study, data of all
time points were used [8]. Subgroup analyses were performed, in case subgroups consisted of more than five experimental groups in 3 studies. Most studies contained more than one experimental group, therefore the total number of studies and number of experimental groups (no. of studies/groups) is also provided in the analysis. No adjustment for multiple testing was applied in the analyses for the sub-questions.

Sensitivity analyses were performed on the main research question to evaluate the effect of language (exclusion of articles published in Chinese, because for these articles the risk of bias was not investigated), and the effect of assumptions (exclusion of articles for which assumptions were made) in the meta-analysis.

SAS/STAT® software version 9.2 for Windows, copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA, was used to perform statistical analyses. R software version 3.0.1 [41] with package meta [42] was used to create the funnel plot, which illustrates effect sizes of all studies versus their precision. I² was used as a measure of heterogeneity. ReviewManager (RevMan, Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) was used to create the forest plot.

Results

Search and study inclusion
Searching the PubMed and EMBASE databases [8] for references regarding cartilage regeneration by implantation of cellular and acellular biomaterials in the knee and ankle joint in combination with bone marrow stimulation resulted in a total of 11,248 references, of which 4,743 came from PubMed and 6,505 from EMBASE. After removal of duplicates, 7,354 references were screened by title and title/abstract, which resulted in the exclusion of 6,744 references. Subsequently, 610 studies were screened by full-text, resulting in 146 included studies. From some studies ([43-45]), the full-text could not be retrieved and these studies were excluded.

In the meta-analysis, studies were used which applied semi-quantitative histology as outcome measure, resulting in 117 included studies. A risk of bias assessment (Fig. 2) was performed for 101 of the 117 studies (excluding Chinese studies). Supplementary Information 2 provides an overview of all included studies after the full-text screening, risk of bias assessment and meta-analysis, as well as detailed information regarding reasons for exclusion and assumptions made for some studies. Supplementary Information 3 contains the reference list and abbreviations of studies used in Supplementary Information 2.
Study characteristics

A large variation between studies was observed regarding animal model characteristics (species, strain, sex, etc.), experimental characteristics (surgery, biomaterial, follow-up, etc.), cell characteristics (cell type, culture conditions, etc.) and outcome characteristics (macroscopic evaluation, histology and semi-quantitative histological scoring, etc.), as can be appreciated from Supplementary Information 2. Various animal species were used including rabbit, dog, sheep, pig, rat, horse, minipig, goat and macaques. A large range was found in animal age, e.g. the age of rabbits ranged from 6 weeks to >2 years. Small animals were generally younger (in the range of months) compared to larger animals (in the range of years). In many studies, no detailed information was provided regarding the absolute age of the animal, but ages were described as e.g. adult or mature.

The method used for bone marrow stimulation was mostly subchondral drilling (142 studies), where only 4 studies used microfracture to connect the cartilage defect to the underlying bone marrow stroma for the recruitment of pluripotential cells. Defects were created at various locations (trochlea, condyles, femur and intercondylar fossa) and with diverse dimensions (e.g. for rabbits: 4-7 mm in diameter and 0.8-9 mm in depth).

Implanted biomaterials were prepared from natural (e.g. alginate and collagen), synthetic (e.g. poly(lactic-co-glycolic acid) and polycaprolactone) or mixtures of both natural and synthetic materials. Additionally, in 27 studies biologics, such as bone morphogenetic protein 2 and transforming growth factor beta, were loaded in the biomaterials. Different cell types were applied, including chondrocytes, bone marrow-derived mesenchymal stem cells (BM-MSCs), bone marrow-derived progenitor cells, synovium-derived stem cells (SD-MSCs), bone marrow-derived mononuclear cells, adipose-derived stem cells, adipose-derived stromal vascular fraction cells, endothelial progenitor cells, embryonic stem cells, umbilical cord blood stem cells, fibroblasts, and periosteal cells, while in some studies undefined cell populations like bone marrow aspirate concentrate were used. Cells were either seeded on biomaterials and implanted in the created defect or cultured in vitro to expand and/or differentiate the cells, followed by seeding on biomaterials and implantation. In some studies, cells were cultured as a monolayer (without biomaterial), followed by seeding of the cells onto the biomaterial and implantation, while in other studies cells were directly cultured on biomaterials in vitro prior to implantation.

In most studies, short-term cartilage regeneration was investigated: the follow-up time was generally less than 6 months and the maximum follow-up time was 12 months.
Risk of bias assessment

The methodological quality was assessed for all studies included in the meta-analysis except Chinese articles. The overview of the results in Figure 3 indicates a general lack of information regarding the experimental setup of the studies, limiting the assessment of the actual risk of bias. Please see Supplementary Information 4 for all scores per individual study.

In the assessed studies, details regarding the application and method of randomization (Q1) were generally lacking. As a result, assessment of the actual risk of selection bias was nearly impossible. Assessment of the actual risk of bias due to differences in baseline characteristics was difficult since no details regarding randomization were described. Differences may have been present in

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Figure 2 PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analysis) flowchart of the systematic search of literature. Of the 117 studies included for the meta-analysis, a risk of bias assessment was performed for 101 studies, excluding Chinese articles.
load-bearing between implantation sites (Q2.1) and age, sex and weight of animals (Q2.2). In most studies, few differences were found between animals at the moment of surgical intervention since animals were treated similarly. For example, harvesting of cells was mostly performed by obtaining cells from all animals (Q2.3). Details regarding blinding of experimental conditions at the moment of implantation were generally not provided, which may have resulted in bias (Q3). Random housing of animals was generally not (well) described (Q4). Caregivers and/or investigators did not know which intervention each animal received during the experiment (Q5). No details were presented regarding the random selection of animals for outcome assessment (Q6). The method of blinding during analysis, however, was well described in most studies (Q7). Incomplete outcome data were identified or described in a few studies only, where these resulted in studies with high risk of bias (Q8). Generally, most studies lacked reporting of important details and therefore an adequate assessment of the actual risk of bias was difficult.

Data synthesis

Semi-quantitative histological scores were used as outcome data to compare biomaterials with cells (experimental group) and without cells (control group).

Figure 3 Results of the risk of bias analysis. Low, unknown or high risk of bias are presented in green, orange and red, respectively, where the percentages indicate the percentage of studies scoring low, unknown or high risk of bias of the total number of investigated studies per question. Low risk of bias was mainly found for addressing incomplete outcome data and baseline characteristics at the moment of surgical intervention. Unknown risk of bias was generally the result of limited details described in the studies regarding the experimental set-up. High risk of bias was only occasionally scored. Questions 4-6 are not depicted graphically, but are described and explained in Supplementary Information 4.
Table 1 Overview meta-analysis results; the effect on cartilage regeneration of (1) the addition of cells to biomaterials, (2) loading of stem cells vs. somatic cells, (3) loading of specific cell types, e.g. chondrocytes vs. all cells except chondrocytes, and (4) culture conditions.

<table>
<thead>
<tr>
<th>Meta-analysis</th>
<th>No. of studies/groups</th>
<th>Subgroups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Overall effect</td>
<td>98/265</td>
<td>Cellular scaffolds</td>
</tr>
<tr>
<td></td>
<td>98/208</td>
<td>Acellular scaffolds</td>
</tr>
<tr>
<td>2. Stem cells or somatic cells</td>
<td>57/148</td>
<td>Stem cells</td>
</tr>
<tr>
<td></td>
<td>36/101</td>
<td>Somatic cells</td>
</tr>
<tr>
<td></td>
<td>30/81</td>
<td>Chondrocytes</td>
</tr>
<tr>
<td></td>
<td>44/117</td>
<td>Bone marrow-derived MSCs</td>
</tr>
<tr>
<td></td>
<td>3/6</td>
<td>Synovium-derived MSCs</td>
</tr>
<tr>
<td>3. Type of cells</td>
<td>11/19</td>
<td>Adipose-derived stem cells</td>
</tr>
<tr>
<td></td>
<td>8/14</td>
<td>Bone marrow aspirate</td>
</tr>
<tr>
<td></td>
<td>3/7</td>
<td>Bone marrow-derived mononuclear cells</td>
</tr>
<tr>
<td>4. Cell manipulation</td>
<td>14/27</td>
<td>During surgery: harvesting, implantation</td>
</tr>
<tr>
<td></td>
<td>59/180</td>
<td>Expansion: harvesting, expansion in vitro, implantation</td>
</tr>
<tr>
<td></td>
<td>27/58</td>
<td>Differentiation: harvesting, differentiation in vitro, implantation</td>
</tr>
</tbody>
</table>

The total number of studies and number of groups included in the meta-analysis are depicted (studies may have >1 experimental group, no. of studies/groups). Results are presented on a 100% cartilage regeneration scale, where 100% indicates ‘maximum’ cartilage regeneration. The addition of cells to biomaterials significantly improved cartilage regeneration compared to acellular biomaterials. The use of stem cells or somatic cells resulted in comparable cartilage regeneration. Cartilage regeneration was significantly lower for biomaterials seeded with adipose-derived stem cells compared to other cell types. Cartilage regeneration was not affected by the method of cell manipulation.
### Table 1
Overview meta-analysis results; the effect on cartilage regeneration of (1) the addition of cells to biomaterials, (2) loading of stem cells vs. somatic cells, (3) loading of specific cell types, e.g. chondrocytes vs. all cells except chondrocytes, and (4) culture conditions.

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>Cartilage regeneration (% [95% CI])</th>
<th>Mean difference (% [95% CI])</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall effect</td>
<td>98/265</td>
<td>61.5 [58.5, 64.5]</td>
<td>18.6% [15.2, 22.0]</td>
</tr>
<tr>
<td>1. Overall effect</td>
<td>98/208</td>
<td>62.8 [58.5, 67.1]</td>
<td>-1.28 [-6.5, 4.0]</td>
</tr>
<tr>
<td>2. Stem cells or somatic cells</td>
<td>57/148</td>
<td>61.5 [58.1, 65.0]</td>
<td>62.8 [58.5, 67.1]</td>
</tr>
<tr>
<td>3. Type of cells</td>
<td>30/81</td>
<td>63.6 [58.1, 69.0]</td>
<td>57/117</td>
</tr>
<tr>
<td>4. Cell manipulation</td>
<td>14/27</td>
<td>56.3 [49.9, 62.6]</td>
<td>59/180</td>
</tr>
<tr>
<td>5. Surgery vs. Expansion</td>
<td></td>
<td></td>
<td>11/19</td>
</tr>
<tr>
<td>6. Surgery vs. Differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Expansion vs. Differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The total number of studies and number of groups included in the meta-analysis are depicted (studies may have >1 experimental group, no. of studies/groups). Results are presented on a 100% cartilage regeneration scale, where 100% indicates 'maximum' cartilage regeneration. The addition of cells to biomaterials significantly improved cartilage regeneration compared to acellular biomaterials. The use of stem cells or somatic cells resulted in comparable cartilage regeneration. Cartilage regeneration was significantly lower for biomaterials seeded with adipose-derived stem cells compared to other cell types. Cartilage regeneration was not affected by the method of cell manipulation.
and to address sub-questions related to the use of type of cells and culture conditions. An overview of all meta-analysis results is provided in Table 1; an overview of all raw data is given in Supplementary Information 5.

Data are presented as the effect (%) with 95% CI, where 100% cartilage regeneration represents healthy tissue and lower percentages indicate less regenerated cartilage tissue.

**Overall effect implantation of cellular and acellular biomaterials**
The meta-analysis indicates that implantation of cellular and acellular biomaterials resulted in 61.5% (95% CI: [58.5, 64.5]) and 43.0% (95% CI: [40.0, 46.0]) cartilage regeneration, respectively. The addition of cells to biomaterials significantly improved cartilage regeneration by 18.6% (95% CI: [15.2, 22.0], p < 0.0001). An overview of the results of each individual study is displayed in the forest plot (Supplementary Information 6), presenting improved cartilage regeneration by loading biomaterials with cells in 66 studies, similar cartilage regeneration in 30 studies, and a negative effect on cartilage regeneration in 2 studies. The heterogeneity (I²) was very high for the comparison between cellular and acellular biomaterials (99.4% [95% CI 99.3%; 99.4%]).

**Stem cells and somatic cells**
No significant differences (p = 0.622) were found between biomaterials loaded with stem cells (61.5% (95% CI: [58.1, 65.0]) and somatic cells (62.8% (95% CI: [58.5, 67.1])).

**Cell type**
Biomaterials were loaded with various cell types, including chondrocytes, bone marrow-derived MSCs, synovium-derived MSCs, adipose-derived stem cells, bone marrow aspirate, periosteal cells, fibroblasts, adipose-derived stromal vascular fraction cells, endothelial progenitor cells, bone marrow mononuclear cells, cartilage, umbilical cord stem cells, embryonic stem cell, or bone marrow aspirate. Subgroup analyses were only performed when subgroups consisted of more than five experimental groups in 3 studies. Seeding biomaterials with adipose-derived stem cells significantly decreased cartilage regeneration, while for the rest of the cell types no significant differences were observed (Table 1).

**Cell manipulation**
Comparing differences in cartilage regeneration between biomaterials loaded with cells which were not cultured in vitro (thus implanted immediately after harvesting of cells), or were expanded and/or differentiated in vitro, indicated that the method of cell manipulation did not affect cartilage regeneration (Table 1).
**Sensitivity analyses**

To investigate the robustness of the meta-analysis, sensitivity analyses were performed regarding the overall effect of the addition of cells to biomaterials. The overall outcome effect for cellular scaffolds was not notably affected by the exclusion of studies (1) with assumptions (2) or written in Chinese (no risk of bias assessment performed). Also for acellular biomaterials, the exclusion of both types of studies had no effect on cartilage regeneration.

**Publication bias**

Publication bias was assessed for all studies included in the meta-analysis comparing cartilage regeneration using acellular versus cell-laden biomaterials. Although the funnel plot (Fig. 4) is rectangular in shape, no major asymmetry was observed, giving no indication for publication bias.

**Figure 4** Funnel plot of the studies included in the meta-analysis comparing cartilage regeneration using cell-laden and acellular biomaterials. No substantial asymmetry was found.
Discussion

Bone marrow stimulation can be applied to induce cartilage regeneration. In a previous systematic review, it has been demonstrated that in animals the quality of newly formed cartilage was improved by the implantation of biomaterials after bone marrow stimulation, which is further enhanced by loading biomaterials with biologics [8]. The aim of this systematic review was (a) to provide a comprehensive, systematic and unbiased overview of all current literature regarding animal studies on cartilage regeneration using cellular versus acellular biomaterials and to identify knowledge gaps, (b) to assess the efficacy of cartilage regeneration using cellular versus acellular biomaterials and to investigate the effect of various parameters (i.e. stem/somatic cells, cell source, cell culture conditions), (c) to gain insight in the methodological quality of animal studies, and (d) to improve the design of future animal models and eventually clinical trials.

In animal studies, the implantation of cellular biomaterials significantly improved cartilage regeneration by 18.6% (95% CI 15.2, 22.0) compared to acellular biomaterials in animal models. This may be explained by the difference in the number of cells and the different contribution of various cell types in the regenerative process. For acellular biomaterials, cartilage matrix is deposited by bone marrow-derived mesenchymal stem cells, while for cellular biomaterials matrix deposition is additionally stimulated by the seeded cells. In the meta-analysis, the heterogeneity (I²) was very high for the comparison between cellular and acellular biomaterials (99.4% [95% CI 99.3%; 99.4%]), and the heterogeneity was approximately similar in the subgroup analyses. Therefore, all results should be interpreted with caution, especially for subgroup analyses with a relatively limited number of studies. Further clinical studies are required to assess the potential beneficial effect of cellular biomaterials versus acellular biomaterials in patients. Marcacci et al. [46] published promising results of a multicenter clinical phase III retrospective cohort study in which patients were treated with an implant consisting of autologous chondrocytes grown on Hyalograft C, a hyaluronic acid derivative, with a 3-year follow-up. Histological assessment of neotissue indicated hyaline-like cartilage for the majority of biopsies as well as major clinical improvements. The therapy was considered safe and effective for the treatment of articular cartilage lesions.

In a subgroup analysis, no significant differences were found between somatic cells (e.g. chondrocytes) and stem cells (e.g. mesenchymal stem cells (MSCs) and adipose-derived stem cells (ADSCs)). Differences were found between various cell types. ADSCs reduced cartilage regeneration in the subgroup analysis. However, cartilage regeneration using biomaterials seeded with ADSCs was still superior to biomaterials without cells. As compared to other cell types, it may be
that the origin of ADSCs from fatty tissue resulted in significantly reduced cartilage regeneration compared to cells derived from cartilage and the subchondral bone. A major drawback of the subgroup analysis evaluating the seeding of various cell types on biomaterials was the limited number of studies, as endothelial progenitor cells, embryonic stem cells, umbilical cord stem cells and fibroblasts were each only researched in one study. The use of MSCs may be favorable since these cells overcome limitations associated with the use of chondrocytes, including donor-site morbidity and limited matrix production after expansion in vitro [47]. Moreover, MSCs can be harvested from numerous sources, maintain their multipotency after expansion in vitro, can differentiate into chondrocytes that produce cartilage matrix and may suppress proinflammatory cytokines by their immunoregulatory properties. A drawback of MSCs may be their potency to terminally differentiate after chondrogenic differentiation, resulting in bone formation [47]. Additionally, MSCs are more difficult to manipulate and regulate than chondrocytes [48]. In clinical trials, the addition of MSCs to biomaterials did not result in a large improvement compared to autologous chondrocyte implantation or microfracture surgery [49, 50]. Besides MSCs, other cell sources are of interest for combined use with biomaterials, including embryonic stem cells and induced pluripotent stem cells (iPSCs). The use of embryonic stem cells, however, is at present associated with ethical issues, and although iPSCs provide pluripotency with less ethical issues and can be derived from various tissues, their safety in vivo has not been proven yet. It has been demonstrated, however, that newly formed cartilage by iPSCs integrates with native cartilage and subchondral bone in rats [47].

Study characteristics were collected from all included studies (also for studies not included in the meta-analysis) to provide a comprehensive overview of all literature regarding the effect of cellular versus acellular biomaterials on cartilage regeneration. A large heterogeneity was found between studies due to differences in animal model (species, strain, weight, age, and sex), performed surgery (bone marrow stimulation technique, implant location and defect size), biomaterials implanted (e.g. natural and synthetic origin) and follow-up period (short-term and long-term). To reduce the influence of possible confounding parameters, we excluded studies using healthy animals in which created defects were not filled during the first surgery (e.g. some studies implanted biomaterials 3 weeks after applying bone marrow stimulation) and osteoarthritis animal models, despite their greater relevance for future applications to treat patients with osteoarthritis. Bone marrow stimulation can be performed by subchondral drilling using a high speed drill or by microfracture surgery using arthroscopic awls [51]. It was remarkable that many more studies applied subchondral drilling (142 studies) compared to microfracture surgery (4 studies), especially since microfracture
surgery overcomes problems associated with thermal necrosis from subchondral drilling [52]. It may be that in animal models subchondral drilling is easier to perform than microfracture surgery. In a previous systematic review and meta-analysis, we found that the applied bone marrow stimulation technique had no effect on cartilage regeneration [8].

Various outcome measures have been used to investigate cartilage regeneration, including MRI, macroscopic and histological evaluation, semi-quantitative macroscopical and histological evaluation using scoring systems, histomorphometry, biomechanical analysis, polymerase chain reaction, biochemical assays, microCT and scanning electron microscopy. We selected data from semi-quantitative histological scoring systems as outcome measure, because histological scores are often used and it allow for quantitative comparisons between studies. However, different histological scoring systems to evaluate histological sections are available (extensively reviewed by Rutgers et al. [37]), including the O’Driscoll and ICRS scoring system, which evaluate cartilage regeneration using different parameters (e.g. cell morphology, matrix staining and defect filling) and looking at different processes, e.g. cartilage regeneration only, cartilage and subchondral bone regeneration, and additionally biomaterial degradation. A limitation of this review and meta-analysis may be that we did not discriminate between these parameters. On the other hand, the use of all data may provide an extensive and complete overview of all aspects affecting the regenerative process.

The methodological quality assessment was performed to evaluate the experimental designs and the reliability of the results of the studies included in the meta-analysis. The methodological quality (internal validity) is of great importance since a low methodological quality may result in an overestimation or underestimation of the effect size [53]. No studies were included in or excluded from the meta-analysis based on methodological quality assessment results. ‘Low risk of bias’ and ‘unknown risk of bias’ were the most found scores, but some studies were marked as having a high risk of bias. Generally, the possibility of assessing of the actual risk of bias was limited due to the absence of important details regarding the experimental set-up in most studies. No details were described related to the method of randomization. It may be that the animal studies were performed well, but that experimental designs were only reported poorly [54]. For the analysis of the histological sections, however, it was described in most studies that sections were randomized and that outcome assessors were blinded. Detection/observer bias may be introduced in case blinding was not performed and can result in an overestimation of the actual effect of the therapy [55]. The overall validity of the study results may be impaired by bias due to the lack of blinding and randomization [56, 57]. Reporting of animal studies may be improved by using standardized protocols, including the ARRIVE guidelines [58]
or golden standard publication checklist [59]. Publication bias may be the result of omission to publish negative results and the inclusion of multiple studies of the same author, and can result in an overestimation of the results. Publication bias can be detected using a funnel scatter plot, but for the data used in the meta-analysis of this study, no asymmetry was observed and no indications for publication bias found.

The power of experimental designs is important when performing experimental studies and insufficient power may result in false negative results. Many studies (30 out of 98) in the meta-analysis with a large CI showed a neutral effect of the implantation of biomaterials loaded with cells on cartilage regeneration. Only in some studies, it was described that power calculations were performed but the actual calculations were never provided, while sufficient power is required to avoid false negative results.

A high translational value of animal studies is crucial to successfully take treatments forward to clinical practice. Therefore, validated and predictive animal models are required. Many challenges and limitations are associated with the use of animal models for cartilage defects. Chu et al. [60] and Ahern et al. [61] extensively described the strengths and shortcomings of different animal models related to e.g. joint size, cartilage thickness, defect size, intrinsic healing potential and animal maturity, in comparison to lesions in clinical studies. In most animal experiments, the follow-up period was maximally 6 months, while in patients clinical improvements are generally observed up to 1.5 - 3 years after microfracture surgery [62, 63]. The translational value and considerations to select animal models were extensively discussed before [8].

In future studies, improved reporting of animal studies is required and studies should strive to resemble the clinical situation to facilitate translation of the results. For clinical application of new regenerative medicine and tissue engineering strategies, including the use of biomaterials, biologics and cells, the effectiveness needs to be proven both in animal models and clinical studies [64]. Moreover, the cost-effectiveness of new interventions in clinical practice may be assessed using early health economic models [65]. Considerations for the addition of cells to biomaterials are of great importance and limitations (including donor-site morbidity, cell culture costs, regulatory issues, limited off the shelf availability, and potentially multiple-stage surgical procedures [8, 66]) should be weighed against potentially superior cartilage regeneration by applying cellular biomaterials. Difficulties in controlling cell culture and the development of novel materials stimulating tissue regeneration may justify the use of acellular biomaterials. Future research focusing on the properties of biomaterials, the source and manipulation of cells, and potentially patient profiling, may allow selection of the best treatment for each individual patient [67].
Conclusion
This systematic review and meta-analysis provides an extensive overview of all studies applying regenerative medicine and tissue engineering approaches to regenerate articular cartilage by implantation of cellular versus acellular biomaterials after applying bone barrow stimulation in animal models. Cartilage regeneration was more effective by implantation of cellular biomaterials compared to acellular biomaterials. The use of stem cells or somatic cells gave comparable results. Only seeding of adipose-derived stem cells negatively affected cartilage regeneration in cell-seeded biomaterials.

Acknowledgements
We thank Jie An (Department of Biomaterials, Radboud Institute for Molecular Life Sciences, Radboud university medical center) and Chunling Tang (Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences, Radboud university medical center) for their contribution to the paper. Gerrie Hermkens from the Radboud university medical center library is greatly acknowledged for help retrieving full-text studies.

Supplemental Information
All supplemental Information will be available upon publication.
References


Supplementary Information 1. Screening exclusion criteria.

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The forest plot illustrates the difference in cartilage regeneration between cellular and acellular biomaterials of each individual study.
Cartilage regeneration using cellular implants

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Versatile wedge-based system for the construction of unidirectional collagen scaffolds by directional freezing: practical and theoretical considerations

Michiel W. Pot | Kaeuis A. Faraj | Alaa Adawy | Willem J.P. van Enckevort
Herman T.B. van Moerkerk | Elias Vlieg | Willeke F. Daamen
Toin H. van Kuppevelt

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Abstract

Aligned unidirectional collagen scaffolds may aid regeneration of those tissues where alignment of cells and extracellular matrix is essential, as for instance in cartilage, nerve bundles and skeletal muscle. Pores can be introduced by ice crystal formation followed by freeze-drying, the pore architecture reflecting the ice crystal morphology. In this study we developed a wedge-based system allowing the production of a wide range of collagen scaffolds with unidirectional pores by directional freezing. Insoluble type I collagen suspensions were frozen using a custom-made wedge system, facilitating the formation of a horizontal as well as a vertical temperature gradient and providing a controlled solidification area for ice dendrites. The system permitted the growth of aligned unidirectional ice crystals over a large distance (> 2.5 cm), an insulator prolonging the freezing process and facilitating the construction of crack-free scaffolds. Unidirectional collagen scaffolds with tunable pore sizes and pore morphologies were constructed by varying freezing rates and suspension media. The versatility of the system was indicated by the construction of unidirectional scaffolds from albumin, poly(vinyl alcohol) (a synthetic polymer), and collagen-polymer blends producing hybrid scaffolds. Macroscopic observations, temperature measurements and scanning electron microscopy indicated that directed horizontal ice dendrite formation, vertical ice crystal nucleation and evolutionary selection were the basis of the aligned unidirectional ice crystal growth, and hence the aligned unidirectional pore structure. In conclusion, a simple, highly adjustable freezing system has been developed allowing the construction of large (hybrid) bioscaffolds with tunable unidirectional pore architecture.
Introduction

Tissue engineering and regenerative medicine aim to restore the structure and function of damaged tissues and organs. A commonly used strategy is the incorporation of cells and effector molecules into supporting structures, also referred to as scaffolds, to induce tissue regeneration. An important parameter for the guidance of tissue formation is the pore architecture of scaffolds. Typical isotropic scaffolds show uniformity in all orientations, but isotropy may be undesirable in tissues displaying an anisotropic extracellular matrix (ECM). In this case, application of isotropic scaffolds may result in structural and mechanical discordance with the surrounding tissue, and the use of scaffolds with anisotropic architecture is therefore favored [1].

Construction of unidirectional scaffolds has been widely investigated, since they provide cues to direct growth of tissues along the aligned structures [2-4]. Aligned growth is important during, e.g., the formation of nerve bundles, skeletal muscle fibers, and cartilaginous tissue. In addition, longitudinal microchannels have been reported to increase cellular influx [5]. Anisotropic pore organization in scaffolds has been applied for the regeneration of a number of tissues, such as intervertebral disc [6], cartilage [7], muscle [8], tendon [9], and nerves [10]. Besides the pore orientation, physical parameters as intrinsic surface topographies influence cellular behavior [11].

ECM proteins are appropriate substrates for scaffolding material because of their biocompatibility, biodegradability, bioactive properties, and, in case of collagen, low antigenicity. Type I collagen is the most abundant scaffolding material in the body, and can be used to construct scaffolds of different architectures [12]. Several methods have been developed to produce anisotropic unidirectional porous collagen-based scaffolds, such as lyophilization [13], electrospinning [14], and application of electrochemical gradients [15] and magnetic fields [16]. The requirement for complex equipment restricts wide applicability of most of these strategies. Moreover, many techniques require harsh operating conditions that can limit the use of biologicals, and the remaining organic solvents may cause in vivo toxicity [17]. From these techniques, lyophilization is a widely established method [18]. The pore architecture of scaffolds obtained by lyophilization reflects the ice crystal morphology obtained during freezing. Controlled growth of ice crystals forms the basis of the development of collagen scaffolds with a unidirectional structure and the final pore structure can be modified by adjusting the freezing regime [12]. Generally, comprehensive setups are employed to obtain unidirectional scaffolds by lyophilization [19]. For those studies reporting simple freezing strategies, one strategy is to dip collagen suspensions in freezing media [20], which may limit control over the freezing
process. Another simple approach is to fill a mold with collagen suspension and subsequently cool the mold with freezing media. Such simple molds typically consist of a metal surface and isolation, but generally result in limited homogeneity due to the lack of constant slow cooling [21]. To our knowledge the construction of collagen scaffolds with unidirectional pores over long distances (>2.5 cm) by controlled unidirectional freezing conditions have not been described before. The development of a simple adjustable system for the production of high quality tailor-made unidirectional collagen scaffolds may allow easy access to unidirectional scaffolds. Porous collagen scaffolds generally have rather weak mechanical strength. An improvement of (unidirectional) collagen scaffolds would be an increase in mechanical strength, such as tensile strength, fracture strength and compressive strength. Reinforced hybrid scaffolds prepared from premixed collagen and water-soluble polymers such as poly(vinyl alcohol) may aid interesting characteristics for tissue engineering applications [22]. In this study, we describe an adjustable directional freezing method to develop porous collagen scaffolds with aligned unidirectional pores, taking into account the physics of ice crystal growth. A custom-made wedge system was developed to aid controlled solidification, especially in the initial phases of the freezing process. The influence of freezing temperature, solvents, collagen concentration, and use of synthetic polymers are described.

Materials and methods

Preparation of scaffolds with aligned pores
Fibrillar insoluble type I collagen was isolated from bovine achilles tendon. The general procedure for scaffold construction comprised the preparation of a 0.7% (w/v) collagen suspension by incubating collagen fibrils overnight at 4 °C in 0.25 M acetic acid (pH 2.7, Scharlau, Spain). The collagen suspension was homogenized on ice using a Teflon glass Potter-Elvehjem device (Louwers Glass and Ceramic Technologies, Hapert, The Netherlands) with an intervening space of 0.35 mm (10 strokes). Air bubbles were removed by centrifugation at 117 g for 30 min at 4 °C. Unidirectional porous collagen scaffolds were produced by directional freezing of the collagen suspension using a custom-made wedge system (for an overview of the system, including dimensions, see Fig. 1).

The system consisted of two wedges of anodized aluminum and polyurethane (Obomodulan®, type 652, Vink, Didam, The Netherlands) with thermal conductivities of 205 and 0.03 W/m·K, respectively. A 16 mm high reservoir of Scotch® tape was attached to the aluminum wedge. Styrofoam insulation (outer dimensions: W x L x H (mm): 45 x 70 x 40; inner dimensions: W x L x H (mm): 15 x 45 x 40) was placed
around the wedge system and reservoir to ensure a vertical temperature gradient without external disturbances. The wedge system was applied to induce a horizontal temperature gradient (see Figure 2B). The collagen suspension was pipetted into the reservoir. The wedge system was placed on a plateau of aluminum in a container and the collagen suspension was subsequently frozen using liquid nitrogen (-196 °C). The freezing medium was only in contact with Obomodulan and not with the aluminum (Fig. 1B). The system was left at ambient temperature during freezing. Scaffolds were prepared by freezing 10 ml of a collagen suspension followed by lyophilization for 2 days in a Zirbus freeze-dryer (Sublimator 500 II, Bad Grund, Germany). Next, scaffolds were stabilized using vapor fixation with 37% formaldehyde under vacuum for 30 min [5].

The effect of different parameters on the pore structure was investigated, i.e., freezing temperature, collagen concentration, acetic acid concentration, addition of detergent, use of other components than collagen, and collagen/PVA blends (see Table 1). To investigate the effect of the freezing temperature and speed, the collagen suspension was also frozen using a mixture of dry ice and ethanol

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**Figure 1** Experimental setup of the wedge system to construct unidirectional scaffolds. **A)** Wedge system used for the preparation of lyophilized unidirectional scaffolds. The gray and brown wedges represent aluminum and Obomodulan, respectively. The suspension is present in the blue reservoir made from Scotch tape. **B)** Front view of the wedge system in its experimental setup. The wedge system is placed on a plateau of aluminum in a styrofoam container. Styrofoam insulation surrounds the wedge system. The freezing medium is in direct contact with the wedge-shaped Obomodulan but not with the wedge-shaped aluminum.
(−78 °C). To study the effect of the volume of the applied collagen suspension, scaffolds were made using volumes up to 30 ml collagen suspension in 0.25 M acetic acid. Additional Scotch® tape was attached to the wedge system for a 32 mm high reservoir. To examine the effect of collagen concentration on pore structure, collagen suspensions of 0.4, 1.0, and 2.0% (w/v) were used. Collagen suspensions of 0.4% and 1% (w/v) were prepared similarly as 0.7% (w/v) while the 2.0% (w/v) collagen suspensions were homogenized by passing the suspension five times through a 50 ml syringe, followed by centrifugation at 2538 g for 45 min to remove air bubbles. To investigate the effect of a lowered surface free energy during ice crystal formation, a solution of 15 mM octyl β-D-glucopyranoside (Sigma-Aldrich), a nonionic detergent, in 0.25 M acetic acid was prepared, after which collagen fibrils were added. The effect of the suspension medium was further studied by altering the concentration of the acetic acid used. Preparation of collagen in 0.025 M acetic acid (0.15 wt%, pH 3.5), 0.25 M acetic acid (1.5 wt%, pH 2.7), and 2 M acetic acid (12 wt%, pH 2.5) were studied. As a reference, collagen fibrils were suspended in Milli-Q water. To investigate the versatility of the system for use of components other than collagen, scaffolds were prepared from 7% (w/v) bovine serum albumin (BSA, PAA Laboratories GmbH, Pasching, Austria) and 5, 10, 15, and 20% (w/v) poly(vinyl alcohol) (PVA, Sulkey of America, Kennesaw, GA, USA), all in 0.25 M acetic acid. Moreover, to investigate reinforcement of collagen scaffolds with synthetic polymers (hybrid scaffolds), mixtures of collagen and PVA (0.7% w/v collagen combined with 0.5, 1.0, 2.5, and 5.0% (w/v) PVA), in 0.25 M acetic acid, were prepared. All suspensions were homogenized using the Potter-Elvehjem device.

**Process of directional solidification**

The process of planar ice dendrite formation was visualized by video capture using a Sony Cybershot DSC-H10 camera. To visualize the process, 10 ml of a solution of 0.25 M acetic acid was frozen using liquid nitrogen. A solution without collagen was used to circumvent interference caused by the cloudy appearance of the collagen suspension.

Temperature measurements were performed to characterize the freezing process. The temperature differences in the collagen suspension during the freezing process with liquid nitrogen and a mixture of dry ice and ethanol were measured n=3, in triplicate, at nine different locations (Fig. 2B) using thermocouples (Testo 922 and 925, Testo AG, Lenzkirch, Germany). Sensors were placed at three locations on the aluminum surface, from the thick aluminum part to the thin aluminum part (left: 0.5 cm, middle: 2.25 cm, right: 4 cm). Sensors were also placed at three heights from the surface of the aluminum (bottom: 0 mm, middle: 5 mm, top: 10 mm) at three different locations (left: 0.5 cm, middle: 2.25 cm, right: 4 cm).
As a control, sensors were placed on the aluminum surface (left: 0.5 cm, middle: 2.25 cm, right: 4 cm) of a non-wedge, flat system (aluminum: W x L x H (mm): 15 x 45 x 15; Obomodulan: W x L x H (mm): 15 x 45 x 3), and measurements were performed during freezing with liquid nitrogen. Temperature measurements were performed for 10 min with time intervals of 10 s for freezing at -196 °C and for 45 min with 1 min time intervals for freezing at -78 °C. The duration of the freezing process was measured from the start of the measurement until collagen suspensions were macroscopically frozen. Freezing times are represented as mean ± standard deviation (min). Cooling rates were calculated from 0 °C until -20 °C for temperature measurements at different heights. Cooling rates are represented as mean ± standard deviation (°C/min). Horizontal temperature differences between the left and the right sensor were calculated from 0 °C until -30 °C and are represented as mean ± standard deviation. The measured temperature difference between the left and the right sensor was divided by the distance between both sensors (3.5 cm) to calculate the temperature difference per cm (°C/cm).

**Scaffold morphology**

The morphology of the scaffolds was evaluated using scanning electron microscopy (JEOL SEM6340F, Tokyo, Japan). Samples were mounted on stubs and sputtered with a thin layer of gold using a Polaron E5100 Coating System [23]. Images were recorded using an accelerating voltage of 10 kV. Scaffolds were cut longitudinally and perpendicularly. High magnification images were taken from the lower parts of scaffolds to assess the development of unidirectional pores. Pilot experiments showed homogeneity in pore size distribution throughout the scaffolds. In a pilot study three scaffolds were extensively analyzed with respect to pore size. The following data were obtained from nine samples per scaffold at three locations - left, middle, right (see figure 2) and heights - 2, 6 and 10 mm. Pore sizes were 54 ± 9 µm, 50 ± 9 µm and 55 ± 9 µm. On the basis of these data we used one longitudinal and cross-section per scaffold for further analysis. Four images were recorded from random locations per cross-section and the lengths of the shortest axis of 50 pores were measured using ImageJ. This experiment was performed n=3 in triplicate.

**Statistics**

Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., version 5, La Jolla, CA, U.S.A.). Horizontal temperature differences between the left and the right sensor were determined by paired t-tests. The effect of the various experimental conditions was assessed by a repeated measures ANOVA with Tukey’s post-hoc test. Pore sizes are shown as mean ± standard deviation. P-values < 0.05 were considered statistically significant.
Results

Construction of unidirectional collagen scaffolds
Collagen scaffolds with unidirectional pore architecture were constructed by directional freezing using the custom-made wedge system (Fig. 1). The system consists of two opposite wedges of aluminum (upper layer) and Obomodulan (lower layer) that differ in thermal conductivity. The Obomodulan wedge is a poor thermal conductor, acting as a mediator between the freezing medium and the aluminum wedge, resulting in slower freezing compared to the situation when the aluminum layer would be in direct contact with liquid nitrogen. After completion of the freezing process, frozen suspensions could easily be removed from the Scotch tape reservoir. No cracks were observed before or after lyophilization.

Temperature measurements during the freezing process indicated a large vertical temperature gradient (Fig. 2), resulting in freezing of the collagen suspension from the aluminum surface upward to where the collagen suspension was exposed to ambient temperature. Freezing at -196 °C and -78 °C resulted in cooling rates of 6.4 ± 1.2 °C/min and 0.3 ± 0.1 °C/min, respectively. Collagen suspensions were frozen after 11 ± 1 min and 50 ± 5 min for freezing at -196 °C for and -78 °C respectively. Macroscopic observations showed that the wedge system induced horizontal dendrite formation by the growth of ice dendrites over the aluminum surface of the wedge (Supporting Information 1, video). To evaluate the presence of a horizontal temperature gradient sensors were placed on the aluminum surface at three different locations. The sensor located on the thick aluminum part of the wedge always showed a lower temperature compared to sensors placed midway and on the thin aluminum part. The temperature difference between the left and right sensor was 1.1 ± 0.9 °C (p <0.0001) and 0.3 ± 0.1 °C (p <0.0001) for -196 °C and -78 °C, respectively, resulting in temperature differences of 0.3 ± 0.3 °C/cm and 0.1 ± 0.0 °C/cm. This indicates the presence of a significant horizontal temperature gradient from left to right over the aluminum surface of the wedge system during the freezing process, for freezing at -196 °C as well as at -78 °C. Temperature measurements performed using the non-wedge, flat system did not reveal an obvious temperature differences between different horizontal locations.

Scaffold characterization
Directional freezing and lyophilization was applied to construct scaffolds with unidirectional pores. The collagen scaffolds constructed using the standard method (with 10 ml collagen suspension) comprised a height of 12 mm. In the lower parts of the scaffolds, SEM images (Fig. 3A) indicated a small area of round pores at the base of the freeze-dried scaffolds. Above this area, there was a small
Figure 2  Experimental setup of the wedge system to construct unidirectional scaffolds. Temperature gradients observed during construction of unidirectional scaffolds using the wedge system. Liquid nitrogen or dry ice/ethanol were used as freezing medium. The position of the sensors is indicated. See Fig. 1 for color coding of the wedge system. Data from representative measurements are shown. A) Vertical temperature gradient with cooling rates of $6.4 \pm 1.2 \, ^\circ\text{C}/\text{min}$ and $0.3 \pm 0.1 \, ^\circ\text{C}/\text{min}$ for liquid nitrogen and dry ice/ethanol respectively. B) Horizontal temperature gradient measured during the entire freezing process. The temperature difference between the left and right sensor was $1.1 \pm 0.9 \, ^\circ\text{C}$ ($p < 0.0001$) and $0.3 \pm 0.1 \, ^\circ\text{C}$ ($p < 0.0001$) for $-196 \, ^\circ\text{C}$ and $-78 \, ^\circ\text{C}$, respectively. C) Horizontal temperature measurements performed using a non-wedge system indicated no obvious temperature differences between the left, middle and right part of the wedge.
area where elongated pores were present in multiple directions. These observations indicate an initial process of nucleation and evolutionary selection (see the discussion). The height of the non-unidirectional area was less for higher freezing speeds, i.e., 2 and 3 mm for -196 °C and -78 °C, respectively. Above the non-unidirectional area (2 mm in height), unidirectional pores were present throughout the scaffold from base to top over a distance of 10 mm (Fig. 3B). The cross-sections of scaffolds (Fig. 3B) showed a honeycomb-like morphology of the pores with small openings in the pore walls. These openings provide interconnectivity between adjacent pores. Furthermore, thin thread-like struts bridging opposite pore walls were observed in both longitudinal and cross-sections.

Scaffolds with a height of over 30 mm were constructed from a 30 ml collagen suspension using freezing at -196 °C and -78 °C (Fig. 3C). These scaffolds contained unidirectional pores over a length of about 28 mm.

Control of pore morphology
For an overview of the parameters examined and results obtained see Table 1.

<table>
<thead>
<tr>
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<td>Freezing temperature</td>
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<td>Dry ice/ethanol</td>
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<td>Collagen concentration</td>
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<tr>
<td>Acetic acid concentration</td>
<td>Pore size larger and wall structure more closed for increased acetic acid concentration</td>
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<td>(Bio)molecule</td>
<td>Unidirectional scaffolds with morphology dependent on type and concentration of molecule</td>
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Figure 3  Scanning electron microscopical characterization of scaffolds constructed by freezing with liquid nitrogen or a mixture of dry ice and ethanol. A) Round pores (arrows) were present at the base of the scaffolds, indicating ice crystal nucleation. Elongated pores in different directions were present above the area of nucleation, indicating evolutionary selection. The area of nucleation and evolutionary selection appears smaller in the case of freezing with liquid nitrogen compared to a mixture of dry ice and ethanol. B) High magnification of the unidirectional pore structures in a longitudinal and cross-sectional view. The pores have a hexagonal/elliptic morphology, and are smaller with higher freezing speed (-196 °C: 66 ± 11 µm and -78 °C: 146 ± 30 µm, p <0.0001). C) Panorama views of longitudinal sections of unidirectional scaffolds made using a 30 ml collagen suspension. Unidirectional pores run from bottom to top over a length of > 25 mm. Scale bars in main figure A are 1 mm and in close-ups 300 µm, in B 100 µm and in C 1 mm.
**Freezing temperature**

Pore sizes in scaffolds could be affected by freezing temperature: a fast freezing process using liquid nitrogen resulted in smaller pores compared to freezing using a mixture of dry ice and ethanol (66 ± 11 µm for -196 °C: 146 ± 30 µm for -78 °C, p < 0.0001).

**Collagen concentration**

Scaffolds with aligned unidirectional pores were constructed using four collagen concentrations. Pore diameters were affected by collagen concentration (Fig. 4), and were 74 ± 13, 66 ± 11, 63 ± 9, and 50 ± 10 µm in 0.4, 0.7, 1.0, and 2.0% scaffolds, respectively (p < 0.0001). For all collagen concentrations, honeycomb-like pores were observed in the scaffolds with no apparent differences in wall thickness.

**Figure 4** Scaffolds constructed from suspensions with different collagen concentrations. Scaffolds with unidirectional pores could be prepared for all applied collagen concentrations. Hexagonal/elliptic pores in the scaffolds were observed, with no apparent differences in wall thickness. An increase in collagen concentration resulted in an increase in the number of pores and a decrease in pore size (***: p < 0.0001). Scale bars represent 100 µm.
Although no mechanical tests were performed, handleability of the scaffolds improved with increased collagen concentration.

**Incorporation of detergents**
Production of scaffolds from a collagen suspension in 15 mM octyl β-D-glycopyranoside in 0.25 M acetic acid also resulted in unidirectional collagen scaffolds after freezing and lyophilization. The wall structure was similar to scaffolds prepared using 0.25 M acetic acid without this non-ionic detergent, but a decrease in pore size from 66 ± 11 µm to 57 ± 10 µm was noted (p < 0.0001, results not shown).

**Concentration of acetic acid**
Unidirectional collagen scaffolds could be constructed from collagen suspensions

![Figure 5](image)

**Figure 5** Unidirectional collagen scaffolds prepared from collagen suspensions with different concentrations of acetic acid. An increase in the concentration of acetic acid resulted in scaffolds with a less thread-like more closed wall structure and increased pore sizes (**: p < 0.0001). Scale bars represent 100 µm.
with different concentrations of acetic acid. For reference, collagen fibrils were incubated in Milli-Q water. The pores in these scaffolds consisted of thread-like structures and thin walls. SEM images indicated that the unidirectional pore structure was present, although somewhat masked by the highly thread-like structure. Morphologically, scaffolds prepared using collagen suspended in 0.025 M acetic acid resembled the structure of scaffolds prepared using collagen suspended in Milli-Q water (Fig. 5). When increasing the concentration of acetic acid from 0.025 M to 0.25 M acetic acid, scaffolds became less filamentary and the wall structure was more closed. A further increase of the acetic acid concentration from 0.25 M to 2 M acetic acid further reduced the fibrillar nature of the scaffolds with an even more closed wall structure. The scaffolds showed no obvious differences in wall thickness. The pore sizes in the scaffolds were 32 ± 7 µm, 49 ± 9 µm, 66 ± 11 µm and 87 ± 15 µm for Milli-Q water, 0.025 M, 0.25 M and 2 M acetic acid (p < 0.0001), respectively.

Application of system to other (bio)molecules
To evaluate the general applicability of the system, scaffolds with unidirectional pores were constructed using another protein (albumin), a synthetic polymer

![Figure 6](image)

**Figure 6** Versatility of the wedge system: unidirectional scaffolds prepared from poly(vinyl alcohol) (PVA, a synthetic polymer), and collagen + PVA. A) Longitudinal sections of unidirectional scaffolds prepared with various concentrations of PVA. The unidirectional pore structure was observed for all concentrations. Pore sizes decreased with increasing concentrations of PVA. B) Longitudinal sections of unidirectional hybrid collagen-PVA scaffolds. A mixed morphology resembling features from both collagen and PVA scaffolds was observed. Scale bars represent 100 µm.
(PVA), or a mixture of a protein + polymer (collagen + PVA). The unidirectional pore orientation was clearly observed in scaffolds constructed from albumin. Albumin-based scaffolds displayed similar characteristics as those made from collagen, including a small area at the base of the scaffold of rounded pores and elongated pores in different directions (data not shown). Above this non-unidirectional area, unidirectional pores were present. The cross-sections showed that the pores were irregular and oblong shaped. In general, albumin scaffolds consisted of smooth wall structures with additional globular structures.

Freezing of PVA suspensions at varying polymer concentration also resulted in scaffolds with unidirectional pores (Fig. 6A). Morphologically, the wall structure resembled a fishbone-like arrangement, and an increase in polymer concentration resulted in a decrease in pore size with an increase in wall thickness.

Hybrid unidirectional scaffolds were constructed using 0.7% (w/v) collagen in combination with different concentrations of PVA: 0.5%, 1.0%, 2.5%, and 5% (w/v) (Fig. 6B). The structure of hybrid scaffolds containing 5% PVA showed a mixed morphology combining the architecture observed for 5% PVA and 0.7% collagen scaffolds. The walls of hybrid scaffolds were thicker compared to those seen in collagen-only scaffolds. Moreover, a more fibred morphology was present in hybrid scaffolds compared to polymer-only scaffolds due to the addition of collagen, especially in the fibers crossing the pores. With lowered concentrations of PVA, the structure shifted to that observed for collagen scaffolds. The handleability of the scaffolds was improved by the incorporation of PVA compared to collagen scaffolds.

Discussion

Large collagen scaffolds with unidirectional pore architecture were constructed by freezing collagen suspensions using liquid nitrogen or a mixture of dry ice and ethanol, applying a wedge-like construct consisting of both a thermal conductor and insulator. The delayed freezing process by the insulator may have aided in the development of crack-free scaffolds, whereas cracks were observed after fast freezing of collagen suspensions in metal casts in direct contact with liquid nitrogen. Moreover, changing the materials of the metal wedge and insulator to materials with different thermal conductivities may allow adaptation of ice crystal growth [24]. The wedge shape induced a small but significant horizontal temperature gradient, likely facilitating local nucleation at the coldest point, followed by laterally directed ice crystal growth over the metal surface which stabilizes unidirectional crystal growth. The horizontal temperature gradient may have introduced small differences in height between adjacent upward
growing ice crystals, thereby blocking inclined growing of ice crystals, and resulting in a stabilized upward growth of ice crystals (Fig. 7). A steeper angle of the wedge may give rise to a larger horizontal temperature gradient and thus improved stabilized unidirectional crystal growth.

Four steps can be identified during the formation of unidirectional collagen scaffolds as based on optical observations, temperature measurements and SEM analyses (Fig. 7): (1) formation of a horizontally oriented network of ice dendrites, (2) development of vertical protrusions and/or nuclei, (3) evolutionary selection of the vertical growth direction, and (4) unidirectional (mainly cellular) ice crystal growth. Step 1: At the interface between the aluminum surface and collagen suspension, the ice nuclei that rapidly become dendrites protrude horizontally. The dendrites initially form at the coldest part of the wedge (the thick aluminum part) and spread over the aluminum surface, owing to the horizontal temperature

**Figure 7** Proposed physical principles underlying the formation of aligned unidirectional scaffolds. A) Schematic representation of the physical processes occurring during directional solidification, resulting in unidirectional ice crystal growth. The direction of the growing ice front is facilitated by the wedges, providing a controlled solidification area for ice dendrites. Nucleation sites develop, which merge into ice crystals by evolutionary selection [27], and grow upward due to the vertical temperature gradient. The horizontal temperature gradient, facilitated by the wedge shape, may stabilize the formation of aligned unidirectional ice crystals. B) The planar ice surface progresses into an unstable surface as the result of physical disturbances in freezing media. Ice crystals grow out from protrusions or nuclei formed by these instabilities. Growth of ice crystals is guided by the vertical temperature gradient. Collagen fibrils and other particles are entrapped between the growing ice crystals. Figure 7B was adapted from Deville et al. [30]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.
gradient realized by the wedge-shaped metal. The horizontal temperature gradient thus provides a controlled solidification area for ice dendrites. Step 2: On top of the initial planar layer of dendrites, upward pointing protrusions may form on the ice dendrite arms as a result of morphological (so-called Mullins-Sekerka) instabilities [25]. An alternative possibility is a preferential heterogeneous nucleation of upward pointing ice crystallites on the dendrite arms. These nucleation sites left a mark as round pored structures at the base of the scaffolds (Fig. 3). The nucleation mechanisms and crystal growth phenomena have recently been reviewed by Pawelec and co-workers [26]. Step 3: Evolutionary selection takes place in which only a selection of growing ice crystals will continue and form the structure of the unidirectional scaffold, protruding upward from the aluminum surface [27]. During evolutionary selection non-perpendicular growing ice crystal needles collide with perpendicular growing needles and stop growing. Only perpendicular growing ice crystals survive and continue growing. This process is likely the result of the combined vertical and horizontal temperature gradient. A similar mechanism was observed by Pawelec et al., demonstrating the transformation from isotropic (nucleation sites), via evolutionary selection, to growth of aligned pores upward. The non-unidirectional area was present throughout the whole sample, which may be addressed to the slow freezing applied, while in our experiments relatively fast-freezing was used [28].

Step 4: the further unidirectional upward cellular growth of the ice crystals after evolutionary selection is supported by the vertical temperature gradient. During freezing of the collagen suspension, ice and collagen/acetic acid/water compartments are formed. Aqueous acetic acid is a eutectic system (eutectic point: -26.7 °C, at a 60% (v/v) acetic acid to water ratio) [29], which results in compartmentalization during the freezing process: a compartment of frozen water and a compartment with concentrated liquid acetic acid in which the collagen fibers can be found (we assume that the collagen fibrils do not affect the eutectic point or the freezing process).

After lyophilization, pores showed a honeycomb-like morphology with thin bridging structures and openings in the wall. The development of the honeycomb-like structure can be explained by stacking of dendrites during cellular growth and the bridging structures by dendrite growth [19]. Openings in the wall structure are explained by the formation of side branches of ice crystals (branching phenomenon). These ice protrusions result in a continuous-interpenetrating network of ice crystals intertwined with collagen [31]. The characterization of the scaffolds indicated homogeneous pore size distributions throughout the scaffolds, whereas other studies often describe a gradual increase in pore size upward [28, 32]. Since the basis of the unidirectional collagen scaffolds preparation is the formation and growth of ice crystals, manipulation of ice crystal growth
offers possibilities to control scaffold parameters such as pore size and wall morphology, thus facilitating the construction of a range of collagen scaffolds with different characteristics.

Fast freezing using liquid nitrogen resulted in smaller pore sizes compared to slower freezing using a mixture of dry ice and ethanol. This complies with the well-known fact that dimensions of growing ice crystals generally decrease with increasing growth velocity [33]. Similar effects of the freezing rate on pore size have been reported extensively in literature [9, 12]. The thermal conductivity of the materials used is an important parameter. For instance, Schoof et al. [19] reported pore sizes of approximately 23 µm for collagen in 1.5 wt% acetic acid (resembling approximately 0.25 M acetic acid) frozen using copper blocks at a temperature of -180 °C, whereas Madaghiele et al. [2] reported pore sizes of 33 µm for collagen in hydrochloric acid frozen using liquid nitrogen-cooled copper blocks, while we measured pore sizes of 66 µm for freezing with liquid nitrogen. The insulator applied in our experiments delayed the freezing process and attributed to the larger pore size.

Unidirectional collagen scaffolds were constructed with various collagen concentrations where an increase in collagen concentration resulted in a slight decrease in pore size. Similar trends were shown by Madaghiele et al. where an increase in collagen concentration from 0.5% to 2% (in hydrochloric acid) frozen with liquid nitrogen resulted in a decreased pore size from 33 to 23 µm after lyophilization [2]. This result is in agreement with data shown by Pawelec et al. where 0.5% and 1% collagen scaffolds contained respectively pore sizes of 120 - 170 µm and 90 - 160 µm [28].

Next to freezing temperature and collagen concentration, ice crystal growth was influenced by detergents, such as octyl β-D-glycopyranoside, which tend to accumulate at the interface between the two phases (the solid (ice) and liquid (suspension) phases) where they decrease the surface energy (tension) to facilitate nucleation, resulting in smaller pores [34].

The properties of the suspension medium also influenced pore size in unidirectional scaffolds. Collagen scaffolds made from increasing concentrations of acetic acid resulted in scaffolds with larger pore sizes, in line with observations made by Schoof et al. who also showed that an increase in acetic acid concentration resulted in an increase in pore size (20 – 40 um for 1.5 - to 3.8 wt%) [19].

Unidirectional scaffolds have been constructed from other proteins than collagen and albumin, e.g. Zhang et al. prepared unidirectional scaffolds from silk fibroin and showed that an increase in silk fibroin concentration resulted in a decreased pore size, an improved pore orientation and an increased wall thickness [10]. Similar trends were described regarding PVA scaffolds by Gutiérrez et al. [35]. In our experiments, the increased wall thickness was only observed for scaffolds
prepared using BSA and PVA but not for collagen scaffolds, suggesting that different proteins/particles can be present varying compactness that influences ice crystal formation.

In this study, hybrid collagen/polymer scaffolds were prepared by mixing collagen and PVA prior to freezing and lyophilization. This method allows the freezing process to be the determining parameter for the final scaffold architecture. Other studies have created hybrid constructs by combining polymer meshes with hydrogels [36] or with a collagen/chitosan suspension prior to freeze-drying [37]. A limitation of such a strategy is that the meshes may have an effect on ice crystal growth during freezing and thus on the final pore structure of the freeze-dried scaffold.

Tissue engineering strategies generally encompass the use of scaffolds as cell-carriers and the incorporation of effector molecules to direct cellular ingrowth and differentiation [24, 38]. Unidirectional collagen scaffolds may provide improved cellular ingrowth as the result of the unidirectional architecture [1, 31]. An application for these scaffolds may be the regeneration of articular cartilage. Stimuli such as SDF-1α and growth factors of the BMP family may be coupled to the scaffold to attract mesenchymal stromal cells from the underlying subchondral bone and induce chondrogenesis, while the architecture facilitates migration of cells throughout the scaffold [39, 40].

The incorporation of (biodegradable) polymers is an option to improve the mechanical strength of collagen scaffolds, as indicated in this study for PVA, a FDA approved, biocompatible, and water-soluble polymer. Especially for load-bearing applications, an improvement of the mechanical strength is required to develop long-lasting implants [36].

The custom-made wedge system presented here allows a wide range of unidirectional scaffolds to be created in a simple manner and at low cost.

**Conclusion**

Porous scaffolds with unidirectional anisotropic pores were constructed by directional freezing using a custom-made wedge system. The mechanism of unidirectional ice crystal growth was elucidated, and ice crystal growth was manipulated to develop a wide range of unidirectional collagen scaffolds with distinctive pore structures.

**Acknowledgements**

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Supplemental Information
The process of planar ice dendrite formation visualized by video capture. This material is available free of charge via the Internet at http://pubs.acs.org.
References


Chapter 5

A comparison of cell distribution in anisotropic versus isotropic collagen scaffolds

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Willeke F. Daamen

Submitted
Abstract

Porous scaffolds constructed from type I collagen fibrils by freezing and lyophilization are frequently used for tissue engineering applications. Varying freezing conditions can adjust pore architecture, e.g. isotropic (random-oriented) vs. anisotropic (aligned) pores. A typical drawback of collagen scaffolds in vitro is the limited cell infiltration which hampers the development of cellular constructs.

In this study, we evaluated cell seeding procedures for both isotropic and anisotropic collagen scaffolds and investigated the effect of pore orientation on cell seeding efficacy, distribution and infiltration, and on tissue formation using C2C12 murine skeletal muscle myoblasts and human adipose-derived stem cells. Larger seeding volumes could only be applied for anisotropic scaffolds and resulted in a more equally distributed population of cells. For isotropic scaffolds cells resided more at the scaffold surface, while in anisotropic scaffolds they infiltrated further into the scaffold. Tissue formation, as judged by e.g. extracellular matrix formation, was also observed deeper in anisotropic scaffolds compared to isotropic scaffolds, and was deposited according to the scaffold template provided. In conclusion, anisotropic scaffolds allow increased cell infiltration, better cell distribution and result in more tissue formation in the scaffold in vitro.
Introduction

Tissue engineering aims to restore structure and function of damaged tissues. The general approach encompasses the development of scaffolds, which act as extracellular matrix (ECM) and provide a 3D environment for cells stimulating tissue regeneration [1]. Scaffolds can be constructed from various materials of which type I collagen, the main scaffolding material of the body, offers a number of valuable properties including biocompatibility, biodegradability, bioactivity and low antigenicity [2]. Type I collagen can be used to prepare hydrogels and porous scaffolds. The application of porous scaffolds prepared from insoluble collagen fibrils may be favorable since they generally provide better mechanical characteristics than hydrogels and collagen fibrils are the structural elements used by nature. For the construction of hydrogels, type I collagen needs to be solubilized [3], allowing homogeneous cell encapsulation [4], which is generally not observed in isotropic collagen scaffolds in vitro [5]. The use of anisotropic rather than isotropic collagen scaffolds may improve cellular infiltration [2], and allow improved development of cellular constructs in vitro. Anisotropic scaffolds can be constructed by freezing and lyophilization, resulting in aligned pores [2]. The pore orientation is crucial for proper functioning of tissues and plays an important role in the biomechanical properties of scaffolds [6]. In addition, the direction of newly formed ECM is dependent on the structure of the provided scaffold template [7, 8].

In this study, we directly compared isotropic and anisotropic collagen scaffolds with respect to cell seeding efficacy, cell distribution and tissue formation.

Materials and methods

Isotropic collagen scaffolds were prepared by freezing 0.7% (w/v) collagen suspensions (in 0.25 M acetic acid) in 6-well plates (7.5 ml/well) at -20 °C, followed by lyophilization [8] (Fig. 1A). To construct anisotropic collagen scaffolds, collagen suspensions were frozen by directional freezing (10 ml/scaffold) using liquid nitrogen [2]. Scaffolds were strengthened by vapor fixation with formaldehyde under vacuum (Fig. 1B), processed into their final dimensions (diameter: 12 mm, height: 4 mm, Fig. 1C), and further crosslinked using N-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS, Fig. 1B). Scaffolds were sterilized with 25 kGy γ-irradiation [9].

Scaffold morphology was assessed using scanning electron microscopy. Sample preparations and recordings were performed as described [2].
C2C12 murine skeletal muscle myoblasts were used to evaluate cell seeding procedures, while both C2C12 cells and human adipose-derived stem cells (ADSCs) were employed to investigate the effect of pore orientation. Cell culture experiments were performed n=3 with each condition in triplicate.

C2C12 myoblasts were expanded [10] and harvested (P23-24) [9], followed by assessing optimal cell seeding volumes and efficacies. Cell suspensions (100 µl and 250 µl, both with 2.5 x 10⁶ cells) were dripped on scaffolds placed on autoclaved Whatman paper to allow infiltration by capillary force (Fig. 1C, [9]). Scaffolds were incubated at 37 °C for 3 h and transferred to new 12-well plates, prior to adding proliferation medium [10] and harvesting for DNA analysis. As controls for DNA analysis, cell pellets (2.5 x 10⁶ cells) were prepared by centrifugation at 104 g for 5 min. Unless stated otherwise, 100 µl cell suspensions (2.5 x 10⁶ cells) were used for further experiments. Seeded scaffolds were cultured for 14 days (7 days proliferation medium and 7 days differentiation medium [10]), with medium refreshed every 3 days.

Human ADSCs were obtained from healthy donors undergoing reconstructive procedure (Radboud university medical center, Nijmegen, The Netherlands) and expanded [11]. Cells (P4) were seeded as described above and cultured with scaffolds for 21 days in α-MEM and chondrogenic medium [12], with medium refreshed every 3 days.

DNA analysis was performed after papain digestion and measured with Hoechst 33342 solution using calf thymus DNA as standard [9] and cell pellets as a reference for the total number of cells. qPCR analysis was performed as described previously [9], using primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), perinatal myosin heavy chain (pMHC), myogenin and actinin [13]. Results were expressed as quantification cycle (Cq) and normalized to GAPDH.

Cells cultured in monolayer (5,000 cells/well in 12-wells plate) were used as a reference. For (immuno)cytochemistry, scaffolds were processed [9] and stained with hematoxylin and eosin (H&E) and Alcian blue [12], or with antibodies against myosin (1:400, MY-32, Sigma-Aldrich) [13] and chondroitin sulfate [9]. Cell infiltration depth was determined using ImageJ using two methods: 1) by measuring the maximum depth where three single cells were located (linear intercept) and 2) by measuring the depth where three groups of at least 5 cells were observed.

GraphPad Prism (GraphPad Software, Inc., version 5, La Jolla, CA, U.S.A.) was used to perform statistical analysis. Results are shown as mean ± standard deviation. Repeated measures ANOVA with Tukey’s post-hoc tests was performed to compare groups, where p-values < 0.05 are considered statistically significant.
Results

Random and directional freezing of collagen suspensions and lyophilization yielded collagen scaffolds with isotropic and anisotropic pore architectures (Fig. 1A). Pore diameters were 89 ± 24 µm [8] and 66 ± 11 µm [2] for isotropic and anisotropic scaffolds, respectively.

Placing scaffolds on Whatman paper prior to cell seeding enhanced the influx of cells into the scaffolds. Without Whatman paper, cell seeding was difficult for both isotropic and anisotropic scaffolds because the scaffolds were wet after the scaffold processing techniques causing cell suspensions to spill over the edges of the scaffolds. Drying of scaffolds by freeze-drying was unfavorable since the additional freezing step damages the pore architecture formed during the initial freezing process.

For both isotropic and anisotropic scaffolds, the distribution of cells over the scaffold surface during the seeding procedure was more equal with larger volumes. Comparing seeding volumes of 250 µl to 100 µl, cells distributed better over the scaffold surface in case of 250 µl, whereas cells were unequally distributed with local high cell densities with 100 µl. For isotropic scaffolds seeded with a volume of 250 µl, the uptake of the cell suspension was limited, while anisotropic scaffolds did allow the infiltration of the whole cell suspension. DNA content was therefore significantly lower (p < 0.001) for isotropic scaffolds (18 ± 4 µg, Fig. 2A) compared to anisotropic scaffolds (51 ± 7 µg) and control cell pellets (2.5 x 10^6 cells; 46 ± 4 µg). For a seeding volume of 100 µl, cell suspensions could fully enter both scaffold types. No differences were found in cell seeding efficiency between isotropic scaffolds (56 ± 5 µg, Fig. 2A), anisotropic scaffolds (54 ± 8 µg) and control cell pellets (56 ± 7 µg). In order to compare isotropic and anisotropic scaffolds, 100 µl was applied as cell seeding volume for in vitro experiments.

Seeded C2C12 myoblasts and ADSCs penetrated further into the anisotropic scaffolds, while the cells mainly resided at the seeding side for isotropic scaffolds (Fig. 2B). Consequently, higher cell densities were present on top of isotropic scaffolds compared to anisotropic scaffolds. Linear depth intercept was 1.4 ± 0.2 mm and 0.9 ± 0.3 mm in anisotropic and isotropic scaffolds, respectively (p < 0.001). Groups of five cells were found almost twice as deep (p < 0.01) in anisotropic (0.9 ± 0.4 mm) compared to isotropic scaffolds (0.5 ± 0.2 mm).

For ADSCs, cartilage matrix formation was deposited according to the provided template. Most staining was found at the seeding side of the scaffolds (control staining were negative, data not shown), and deeper into anisotropic scaffolds compared to isotropic scaffolds (Fig. 2C). Therefore, the use of anisotropic scaffolds may be beneficial in vivo for the regeneration of aligned tissues, including tendon, muscle, nerves and cartilage [2].
Figure 1  Schematic illustration of the process from scaffold construction to cell seeding. 

A) Collagen suspensions were frozen by random or directional freezing (A1), yielding scaffolds with isotropic or anisotropic pores (A2). Scale bars represent 100 µm.  

B) Scaffolds were strengthened by vapor fixation with formaldehyde (B1), processed into their final dimensions (B2), and crosslinked using EDC/NHS (B3).  

C) Cell suspensions were dripped on top of wet, sterilized scaffolds, while the scaffolds were placed on Whatman paper, to allow infiltration of the cell suspension in the scaffold by capillary force.
For C2C12 myoblasts, the anisotropic pore architecture facilitated the formation of aligned elongated cellular structures, indicating fusion of myoblasts, while only randomly-oriented staining was observed in isotropic scaffolds (Fig. 2D). PCR analysis, however, indicated no differences in actinin, myogenin and pMHC gene expressions between isotropic and anisotropic scaffolds (data not shown).

In conclusion, anisotropic scaffolds facilitated cell infiltration best, and may be the scaffolds of choice to develop cellular constructs in vitro. Additionally, the anisotropic pore architecture enabled aligned tissue formation, important for the regeneration of tissues with an anisotropic extracellular matrix.

Figure 2 Seeding volume affects seeding efficacy, pore orientation affects cell infiltration and matrix deposition. A) The number of cells seeded was significantly (***: p < 0.001) reduced for isotropic scaffolds using 250 µl as seeding volume. Cell pellet reflects the total number of cells seeded. B) The anisotropic pore orientation facilitated the influx of cells, while cells seeded on isotropic scaffolds were largely present at the scaffold surface. C) Cartilage matrix produced by ADSCs was deposited deeper in anisotropic scaffolds compared to isotropic scaffolds. D) Using C2C12 myoblasts, alignment of cells (indicated by arrows) was observed in anisotropic scaffolds only. Scale bars represent 400 µm in (B) and 100 µm in (C) and (D). ADSCs: adipose-derived stem cells.
Acknowledgements

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References

Chapter 6

Introduction of specific 3D micro-morphologies in collagen scaffolds using even/uneven dicarboxylic acids

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Egbert Oosterwijk | Willeke F. Daamen | Toin H. van Kuppevelt
Abstract

The construction of scaffolds and subsequent incorporation of cells and biologics have been widely investigated to regenerate damaged tissues. Scaffolds act as a template to guide tissue formation and their properties have considerable impact on the regenerative process. Whereas many technologies exist to induce specific 2D morphologies into biomaterials, the introduction of 3D micro-morphologies into scaffolds produced from biological molecules poses a challenge. Scaffolds based on collagen are generally prepared by freezing and lyophilization. Variation in the speed of freezing allows for specific overall pore architecture, but cannot be used for the introduction of specific micro-morphologies into each individual pore wall. We here report the use of dicarboxylic acids to induce specific micro-morphologies in collagen scaffolds, and evaluate the effect of micro-morphologies on cellular migration and differentiation. Insoluble type I collagen fibrils were suspended in monocarboxylic acids and dicarboxylic acids of different concentrations, and unidirectional and random scaffolds were constructed by freezing/lyophilization. Scanning electron microscopy was used to investigate scaffold morphology and pore sizes. C2C12 cells were seeded on scaffolds with smooth and frayed-like pores and cultured for two weeks. Readout parameters included (immuno)cytochemistry and molecular biological analysis. Applying various acids and concentrations resulted in variations in 3D micro-morphologies, including wall structure, wall thickness and pore size. The use of dicarboxylic acids resulted in acid specific differences in pore structures, whereas monocarboxylic acids did not result in remarkable structural differences. Dicarboxylic acids with an odd or even number of C-atoms resulted in frayed or smooth wall structures, respectively, although with varying appearances. Formation of micro-morphologies was concentration dependent. In vitro analysis indicated cytocompatibility of scaffolds and enhanced myosin staining and myosin heavy chain gene expression levels for C2C12 cells cultured on scaffolds with frayed-like micro-morphologies compared to smooth micro-morphologies. In conclusion, porous collagen scaffolds with various 3D micro-morphologies can be constructed, acid crystal formation is key to the specific micro-morphologies observed and modulation of crystal growth by dicarboxylic acids allows generation of micrometer-defined topographies. These 3D micro-morphologies may be used as a screening platform to select optimal substrates for the regeneration of specific tissues.
Introduction

The field of tissue engineering strives to reconstruct structural and functional properties of damaged, diseased or lost tissues [1]. The general strategy includes the fabrication of supportive biomaterials, so-called scaffolds, which provide a template guiding tissue formation, and subsequently loading of these scaffolds with biological compounds and cells [2]. The selection of appropriate scaffolding materials is key since scaffolds have considerably impact in the regenerative process [3]. The interaction between cells and scaffolds is a complex interplay where both affect one another: the cells by remodeling the microenvironment and the scaffold by influencing cell fate. Properties such as mechanical strength, degradation rate, pore size and interconnectivity steer cellular growth and function [4, 5]. Moreover, micro-morphologies in the scaffold may regulate cellular behavior since 3D topographical features have been shown to regulate cellular migration, attachment, viability and differentiation [6, 7]. It has for instance been shown that bone marrow-derived mesenchymal stem cells preferentially adhere to ridged surfaces [8], that embryonic stem cells differentiate towards the neuronal lineage without the use of any differentiation-inducing agents on ridged or grooved surfaces [9], and that attachment and proliferation of chondrocytes is enhanced by incorporation of ridged or grooved surfaces [10].

Currently, several physical and chemical micro- and nanofabrication patterning techniques are available to introduce morphologies (e.g. ridges, pillars, pits and grooves) and distributions (e.g. random and regular features). These techniques encompass e.g. soft lithography, photolithography, electrospinning, polymer phase separation, layer-by-layer microfluidic patterning, three-dimensional printing, chemical vapor deposition, ion milling, salt leaching and reactive ion etching [11-13]. These techniques are generally not applicable to vulnerable biological molecules. Therefore, the construction of scaffolds consisting of biological structures as collagen fibrils, the structural elements used by nature, and the incorporation of micro-morphologies is limited by these techniques, but crucial to develop biologically relevant biocompatible systems for regeneration of tissues in vitro and in vivo.

Type I collagen is a promising biomaterial due to its biocompatibility, biodegradability, and bioactivity. Porous collagen scaffolds can be constructed by freezing-lyophilization, the ice crystals formed during the freezing process forming a negative blueprint for the final pore architecture [14]. Variations in the overall architecture of collagen scaffolds prepared by lyophilization have generally been introduced by adjustments of the freezing conditions, such as the freezing temperature, which are known to affect pore sizes [15-17]. Moreover, the pore orientation and structure can be adapted by applying temperature gradients.
and changing the concentration of acetic acid in which collagen fibrils were suspended, respectively. An increase in acetic acid concentration resulted in less thread-like and more closed wall structures [18]. Pawelec et al. showed that the incorporation of ionic or non-ionic solutes in a suspension of collagen in aqueous acetic acid changed pore size and influenced cell attachment [19]. These results imply that variations in crystal formation, due to the solute in which collagen fibrils are suspended, may result in adaptable micro-morphologies. Therefore, we hypothesize that scaffolds prepared using different acids (monocarboxylic acids and dicarboxylic acids) and concentrations may result in scaffolds with various micro-morphologies. Adjustable collagen scaffolds with variations in micro-morphology may be used as a screening platform to study the effect of such properties on cellular behavior in order to select optimal substrates for the regeneration of specific tissues.

Materials and methods

Scaffold construction

Type I collagen fibrils were isolated from bovine achilles tendon as described [20]. Collagen suspensions of 0.7% (w/v) were prepared using monocarboxylic and dicarboxylic acids. Monocarboxylic acids included formic acid (Merck, Darmstadt, Germany), acetic acid (Scharlau, Barcelona, Spain) and propionic acid (Merck). Dicarboxylic acids included oxalic acid (Merck), malonic acid (Sigma-Aldrich, St. Louis, MO, USA), succinic acid (Sigma-Aldrich), glutaric acid (Sigma-Aldrich), maleic acid (Sigma-Aldrich), and fumaric acid (Sigma-Aldrich). An overview of used acids (including their IUPAC names and chemical formula) and molarities is provided in Table 1. The acid solubility determined the maximum acid concentration applied to prepare collagen suspensions. After overnight incubation at 4 °C, collagen suspensions were homogenized on ice using a Teflon glass Potter-Elvehjem (Louwers Glass and Ceramic Technologies, Hapert, The Netherlands) with an intervening space of 0.35 mm (10 strokes), and deaerated by centrifugation at 117g for 30 min at 4 °C [18].

To investigate the effect of the various diluted acids on the micro-morphology of collagen scaffolds, scaffolds with unidirectional and random-oriented pores were constructed. Briefly, unidirectional collagen scaffolds were prepared by freezing collagen suspensions by directional freezing (10 ml/scaffold) using liquid nitrogen and a custom-made wedge system, followed by lyophilization in a Zirbus freeze dryer (Sublimator 500 II, Bad Grund, Germany) [18]. Random collagen scaffolds were prepared by freezing collagen suspensions in 6-wells plates (5 ml/well) at -20 °C, followed by lyophilization [16]. Scaffolds were then strengthened
Table 1  Overview of concentrations of acids used to induce micro-morphologies in unidirectional and random collagen scaffolds.

<table>
<thead>
<tr>
<th>Type of carboxylic acid</th>
<th>Acid</th>
<th>IUPAC</th>
<th>Chemical formula</th>
<th>Unidirectional scaffolds</th>
<th>Random scaffolds</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Concentration of acid used:</td>
<td>Concentration of acid used:</td>
</tr>
<tr>
<td>Monocarboxylic acid</td>
<td>Formic acid</td>
<td>Methanoic acid</td>
<td>HCOOH</td>
<td>0.05 M, 0.25 M, 1 M</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Acetic acid</td>
<td>Ethanoic acid</td>
<td>COOH</td>
<td>0.05 M, 0.25 M, 1 M</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Propionic acid</td>
<td>Ethanecarboxylic acid</td>
<td>COOH</td>
<td>0.05 M, 0.25 M, 1 M</td>
<td>-</td>
</tr>
<tr>
<td>Dicarboxylic acid</td>
<td>Oxalic acid</td>
<td>Ethanedioic acid</td>
<td>HOOCCOOH</td>
<td>0.05 M, 0.25 M, 1 M</td>
<td>0.05 M, 0.25 M, 1 M</td>
</tr>
<tr>
<td></td>
<td>Malonic acid</td>
<td>Propanedioic acid</td>
<td>HOOCCOOH</td>
<td>0.05 M, 0.25 M, 1 M</td>
<td>0.05 M, 0.25 M, 1 M, 2 M</td>
</tr>
<tr>
<td></td>
<td>Succinic acid</td>
<td>Butanedioic acid</td>
<td>HOOCCOOH</td>
<td>0.05 M, 0.25 M</td>
<td>0.05 M, 0.25 M</td>
</tr>
<tr>
<td></td>
<td>Glutaric acid</td>
<td>Pentanedioic acid</td>
<td>HOOCCOOH</td>
<td>0.05 M, 0.25 M, 1 M</td>
<td>0.05 M, 0.25 M, 1 M, 2 M</td>
</tr>
<tr>
<td></td>
<td>Maleic acid</td>
<td>(Z)-Butenedioic acid</td>
<td>HOOCCOOH</td>
<td>-</td>
<td>0.05 M</td>
</tr>
<tr>
<td></td>
<td>Fumaric acid</td>
<td>(E)-Butenedioic acid</td>
<td>HOOCCOOH</td>
<td>-</td>
<td>0.05 M</td>
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by vapor fixation crosslinking using 37% formaldehyde (Scharlau) under vacuum for 30 min to improve the handling [18].

**Scanning electron microscopy**
Scanning electron microscopy (SEM; JEOL SEM6340F, Tokyo, Japan) was used to investigate scaffold morphology. Scaffolds were cut longitudinally and perpendicularly and samples were placed on stubs and sputtered with a thin layer of gold using a Polaron E5100 Coating System [18]. Images were recorded using an accelerating voltage of 10 kV. For pore size quantifications, images were recorded from four random locations of perpendicular samples. The lengths of the shortest axis of 50 pores were measured using ImageJ [18].

**Cell culture**
To evaluate the effect of micro-morphologies on the differentiation of C2C12 murine skeletal muscle myoblasts, unidirectional collagen scaffolds with frayed-like (prepared using 0.25 M oxalic acid) and smooth (prepared using 0.25 M malonic acid) micro-morphologies (based on the SEM results) were selected.

Scaffolds were prepared as above and processed as described [21]. Briefly, scaffolds were strengthened by vapor fixation with 37% formaldehyde (Scharlau) under vacuum for 30 min, processed into their final dimensions (diameter: 12 mm, height: 4 mm, top and bottom were removed), then remaining aldehydes were quenched, and scaffolds were crosslinked using 33 mM N-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Fluka Chemica AG, Buchs, Switzerland) and 6 mM N-hydroxysuccinimide (Fluka Chemica AG) in 50 mM 2-morpholinoethane sulfonic acid (MES buffer, pH 5.0; Sigma-Aldrich) containing 40% (v/v) ethanol for 4 h. Subsequently, scaffolds were washed with 0.1 M Na₂HPO₄ (Merck), 1 M NaCl (Merck), 2 M NaCl, demineralized water and phosphate buffered saline (PBS, pH 7.4), and sterilized while wet with 25 kGy γ-irradiation [21].

Cell culture experiments were performed n=4 with each condition in triplicate. C2C12 murine skeletal muscle myoblasts were expanded in monolayer until 90% confluency in proliferation medium; Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Carsbad, CA, USA), supplemented with 10% fetal calf serum (FCS, Pan-Biotech, Aidenbach, Germany) and 100 IU/ml penicillin and 100 µg/ml streptomycin (Amresco, Solon, OH, USA). C2C12 cells were trypsinized using 0.05% trypsin-EDTA (Corning, Manassas, VA, USA), followed by seeding of 2.5 x 10⁶ cells per scaffold. In short, 100 µl cell suspensions (25 x 10⁶ cells/ml) were dripped on the scaffolds while the scaffolds were placed on autoclaved Whatman™ chromatography paper (3030-917, GE Healthcare Life Sciences, Pittsburg, PA, USA) to allow the cell suspension to infiltrate the scaffold by capillary force. Scaffolds were then incubated at 37 °C for 3 h to let the cells adhere to the scaffolds, after
which scaffolds were transferred to new 12-well plates and proliferation medium was added. The scaffolds were cultured for 7 days in proliferation medium and subsequently for 7 days in differentiation medium (DMEM, supplemented with 1% horse serum (PAA Laboratories, Cölbe, Germany) and 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells cultured in monolayer (5,000 cells/well in 12-wells plate) were used as a reference for quantitative polymerase chain reaction (qPCR). Medium was changed every 3 days. Scaffolds were harvested after 14 days and divided as follows: ½ scaffold for histological analysis and ½ scaffold for qPCR.

**Histology and immunohistochemistry**

For histological evaluation, samples were washed in 0.1 M PBS pH 7.4, fixed in 4% formaldehyde in 0.1 M PBS pH 7.4, dehydrated through graded ethanol series, cleared in xylene, embedded in paraffin, and cut into 5 µm thick sections. Sections were stained with haematoxylin and eosin (H&E) and immunofluorescent staining against myosin [22]. Briefly, sections were incubated in 0.1 M citrate buffer for 60 min, blocked in 0.15% glycine in 0.1 M PBS pH 7.4 for 30 min and in 1% (w/v) bovine serum albumin (BSA), 5% (v/v) normal goat serum, 0.1% (w/v) cold water fish gelatin (Sigma-Aldrich), 0.1% (v/v) Triton X-100 in 0.1 M PBS pH 7.4 for another 30 min. Sections were incubated with a primary antibody against myosin (1:400, MY-32, Sigma-Aldrich) for 60 min, secondary antibody goat anti-mouse IgG (H+L) Alexa 488 for 60 min, and 10 µg/ml 4′,6-diamidino-2-phenylindole (DAPI; Roche Diagnostics GmbH, Mannheim, Germany) to stain nuclei. Control sections were incubated with secondary antibody only. After each step, sections were washed with PBS. Sections were enclosed in Mowiol mounting medium (Sigma-Aldrich).

**qPCR**

Isolation of RNA, evaluation of RNA quality, synthesis of cDNA and qPCR analysis were performed as described previously [21]. Briefly, RNA was isolated in TRIzol® (Life Technologies, Carlsbad, CA, USA) using the RNeasy Mini Kit (Qiagen GmbH., Hilden, Germany, 74106), followed by measuring RNA quality using a NanoDrop instrument (Thermo Scientific, Rockford, IL, USA). Then, the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to convert 500 ng RNA into cDNA, using a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc.) for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. MilliQ water was included as no template control (NTC). Obtained cDNA (20 µl) was diluted 20 times in Milli-Q water. Gene expressions were measured using SYBR Green qPCR, using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) and 2 µL cDNA as a template, including NTC and MilliQ water controls. The following primer sequences were used in real-time PCR analyses: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward
5'-TGATGGGTGTGAACCACGAG-3'; reverse 5'-GGGCCATCCACAGTCTTCTG-3'; actinin forward 5'-TCATCCTCCGCTTCGCCATTC-3'; reverse 5'-CTTCAGCATCCAACATCTT-3'; and Myosin Heavy Chain (pMHC) forward 5'-TCGCTGGGCTGGGTGTTAG-3'; reverse 5'-TGTCTGTCAGGCTGGGTGTG-3'. qPCR was performed on a CFX96™ Real-Time System (Biorad). The following amplification settings were used: 5 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 20 s at 60°C and 20 s at 72 °C. Crossing-point (Cp) values, expressed as quantification cycle (Cq), were obtained using Bio-Rad CFX Manager 3.1 software (Bio-Rad) [21]. Gene expressions were normalized to GAPDH and were expressed as $2^{-\Delta Cq}$.

**Statistics**

Results of pore size measurements and qPCR are shown as mean ± standard deviation. Statistical differences were assessed using GraphPad Prism (GraphPad Software, Inc., version 5, La Jolla, CA, USA) by unpaired t-tests and repeated measures ANOVA with Tukey’s post-hoc tests to compare different groups, where p-values < 0.05 were considered statistically significant.

**Results**

**Scaffold characterization**

*Collagen scaffolds constructed with dicarboxylic acids*

Construction of unidirectional collagen scaffolds using various dicarboxylic acids resulted in different micro-morphologies of the pore wall (Fig. 1A). Scaffolds constructed using oxalic acid and succinic acid showed morphologically open inter-connective frayed-like wall structures, while the morphology in scaffolds constructed from malonic acid and glutaric acid showed smooth wall surfaces. Differences in frayed-like wall structures were observed, where scaffolds prepared using oxalic acid and succinic acid resulted in honeycomb-like and fibrillar micro-morphologies, respectively. Wall thickness also varied among the applied acids, where oxalic acid and succinic acid resulted in thick walls, whereas the use of malonic acid and glutaric acid resulted in thinner walls.

For random collagen scaffolds constructed using dicarboxylic acids with different concentrations (0.05-2 M, see Table 1), an increase in acid concentration resulted in more fibrillar wall structures (Fig. 2A). For dicarboxylic acids with an even number of C-atoms, oxalic acid (C2) and succinic acid (C4), the pore structure became more fibrillar from 0.25 M acid onwards. For dicarboxylic acids with an odd number of C-atoms, malonic acid (C3) and glutaric acid (C5), fibrillar structures appeared when using 1 M acid to construct scaffolds, which became even more apparent for acid concentrations of 2 M. SEM images showed smooth wall
Figure 1 Unidirectional scaffolds prepared using dicarboxylic acids. A) Scanning electron micrographs of unidirectional collagen scaffolds prepared using different dicarboxylic acids (all 0.25 M) showed variations in micro-morphologies. The use of oxalic acid and succinic acid resulted in a frayed-like morphology, while malonic acid and glutaric acid resulted in smooth wall structures. Differences in frayed-like micro-morphologies, honeycomb-like and fibrillar, were observed for scaffolds prepared using oxalic acid and succinic acid, respectively. B) Scanning electron micrographs of longitudinal cross-sections of unidirectional collagen scaffolds prepared using 0.05 M and 1 M oxalic acid indicated that the pore morphology depends on applied concentration, where a higher acid concentration increased wall thickness and fibrillarity. All scale bars represent 50 µm.
structures in scaffolds prepared using 0.05 M oxalic acid, malonic acid, succinic acid and glutaric acid. These results indicate that the formation of smooth or fibrillar structures depends on the type of dicarboxylic acid and acid concentration used (also see 3.1.3). An increase in acid concentration from 0.05-2 M significantly reduced pore size in scaffolds (Fig. 2B).

**Figure 2** Effect of dicarboxylic acid concentration on the formation of micro-morphologies in random collagen scaffolds. **A)** Scanning electron micrographs showed that all scaffolds prepared using 0.05 M dicarboxylic acid have smooth wall structures. The pore wall became more fibrillar upon an increase in acid concentration. For dicarboxylic acids with an even number of C-atoms (oxalic acid and succinic acid), the pore structure became more fibrillar at 0.25 M already, while for ‘odd’ acids (malonic acid and glutaric acid) fibrillar structures were observed starting at 1 M and were most apparent at 2 M. Scale bars represent 100 µm. **B)** An increase in acid concentration resulted in decreased pore sizes (*: p < 0.05, **: p < 0.0001). Note: scaffolds prepared using 1 M oxalic acid were too heterogeneous to determine pore sizes.
**Unidirectional collagen scaffolds constructed with monocarboxylic acids**

No morphological differences in pore wall structure and wall thickness were found between scaffolds prepared using formic acid, acetic acid and propionic acid (Fig. 3A; 0.25 M acid concentration). An increase in acid concentration generally increased the pore size (Fig. 3B) and scaffolds became less fibrillar with a more closed wall structure. Differences in pore size between acid concentrations applied were more apparent for higher acid concentrations. For 1 M acid concentrations, pore sizes were significantly different between formic acid, acetic acid and propionic acid (**: p < 0.0001). For 0.05 M and 0.25 M acid concentrations, pore sizes were significantly larger in scaffolds prepared using formic acid compared to acetic acid and propionic acid (\*: p < 0.05, ***: p < 0.0001). Pore sizes of scaffolds prepared using acetic acid were obtained from a previous study [18].

**Figure 3** Unidirectional scaffolds prepared using monocarboxylic acids. A) Scanning electron micrographs of unidirectional collagen scaffolds prepared using different monocarboxylic acids (all 0.25 M) showed no variations in micro-morphologies. Scale bars represent 50 µm. B) Pore sizes of scaffolds prepared from formic acid differed from those made with acetic acid [18] and propionic acid (\*: p < 0.05, **: p < 0.0001). For scaffolds constructed using 1 M acids, pore sizes were significantly different between formic acid, acetic acid and propionic acid (**: p < 0.0001).
**Effect of acid solubility on micro-morphology formation in random collagen scaffolds**

To further investigate the effect of acid solubility, independently of carbon chain length, random collagen scaffolds were constructed using dicarboxylic acids maleic acid (*cis*) and fumaric acid (*trans*), both C4. For maleic acid, smooth pore walls were visible, while a more thread-like morphology was observed for fumaric acid (Fig. 4).

![Figure 4](image)

**Figure 4** Effect of acid solubility on the formation of micro-morphologies. Scanning electron micrographs of the pore structure of scaffolds prepared from *cis/trans* isomers (both C4) maleic acid (A, *cis*) and fumaric acid (B, *trans*) showed smooth and a more fibrillar pore structure for maleic acid and fumaric acid, respectively. Scale bars represent 100 µm.

**Cytocompatibility and cellular differentiation**

**General morphology and immunocytochemistry**

To evaluate cytocompatibility of the scaffolds and to investigate the effect of incorporated micro-morphologies on cell differentiation, C2C12 cells were cultured on unidirectional collagen scaffolds prepared using 0.25 M oxalic acid (frayed-like morphology) and 0.25 M malonic acid (smooth morphology) for 14 days. C2C12 cells infiltrated deeply into the scaffolds, which was facilitated by the unidirectional pore architecture. Cells resided more at the outside of scaffolds prepared using oxalic acid, while they infiltrated further into scaffolds prepared using malonic acid (Fig. 5A). Myosin staining (Fig. 5B) indicated aligned elongated cellular structures for C2C12 cells cultured in scaffolds with both smooth and frayed-like pores, but the staining was more intense for C2C12 cells cultured in scaffolds prepared using oxalic acid (frayed morphology).
Figure 5 Differentiation of C2C12 cells seeded on scaffolds prepared using oxalic acid and malonic acid. A) The unidirectional pore architecture facilitated infiltration of cells, but cells were found deeper in scaffolds prepared using malonic acid compared to oxalic acid. Scale bars represent 200 µm (top panel) and 100 µm (bottom panel). B) Myosin staining indicated aligned elongated cellular structures for C2C12 cells cultured in oxalic acid and malonic acid scaffolds. Staining was more intense for oxalic acid. Scale bars represent 50 µm. C) Analysis of gene expression levels of actinin (p = 0.081), myogenin (p = 0.064) and pMHC indicated that only pMHC gene expression levels were significantly increased (p = 0.013) for C2C12 cells cultured on scaffolds prepared using oxalic acid (frayed morphology) compared to malonic acid (smooth morphology). A general trend was higher gene expression levels for C2C12 cells cultured on frayed morphologies compared to smooth morphologies.
**mRNA analysis**

Gene expressions of actinin, myogenin and perinatal myosin heavy chain (pMHC) were analyzed in C2C12 cells and compared to the expression of the C2C12 cells cultured in monolayer as positive control. mRNA analysis indicated that pMHC gene expression was significantly upregulated for C2C12 cells cultured on scaffolds prepared using oxalic acid (frayed morphology) compared to malonic acid (smooth morphology). No differences were observed in actinin ($p = 0.081$) and myogenin ($p = 0.064$) gene expression levels between scaffolds prepared using oxalic acid and malonic acid, and positive controls (Fig. 5C). A general trend of higher gene expression levels of C2C12 cells was found when cultured on frayed morphologies compared to smooth morphologies.

**Discussion**

In general, *in vitro* studies have investigated the effect of micro-morphologies using 2D monolayer culture models, where substrates have been modified with topographical features, including morphologies (e.g. ridges, pillars, pits and grooves) and distributions (e.g. random and regular features) [11-13]. Although this is informative for the effect of specific micro-morphologies on cellular behavior, the ability to incorporate specific 3D micro-morphologies in scaffolds is key to steer cell behavior. The novel methodology developed here allows both the use of biological macromolecules and the incorporation of 3D micro-morphologies in scaffolds, to guide tissue formation in collagen scaffolds.

It was observed that dicarboxylic acids with an even number of C-atoms (referred to as “even acids”) led to fibrillar scaffold wall structures, whereas dicarboxylic acids with an odd number of C-atoms (referred to as “odd acids”) led to scaffolds with a solid wall structure. It is known that series of alkane derivatives often show alternating physicochemical characteristics between compounds with an even and odd number of carbon atoms, *e.g.* with respect to solubility [23]. The use of dicarboxylic acids resulted in acid specific differences in pore structures, whereas monocarboxylic acids did not result in remarkable differences in micro-morphology. Dicarboxylic acids with an even or odd number of C-atoms resulted in frayed or smooth wall structures, respectively, although with different appearances, which was dependent on the acid concentration applied. The scaffold morphology is likely related to the formation of crystals. In our experiments, the use of poorly soluble acids resulted in a higher degree of fibrillarity. The effect of acid solubility was confirmed by constructing scaffolds using dicarboxylic acids maleic acid and fumaric acid, which both contain four carbon atoms including a double bond between C2 and C3, representing the well dissolvable *cis*
and poorly dissolvable trans isomer, respectively. For the cis and trans isomer, a smooth and thread-like micro-morphology were observed, respectively, which could be explained by the cis-conformation enabling the formation of an intramolecular hydrogen bond in maleic acid between the two carboxylic acid groups, which is not present in fumaric acid [24]. This results in a strongly increased solubility (6.16 mol/kg at 298.15 K [25]) for maleic acid compared to fumaric acid (0.05 - 0.06 mol/kg) [26]. Based on these observations, the scaffold

![Figure 6](image)

**Figure 6** Relation between the number of C-atoms and dicarboxylic acid solubility, and crystal packing of even and odd acids. **A** Solubility of dicarboxylic acids in water. There is an even-odd effect with respect to the number of C-atoms on solubility [27, 28]. The effect of solubility was also found by comparing fumaric acid and maleic acid (both 4 C-atoms), which have a low and high solubility, respectively. For poorly soluble acids, readily forming crystals, frayed surface structures were observed in the collagen scaffolds, whereas for highly soluble acids smooth wall structures were found. **B** Overall structure of acids with an even and odd number of C-atoms (parallelogram versus trapezoid-like). The difficulty to form crystals in case of trapezoid shaped acids results in smooth pore walls, while the ease of crystallization of parallelogram shaped acids results in frayed pore walls.
structure at a given acid concentration appears to correlate to the length of the carbon skeleton of the dicarboxylic acid (Fig. 6A). The solubility of a compound is inversely correlated to the ease of crystal formation. The crystal structures of both even and odd dicarboxylic acids were shown to be mainly based on a combination of intermolecular hydrogen bonding and hydrophobic interactions [23]. The carboxylic acid groups on each side of the molecule form hydrogen-bonded dimers with successive molecules, resulting in the formation of continuous acid chains. Furthermore, the hydrophobic methylene backbones stack on top of each other, perpendicular to the chain axis. Starting from glutaric acid (C5), the packing modes of the acids were shown to be regular within both series of odd and even acids. Thalladi et al. [23] proposed a model which explains the lower packing stability of odd acids by the repulsive effect of carboxylic acid groups of adjacent chains. In even acids, the distance between these moieties can be increased by slightly shifting each chain along the chain axis. In odd acids, shifting the chain would increase the distance on one side of each molecule, but decreases the distance on the other side. The crystal packing of odd dicarboxylic acids therefore cannot be stabilized in this way. Although this model is not directly translatable to the lower dicarboxylic acids (< C5), similar mechanisms may account for the solubility alternation found for these acids. Also the morphology of the crystal lattice of odd and even acids may be responsible for the micro-morphologies observed. Acids with an odd and even number of C-atoms have a trapezoid-like and parallelogram-like structure, respectively (Fig. 6B). The ease or difficulty to form crystals from even (parallelogram) and odd (trapezoid) acids, respectively, gives rise to the formation of micro-morphologies observed in this study.

In vitro analysis was performed to investigate cytocompatibility and the effect of different micro-morphologies on cellular differentiation. C2C12 cells were cultured on unidirectional type I collagen scaffolds, since these scaffolds facilitate cell infiltration due to the aligned pore architecture [18]. Incorporation of frayed-like micro-morphologies in unidirectional collagen scaffolds was favorable over the use of smooth micro-morphologies, as indicated by myosin staining and pMHC gene expression levels, confirming the preference of cells for irregular structures [8-10]. The underlying mechanism of the effect of frayed-like micro-morphologies on cell differentiation may be explained by focal adhesion formation and subsequent signal transduction cascades. Yang et al. [29] described that micro-morphologies play important roles in the formation and dissociation of focal adhesions, which connect intracellular compartments of the cell with the extracellular matrix. Adaptation of focal adhesions due to the provided scaffold template, as a result of mechanotransduction, may result in physical changes and reorganization of intracellular compartments [30, 31]. Subsequently, this may result in differences in protein expression, interaction and concentration, and
intracellular signaling, including of focal adhesion kinase [32, 33]. As a proof-of-principle, we investigated the differentiation of C2C12 cells on frayed-like and smooth scaffolds, for which we selected scaffolds prepared using 0.25 M oxalic acid and malonic acid. Further investigations of other dicarboxylic acids and concentrations, and intra- and extracellular mechanisms resulting in specific cell responses to the micro-morphologies applied may provide insights in the selection of substrates for specific purposes.

Unidirectional collagen scaffolds with incorporated micro-morphologies may be applied for the regeneration of various tissues. Screening for the effect of micro-morphologies on cellular behavior can be performed with the knowledge gained here, thereby making it possible to select optimal substrates for the regeneration of specific tissues.

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References

Chapter 7

Unidirectional BMP2-loaded collagen scaffolds induce chondrogenic differentiation

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Submitted
Abstract

Microfracture surgery may be improved by implantation of unidirectional collagen scaffolds that provide a template for mesenchymal stem cells to regenerate cartilage. Incorporation of growth factors in unidirectional scaffolds may further enhance cartilage regeneration. In scaffolds, immobilization of growth factors is required to prolong in vivo activity, to limit diffusion and to reduce the amount of growth factor needed for safe clinical application. We investigated the immobilization of bone morphogenetic protein 2 (BMP2) to unidirectional collagen scaffolds and the effect on chondrogenesis in vitro. C3H10T1/2 cells were seeded on unidirectional collagen scaffolds with and without covalently attached heparin, and with and without incubation with BMP2 (1 and 10 µg), or with BMP2 present in the culture medium (10-200 ng/ml). Culturing was for 2 weeks and readout parameters included histology, immunohistochemistry, biochemical analysis and molecular biological analysis. The unidirectional pores facilitated the distribution of C3H10T1/2 cells and matrix formation throughout scaffolds. The effective dose of medium supplementation with BMP2 was 100 ng/ml (total exposure 1 µg BMP2), and similar production of cartilage-specific molecules chondroitin sulfate and type II collagen was found for scaffolds pre-incubated with 10 µg BMP2. Pre-incubation with 1 µg BMP2 resulted in less cartilage matrix formation. The conjugation of heparin to the scaffolds resulted in more chondroitin sulfate and less type II collagen deposition compared to scaffolds without heparin. In conclusion, unidirectional collagen scaffolds pre-incubated with 10 µg BMP2 supported chondrogenesis in vitro and may be suitable for prolonged cartilage matrix synthesis in vivo.
Introduction

Articular cartilage covers bone surfaces within synovial joints and provides a low-friction and load-bearing surface for efficient and flexible joint motion [1]. Traumatic injuries and osteoarthritis can result in cartilage damage. Restoring cartilage defects is challenging due to the limited self-renewal capacity. Patients with cartilage defects often suffer from progressive joint dysfunction and pain [2]. To treat patients with localized cartilage defects, microfracture surgery [3], mosaicplasty [4] and autologous chondrocyte implantation [5] are applied. Although satisfactory short-term clinical results have been described [6], these treatments can result in donor site morbidity [4] and fibrocartilage formation [7]. Consequently, newly formed tissue often lacks the mechanical and biological properties of native hyaline cartilage and gradually degenerates over time [8].

Regenerative medicine and tissue engineering strive to find alternatives to restore the structure and function of damaged articular cartilage. A promising strategy may be the implantation of scaffolds in combination with microfracture surgery, making use of bone marrow-derived mesenchymal stem cells (MSCs) that penetrate into the scaffold and produce new cartilage tissue [9, 10]. The use of unidirectional collagen scaffolds is promising for this application since the anisotropic pore organization facilitates the influx of cells [11] and steers the arrangement of cells in vertical columns, resembling the organization of chondrocytes in native articular cartilage [12]. Additionally, immobilization of growth factors to the scaffolds may allow for sustained delivery of growth factors to stimulate cartilage regeneration in vivo [13]. The efficacy of the implantation of scaffolds and incorporation of biologics have been studied before; implantation of scaffolds resulted in improved cartilage regeneration, which was further enhanced by loading the scaffolds with biologics [14]. Because cartilage tissue mainly consists of type II collagen, the use of scaffolds prepared from type II collagen seems a logical choice, but Ohno et al. [15] showed similar type I, type II and aggrecan gene expressions for chondrocytes cultured in type I and type II collagen scaffolds in vitro. Moreover, the ability for dedifferentiated chondrocytes to redifferentiate has been described in PLGA-type I collagen hybrid meshes [16], suggesting that type I collagen scaffolds are suitable for cartilage regeneration. This was confirmed by Buma et al. [17], who found less cell migration in type II collagen scaffolds in vivo because cells were directed into a chondrogenic phenotype upon arrival in the scaffolds, while type I collagen scaffolds facilitated the influx of progenitor cells from the subchondral bone towards the defect site and throughout the implanted scaffolds. These scaffolds may be further improved by bioactive molecules that stimulate cartilage regeneration.
Bone and cartilage development are guided by various growth factors, in a distinct spatiotemporal setting [18]. Bone morphogenetic protein (BMP), transforming growth factor beta (TGF-β) and fibroblast growth factor (FGF) have been used to stimulate cartilage regeneration in vitro [19]. These growth factors are generally added to the culture medium to induce a cellular response, but for in vivo applications growth factors need to be immobilized since no biologics can be added after implantation of scaffolds and diffusion of growth factors away from the defect should be prevented. For clinical application, the use of growth factors is constrained by rapid degradation and clearance and therefore excessive doses exceeding 10,000 times the physiological dose have been applied. However, these high doses resulted in side effects including infections, hematomas and cancer [20]. Immobilization may reduce the amount of growth factors needed, necessary for safe clinical application. Growth factors can be incorporated in collagen scaffolds by non-covalent interaction with glycosaminoglycans (GAGs), which can be covalently immobilized to collagen scaffolds [21]. In vivo, GAGs are important for growth factor signaling, controlling the dose and bioactivity, and protecting growth factors from degradation [22, 23]. Heparin is a highly negatively charged glycosaminoglycan that has been used for sustained delivery of BMP2 [13, 24]. Therefore, heparin-conjugated scaffolds were applied as sustained delivery vehicles for the release of growth factors.

The aim of this study was to enhance the bioactivity of unidirectional type I collagen scaffolds for potential in vivo applications, which has to our knowledge not been investigated before. Unidirectional collagen scaffolds were chosen as a model to study in vitro chondrogenesis since these scaffolds aid cellular infiltration [11]. C3H10T1/2 cells, resembling mesenchymal cells, were used as a cell culture model to study chondrogenic differentiation [25]. We first studied chondrogenic differentiation of C3H10T1/2 cells using soluble BMP2 supplemented to the culture medium as a reference to subsequently investigate whether cartilage regeneration could be resembled by (heparin-conjugated) unidirectional collagen scaffolds pre-incubated in BMP2.

Materials and Methods

Scaffold construction
Type I collagen fibrils were isolated from bovine achilles tendon [26]. Scaffolds were prepared by lyophilization of a 0.7% (w/v) collagen suspension in diluted acetic acid that was directionally frozen using a custom-made wedge system using liquid nitrogen [11]. Constructed unidirectional collagen scaffolds were strengthened by crosslinking using vapor fixation with 37% formaldehyde.
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(Scharlau, Barcelona, Spain) under vacuum for 30 min [27]. Subsequently, punches (diameter: 12 mm, height: 8 mm) were taken from the top of the scaffolds using 12 mm punch devices (Acuderm Inc., Ft. Lauderdale, FL, USA), and processed to final dimensions of 12 mm diameter and 4 mm height by removal of the top and bottom of the punched scaffolds and subsequently dividing the scaffold in two parts. The scaffolds were wetted in demineralised water and remaining aldehydes were quenched by incubation in 1 M sodium phosphate buffer pH 6.5 (Merck, Darmstadt, Germany) containing 30 mM NaBH₄ (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 4 °C. Scaffolds were washed six times for 30 min with demineralised water, and crosslinked using 33 mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC, Fluka Chemie, Buchs, Switzerland) and 6 mM N-hydroxysuccinimide (NHS, Fluka Chemie) in 50 mM 2-morpholinoethane sulfonic acid (MES buffer, pH 5.0; Sigma-Aldrich) containing 40% (v/v) ethanol for 4 h in the presence and absence of 0.25% (w/v) heparin (Organon, Oss, The Netherlands). Scaffolds were washed with 0.1 M Na₂HPO₄ (Merck), 1 M NaCl (Merck), 2 M NaCl and demineralized water [28]. Finally, scaffolds were washed in phosphate buffered saline (PBS, pH 7.4) and sterilized with 25 kGy γ-irradiation from a 60Co source (Synergy Health Ede B.V., Ede, The Netherlands). Scaffolds crosslinked with EDC/NHS only and heparin-conjugated scaffolds are abbreviated as “Col” and “Col-Hep”, respectively.

Scanning electron microscopy
Scaffold morphology was analyzed using scanning electron microscopy (SEM, Gemini Sigma 300, Zeiss, Oberkochen, Germany). Scaffolds were cut longitudinally, mounted on stubs, and sputtered with an ultrathin layer of gold using a Polaron E5100 Coating System. Images were recorded at an accelerating voltage of 10 kV [11].

Cell culture
Murine mesenchymal C₃H10T1/2 Clone 8 cells (American Type Culture Collection, Rockville, MD, USA) were seeded (2500 cells/cm²) and expanded in monolayer culture until 90% confluency in proliferation medium (BME, Gibco, Carsbad, CA, USA), supplemented with 10% fetal calf serum (FCS, Perbio Science, Belgium), 1% GlutaMAX-I (Gibco), 100 IU/ml penicillin and 100 µg/ml streptomycin (P/S; Amresco, Solon, OH, USA). Cells were harvested using 0.05% trypsin-EDTA (Gibco). Prior to cell seeding on the scaffolds, scaffolds were incubated in proliferation medium. Then, 250 µl cell suspensions (10⁷ cells/ml in proliferation medium) were pipetted on the scaffolds while placed on an autoclaved Whatman™ chromatography paper (3030-937, GE Healthcare Life Sciences, Pittsburg, PA, USA) to extract fluids from the scaffolds and allow the cell suspension to infiltrate the scaffold by capillary force. Scaffolds were placed in a 12-well plate without additional medium to allow adherence of the cells to the scaffolds. After 3 h,
scaffolds were transferred to a 12-well plate and 2 ml chondrogenic differentiation medium was added (Dulbecco’s modified Eagle’s medium (DMEM) + GlutaMax-I, Gibco), supplemented with 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml sodium selenite, 0.4 mg/ml proline, 1 mg/ml sodium pyruvate, 5.35 µg/ml linoleic acid, 10^{-4} M ascorbic acid 2-phosphate, 1.25 mg/ml bovine serum albumin and 10^{-7} M dexamethasone (all from Sigma-Aldrich)). To investigate the effective dose of BMP2 (see section 2.2.1), the chondrogenic differentiation medium was supplemented with bone morphogenetic protein 2 (BMP2, carrier-free, R&D Systems, Minneapolis, MN, USA). To assess cartilage formation in scaffolds (see section 2.2.2), scaffolds with immobilized BMP2 were used, where scaffolds cultured in medium supplemented with BMP2 served as controls. To load scaffolds with BMP2, the scaffolds were placed on Whatman tissue to extract fluid from the scaffolds and subsequently the scaffolds were incubated in an Eppendorf tube with 100 µl PBS containing 1 µg or 10 µg BMP2 for 3 h. Next, scaffolds were washed to remove unbound BMP2 according to the following protocol: 1) scaffold were placed on Whatman tissue, 2) scaffolds were washed in proliferation medium, and 3) scaffold were placed on Whatman tissue. Medium was refreshed every 3 days. Scaffolds were harvested after 2 weeks.

**Evaluation of the required BMP2 dose to induce chondrogenic differentiation of C3H10T1/2 cells**

The effective BMP2 dose to induce chondrogenic differentiation was investigated by culturing the scaffolds in chondrogenic differentiation medium supplemented with different BMP2 concentrations: 10, 25, 50, 100 and 200 ng/ml. Control scaffolds were cultured without BMP2 in the medium. The following experimental conditions were included: 1) scaffold (Col), 2) heparin-conjugated scaffold (Col-Hep), 3) Col-Hep + 10 ng/ml soluble (sol.) BMP2, 4) Col-Hep + 25 ng/ml sol. BMP2, 5) Col-Hep + 50 ng/ml sol. BMP2, 6) Col-Hep + 100 ng/ml sol. BMP2, and 7) Col-Hep + 200 ng/ml sol. BMP2. Each condition was included in triplicate. The experiment was performed n=3.

**Chondrogenic differentiation of C3H10T1/2 cells on collagen and collagen-heparin scaffolds with non-covalently bound BMP2**

The effect of immobilizing BMP2 to unidirectional collagen scaffolds on chondrogenic differentiation of C3H10T1/2 cells was investigated by pre-incubating scaffolds with 1 or 10 µg BMP2 per scaffold. Based on results of 2.3.1, scaffolds cultured in chondrogenic differentiation medium supplemented with 100 ng/ml sol. BMP2 were used as controls. The following conditions were included: 1) Col, 2) Col-Hep, 3) Col + 100 ng/ml sol. BMP2, 4) Col-Hep + 100 ng/ml sol. BMP2, 5) Col + 1 µg pre-incubated (pre-inc.) BMP2, 6) Col + 10 µg pre-inc. BMP2, 7) Col-Hep + 1 µg pre-inc.
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BMP2, and 8) Col-Hep + 10 µg pre-inc. BMP2. Each condition was included in triplicate. The experiment was performed n=2.

**Histology and immunohistochemistry**

Scaffolds were fixed in 4% formaldehyde in 0.1 M phosphate buffered saline (PBS) pH 7.4 and processed for paraffin embedding via dehydration through graded ethanol series and clearing in xylene. Scaffolds were sectioned to yield 5 µm slices. Sections were stained with haematoxylin and eosin (H&E) and Safranin-O with fast green counterstaining [29]. For immunohistochemical staining, endogenous peroxidase was blocked using a 0.3% hydrogen peroxide solution for 10 min. Sections were washed with phosphate buffered saline (PBS) for 5 min, and blocked with 5% bovine serum albumin in PBS for 30 min, followed by incubation with primary antibodies against type II collagen (1:200, II-6B3II, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), chondroitin sulfate (1:200, CS56, Sigma-Aldrich), or heparan sulfate (1:10, AO4Bo8 VSV tagged single chain variable fragment antibody, cross-reacting with heparin [30]) at room temperature for 60 min. Subsequently, sections were washed three times in PBS for 5 min, followed by incubation at room temperature for 60 min with secondary antibodies; peroxidase-conjugated goat anti-mouse IgG H+L (1:250, Pierce) for II-6B3II, peroxidase-conjugated goat anti-mouse polyvalent immunoglobulins IgG, IgA, IgM (1:200, Sigma) for CS56, and monoclonal mouse anti-VSV peroxidase-conjugated IgG from mouse hybridoma cell line P5D4 (1:200, American Type Culture Collection) for AO4Bo8. As a control, sections were incubated with secondary antibody only. Sections were washed three times in PBS for 5 min and diaminobenzidine solution (Sigma-Aldrich) was added for 10 min to observe staining. Sections were counterstained using Mayer’s haematoxylin solution (Sigma-Aldrich), dehydrated through graded ethanol series, cleared in xylene and enclosed in Entellan™ mounting medium (Merck). Sections were scored with - in case of the absence of staining, ± for little positive staining, + for moderate positive staining and ++ for strong positive staining.

**Biochemical analysis**

Scaffolds were digested with 2.5 U/ml papain (Sigma Aldrich) in digestion buffer (50 mM NaPO₄, pH 6.5, 2 mM EDTA (Merck), 2 mM cysteine (Sigma-Aldrich)) [28] until no remnants of the scaffolds were visible. DNA content was measured using Hoechst 33342 solution (ThermoFisher Scientific) and calf thymus DNA as standard and a spectrofluorometer (Synergy 2, BioTek®, Winooski, VT, USA) [31]. A dimethylmethylen blue (DMMB) dye (Sigma-Aldrich, pH 3) was used to spectrophotometrically quantify heparin conjugated to the collagen scaffolds and GAGs formed by C3H10T1/2 cells. Absorbances were measured at 525 nm with a BioTek
plate reader. A heparin standard was used to calculate GAG content [28]. Results are expressed as GAG per DNA (µg/µg).

qPCR
Total RNA was isolated in TRizol® (Life Technologies, Carlsbad, CA, USA) using the RNeasy Mini Kit (Qiagen GmbH., Hilden, Germany, 74106) according to the manufacturer’s protocol. The quality of the extracted RNA was evaluated by measuring the A260/280 absorbance ratio using a NanoDrop instrument (Thermo Scientific, Rockford, IL, USA). TRizol-extracted total RNA (500 ng) was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc.) for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. MilliQ water was used as a no template control (NTC). The reverse transcriptase reaction mixture (20 µL) was diluted 20 times in H2O (MilliQ). Gene expression was determined by SYBR Green qPCR (quantitative polymerase chain reaction) using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) and 2 µL cDNA as a template, and NTC and MilliQ water controls. The following primer sequences were used in real-time PCR analyses: GAPDH forward 5’-GGCAAATTCAACGGCACACA-3’; reverse 5’-GTTAGTGGGCTCTCGTCTCTG-3’; type II collagen forward 5’-TTCCACTTCAGCTATGGGAG-3’; reverse 5’-GACGTTAGCGGTGTGAGGAG-3’; and aggrecan forward 5’-TACCGTCTGAACTGATGTC-3’; reverse 5’-AGCGTGTGGAAATAGCTCTG-3’. qPCR was performed on a CFX96™ Real-Time System (Biorad) using the following amplification settings: 5 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C. Crossing-point (Cp) values were determined by using Bio-Rad CFX Manager 3.1 software (Bio-Rad) and are expressed as quantification cycle (Cq). In case gene expression was too low to obtain a Cq value, these Cq values were set to 40 to be able to perform calculations. Gene expressions were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and expressed as 2−ΔCq.

Statistics
Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., version 5, La Jolla, CA, USA). Results are shown as mean ± standard deviation. To compare biochemical and qPCR results of different conditions one-way ANOVA with Tukey’s post-hoc tests were performed. P-values < 0.05 were considered to be statistically significant.
Results

Construction of unidirectional collagen scaffolds with and without heparin
Directional freezing of collagen suspensions and lyophilization yielded collagen scaffolds with a unidirectional pore architecture (Fig. 1A). Scaffolds were strengthened by vapor fixation with formaldehyde and further crosslinked with EDC/NHS with and without heparin conjugation. The amount of heparin conjugated to the scaffolds was 31 ± 10 µg/mg collagen, thus 3.1% of the dry weight of the scaffold. In Col only scaffolds, no heparin was detectable (p < 0.001). Staining for heparin indicated that heparin was distributed evenly throughout the unidirectional Col-Hep scaffolds and was not found in Col scaffolds (Fig. 1B).

Establishment of BMP2 dose to induce chondrogenic differentiation of C3H10T1/2 cells
Before investigating unidirectional scaffolds pre-incubated with BMP2, the BMP2 concentration in culture medium needed to induce chondrogenic differentiation

Figure 1 Scaffold characterization. A) Scanning electron micrograph of unidirectional pore architecture in collagen scaffolds after lyophilization and crosslinking with(out) heparin conjugation, showing an intact unidirectional pore architecture after vapor fixation with formaldehyde and further crosslinking with EDC/NHS with and without heparin conjugation. The scale bars represent 100 µm. B) Immunohistochemical staining for heparin showed that heparin was evenly distributed in Col-Hep scaffolds, whereas no heparin staining was observed in Col scaffolds. The scale bars represent 100 µm.
was investigated. This concentration will be used as a reference when pre-
incubating unidirectional collagen scaffolds with BMP2.

After 2 weeks of culture, cells had infiltrated the scaffolds and were found
throughout the scaffolds. The highest cell densities were present at the seeding
side of the scaffolds and the cells were found distributed over the surface. Newly
formed cartilage matrix, containing GAGs (Fig. 2A) and type II collagen (Fig. 2B),
was present mainly at the cell seeding side of the scaffolds, but only for scaffolds
cultured in the presence of BMP2. Some staining was found deeper in the scaffolds.
In the presence of soluble BMP2, cells were larger and stained red (Fig. 2A), possibly
indicating intracellular production of GAGs in the cells. An increase in BMP2
concentration resulted in increased tissue formation. For C3H10T1/2 cells cultured
in the presence of 10 and 25 µg/ml BMP2 little GAG and type II collagen staining
was observed. Increasing the concentration up to 100 ng/ml clearly increased
matrix formation. A further increase up to 200 ng/ml BMP2 only slightly increased
the amount of staining compared to 100 ng/ml BMP2. Newly formed tissue was
found deeper in the scaffold with increasing BMP2 concentration.

The biochemical analyses (Fig. 2C) indicated the presence of GAGs when
C3H10T1/2 cells were cultured in medium supplemented with BMP2. The general
trend was that an increase in BMP2 concentration resulted in increased GAG/DNA
levels. Only a little more GAGs were produced when increasing the BMP2
concentration from 100 ng/ml to 200 ng/ml. GAG deposition was not significantly
different between 50, 100 and 200 ng/ml BMP2.

The optimum dosage of BMP2 to induce chondrogenic differentiation of C3H10T1/2
cells was 100 ng/ml, and this concentration was therefore used as a control for
scaffolds pre-incubated with BMP2 in the next experiment.

**Chondrogenic differentiation of C3H10T1/2 cells on collagen and
collagen-heparin scaffolds with immobilized BMP2**

For scaffolds pre-incubated with BMP2 and control scaffolds cultured for 2 weeks
in chondrogenic differentiation medium supplemented with 100 ng/ml sol. BMP2,
positive matrix staining for type II collagen (Fig. 3A) and chondroitin sulfate (CS,
Fig. 3B) was observed, indicating the presence of cartilage-specific molecules.
Cells were found throughout the scaffolds and tissue formation was mainly
found at the cell seeding side of the scaffolds. Also fields of newly formed matrix
were found throughout the scaffolds. In the absence of BMP2, matrix staining
was negative for both type II collagen and CS. Only some intracellular staining
was observed for CS. Similar results were found for scaffolds cultured in 100 ng/ml sol.
BMP2 as in the previous experiment. For scaffolds pre-incubated with 1 µg BMP2
before cell seeding, only little positive type I collagen and CS staining was
observed. Pre-incubating scaffolds only once with 10 µg BMP2 before cell seeding
Figure 2  Scaffolds seeded with C3H10T1/2 cells cultured in chondrogenic differentiation medium with different BMP2 concentrations reveal that the lowest effective BMP2 dose is 100 ng/ml. (A) Safranin-O and (B) type II collagen staining indicated increasing staining from 10-100 ng/ml BMP2, and GAGs and type II collagen were absent in scaffolds cultured in chondrogenic differentiation medium without supplemented BMP2. Scale bars represent 100 µm. (C) Biochemical assays showed that GAG/DNA levels increased with increasing BMP2 concentrations up to 100 ng/ml. *: p < 0.05, **: p < 0.001, ***: p < 0.0001. Sol. represents soluble, where BMP2 was added to the culture medium.
Figure 3  Similar matrix formation between (heparin-conjugated) collagen scaffolds pre-incubated with 10 µg BMP2 and cultured in 100 ng/ml soluble BMP2. C3H10T1/2 cells were distributed over the scaffold surface, penetrated into the scaffolds, and cartilage matrix staining was observed throughout the scaffolds. (A) Type II collagen and (B) CS staining indicated little staining for scaffolds pre-incubated in 1 µg BMP2 and increased staining for scaffolds pre-incubated in 10 µg BMP2, resembling staining for scaffolds
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...resembled the results of supplementing the culture medium every 3 days for 2 weeks with 100 ng/ml sol. BMP2 (total 1 µg BMP2).

Heparin-conjugation to the scaffolds resulted in less intense type II collagen staining and more intense CS staining compared to Col scaffolds. An overview of the histological scoring per conditions is provided in Fig. 3C.

At the mRNA level, Col and Col-Hep scaffolds pre-incubated with 1 and 10 µg BMP2 showed comparable gene expression levels for type II collagen (Fig. 3D) and aggrecan (Fig. 3E) after culturing for 2 weeks. No differences were observed in type II collagen and aggrecan expression between Col-Hep + sol. BMP2 and scaffolds pre-incubated with 1 and 10 µg BMP2. Only for Col + 100 ng/ml sol. BMP2, a significant upregulation of type II collagen was observed compared to Col and Col-Hep scaffolds pre-incubated with 1 and 10 µg BMP2. For aggrecan gene expression levels, the only significant difference was found between Col + 100 ng/ml sol. BMP2 and all other experimental conditions. This may be due to the addition of growth factor during refreshing the culture medium every 3 days, while no growth factor was added during cell culture for scaffolds pre-incubated with BMP2. Low gene expression levels were found (Cq value set at 40) for Col and Col-Hep scaffolds cultured without BMP2.

Discussion

Cartilage regeneration by implantation of acellular biomaterials has been shown to be more effective than microfracture surgery alone, further improved by the incorporation of biologics [14]. When biologicals are incorporated in scaffolds, immobilization is required because biologics cannot be added after implantation of scaffolds. Hence, we aimed to enhance the bioactivity of collagen scaffolds by the incorporation of BMP2 to yield implants stimulating cartilage regeneration in vivo.

Cell infiltration is a typical limitation of in vitro studies [32], while in vivo cells are able to migrate through collagen scaffolds [32]. Unidirectional type I collagen...
scaffolds were used as a model to study in vitro chondrogenesis because the anisotropic pore organization facilitates the influx of cells [11, 27]. Native human articular cartilage has an average thickness of 2.4 mm [33] and the unidirectional pore architecture facilitated the distribution of cells throughout the scaffolds with a thickness of 4 mm in vitro. Cells were distributed over the scaffold surface and completely penetrated the scaffold. Cartilage formation was found deep into the scaffold. This makes the unidirectional collagen scaffolds a suitable model for in vitro studies. Additionally, the unidirectional architecture mimics the orientation of unidirectional collagen fibrils and steers the arrangement of chondrocytes in vertical columns, while joint loading should ultimately remodel the tissue to resemble the complex organization of native cartilage [12, 33]. The use of bioactive implants may more effectively promote chondrogenic differentiation of MSCs [34].

The chemical characteristics of scaffolds play an important role in cell-matrix interactions and cell differentiation [35]. In this study, the GAG heparin was conjugated to the scaffolds because of its high affinity binding to many growth factors, including basic fibroblast growth factor, vascular endothelial growth factor and BMP2 [36]. The incorporation of the GAGs in scaffolds yields negatively charged scaffolds, similar to the charge of proteoglycans in the cartilage matrix. In native tissue this results in load-dependent deformation due to the high water content [1]. As such, adjusting the heparin content conjugated to the scaffolds may provide control over the compressive modulus of the scaffolds [37]. Heparin-conjugation yielded the incorporation of 31 µg heparin per mg collagen. Lin et al. [37] described that the conjugation of 1.84 µg heparin per mg collagen scaffold already increased the compressive modulus seven-fold compared to control scaffolds. Immobilization of BMP2 to collagen scaffolds with (Col-Hep) and without (Col) conjugated heparin resulted in little cartilage matrix formation for scaffolds pre-incubated with a low dose of BMP2 (1 µg) and considerable cartilage matrix formation for scaffolds pre-incubated with a high dose of BMP2 (10 µg). Similar matrix formation between scaffolds incubated once in 10 µg BMP2 and 100 ng/ml sol. BMP2 repeatedly supplemented to the culture medium was observed after two weeks. Also in other studies 100 ng/ml sol. BMP2 was an adequate concentration to induce chondrogenic differentiation of C3H10T1/2 cells [38, 39]. Our results are in accordance with a study by Li et al. [40], where BMP2 loaded poly(ethylene argininylaspartate diglyceride scaffolds were compared to BMP2 added multiple times during culture. Although both Col and Col-Hep scaffolds induced cartilage matrix formation, heparin-conjugation resulted in more intense CS staining and less intense type II collagen staining compared to Col scaffolds. Tan et al. [41] described a similar divergent effect of heparin, where the presence of heparin induced an increase in proteoglycans and a decrease in
Unidirectional BMP2-loaded collagen scaffolds induce chondrogenic differentiation

Collagen. The highest amount of BMP2 used for scaffold pre-incubation in our study was 10 µg. In other studies similar amounts of growth factor were applied to induce cell differentiation [42] and tissue regeneration [37, 43]. The BMP2 amounts applied in our study (micrograms range) were relatively little compared to amounts applied in clinical studies (milligrams range) to induce an effect in vivo, which is important for safe medical application since high non-physiological doses of growth factors are associated with adverse effects [20].

The C3H10T1/2 cell line is widely used as a relevant cell culture model to study chondrogenic differentiation [25]. The cells display a fibroblastic morphology and can be differentiated towards osteoblasts, myoblasts, adipocytes and chondrocytes under specific stimuli [44]. We were able to induce chondrogenic differentiation of C3H10T1/2 cells and the formation of cartilage matrix under the influence of BMP2. We seeded 2.5 x 10^6 cells per scaffold to obtain high cell densities that may favor prechondrogenic cellular condensation [45-47]. A large number of cells was needed since the unidirectional pore architecture of the scaffolds facilitated the infiltration and distribution of the cells throughout the scaffolds. C3H10T1/2 cells are suitable as a first model to study in vitro chondrogenesis, but the use of bone marrow-derived MSCs may be of interest as they more closely resemble the clinical situation [48].

In future studies, loading scaffolds with different biologics can be investigated. Although BMP2 may be a suitable candidate for chondrogenic differentiation of murine C3H10T1/2 cells, the addition of different growth factors may improve chondrogenesis. For example, TGF-β can be used to stimulate the formation of cartilage-specific ECM, such as inducing type II collagen production [25]. The use of small molecules and synthetic peptides mimicking growth factor functions may be promising since growth factors are expensive. Small molecules are easier to deliver, more stable and less immunogenic than growth factors. Isoliquiritigenin and 4′-hydroxychalcone are such small molecules mimicking BMP2 signaling [49]. Moreover, Saito et al. prepared a synthetic peptide consisting of the BMP2 amino acids 73-92, which promotes chondrogenic differentiation of MSCs [50].

**Conclusion**

In conclusion, unidirectional collagen and heparin-conjugated collagen scaffolds supported the in vitro differentiation of the pluripotent stem cell line C3H10T1/2 towards the chondrogenic lineage under the influence of BMP2. Unidirectional Col and Col-Hep scaffolds pre-incubated with BMP2 may be suitable to induce prolonged cartilage repair in vivo.
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Chapter 8

Scaffolds for whole organ tissue engineering: Construction and in vitro evaluation of a seamless, spherical and hollow collagen bladder construct with appendices

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Abstract

The field of regenerative medicine has developed promising techniques to improve current neobladder strategies used for radical cystectomies or congenital anomalies. Scaffolds made from molecularly defined biomaterials are instrumental in the regeneration of tissues, but are generally confined to small flat patches and do not comprise the whole organ. We have developed a simple, one-step casting method to produce a seamless large hollow collagen-based scaffold, mimicking the shape of the whole bladder, and with integrated anastomotic sites for ureters and urethra. The hollow bladder scaffold is highly standardized, with uniform wall thickness and a unidirectional pore structure to facilitate cell infiltration in vivo. Human and porcine bladder urothelial and smooth muscle cells were able to attach to the scaffold and maintained their phenotype in vitro. The closed luminal side and the porous outside of the scaffold facilitated the formation of an urothelial lining and infiltration of smooth muscle cells, respectively. The cells aligned according to the provided scaffold template. The technology used is highly adjustable (shape, size, materials) and may be used as a starting point for research to an off-the-shelf medical device suitable for neobladders.
Introduction

In case of muscle-invasive and refractory superficial bladder cancer and end stage (congenital) bladder disease, the current clinical standard is radical cystectomy in combination with urinary diversion [1, 2]. The method of diversion depends on, amongst others, the nature of the defect, and the patient’s needs and wishes. Orthotopic bladder reconstruction is increasingly applied for urinary tract reconstruction [3]. However, current methods rely on autologous tissues that are harvested from the gastrointestinal tract. This can lead to severe complications including anastomotic leakages, enteric fistulae, bowel obstruction, prolonged episodes of ileus, life-threatening infections, nutritional mal-absorption, and/or intestinal failure [1, 4].

New techniques and materials generated in the field of regenerative medicine may provide useful alternatives. Regenerative medicine (RM) aims to regenerate tissues and organs by creating biological equivalents through the supplementation of scaffolding materials, bioactive components, cells or a combination thereof [5]. Within the field of RM different attempts have been made to reconstruct the bladder in both animal and human studies [6]. In 2006, a promising avenue for RM in producing a neobladder was published by Atala et al. where a collagen/polyglycolic acid composite was used which was sutured together into a partial bladder/cup shape and seeded with urothelial and smooth muscle cells [7]. Initial clinical results were promising, but a recent related phase II clinical trial demonstrated that an autologous cell cultured scaffold composed of synthetic polymers did not improve bladder compliance and was associated with serious adverse events that surpassed the acceptable safety standard [8]. In addition, the complicated and expensive nature of the procedure may not be feasible in most clinical centers [9-11]. Alternatively, a well-structured molecularly defined acellular scaffold resembling the whole bladder may be an option, using the body as a bioreactor. Previously, flat acellular collagen scaffolds have been used for bladder augmentation in patients with exstrophy-epispadias complex and were found to be completely lined with urothelial cells after implantation [12]. A tubular acellular collagen-based urostomy implanted in a pig model showed good results with respect to the re-urothelialization of the construct using the body as a bioreactor [13]. Flat scaffolds can be manually shaped into a sphere to create a bladder-like construct using sutures and a silicon breast prosthesis, as was shown by Baumert et al., who also pre-seeded the scaffold with urothelial and smooth muscle cells, and wrapped the construct in omentum for further cell differentiation in vivo [14]. Omental wrapping of tubular acellular collagen scaffolds resulted in good vascularization and tissue integration of the scaffold [15]. Bladder shaped acellular scaffolds for in vivo cellularization may be an option for urinary
diversions and neobladder reconstructions and would be in line with statements from recent proceedings from the "2nd international consultation on bladder cancer: urinary diversion", which indicated that widespread acceptance and success of a new technique is based on its simplicity [16]. For this, new methods in construct design are necessary. In this study, we have focused on the design of a novel, simple, standardized and adjustable process to produce resorbable seamless hollow scaffolds that mimic the size and shape of a human bladder and include appendices for anastomosis of the ureters and urethra. Cytocompatibility of bladder scaffolds, cellular influx and cell alignment were investigated using histology, immunohistochemistry, quantitative polymerase chain reaction, scanning electron microscopy and transmission electron microscopy.

**Materials and Methods**

**Construction of bladder scaffolds**
A 0.7 % (w/v) suspension of highly purified bovine tendon type I collagen fibrils in 0.25 M acetic acid (Scharlau, Spain) was prepared by overnight incubation at 4 °C [17]. The suspension was homogenized on ice using a Teflon glass Potter-Elvehjem device (Louwers Glass and Ceramic Technologies, Hapert, The Netherlands) with an intervening space of 0.35 mm (10 strokes). The suspension was deaerated by centrifugation at 117 g for 30 min at 4 °C. 500 mL of collagen suspension was poured into a custom-made aluminum (type 6082 T6) mold with a thermal conductivity of 205 W/m·K. The mold (inner and outer diameter of the spherical part: 8.5 and 9.2 cm, Fig. 1A) was specifically designed to mimic the shape of an adolescent human bladder [18], and three appendices were included to provide anastomosis sites for the ureters and urethra. The mold containing 500 mL collagen suspension was placed in a computer-controlled freezing bath (Proline RP890, Lauda GmbH, Lauda-Königshofen, Germany). To obtain a scaffold with a wall thickness of 10 mm, the mold was frozen at -20 °C for 30 min or -73 °C for 12 min, respectively, after which non-frozen collagen was removed from the mold (a schematic representation can be found in Fig. 1B and 1C). The mold was subsequently placed in a -20 °C freezer for complete solidification of the frozen and non-frozen collagen interface. Frozen constructs were lyophilized in a freeze dryer (Sublimator 500 II, Zirbus, Bad Grund, Germany) and subsequently γ-irradiated (25 kGy, Synergy Health B.V., Ede, The Netherlands).

**Temperature measurements**
Temperature differences in the collagen suspension were measured during the freezing process in triplicate using two thermocouples (Testo 922, Testo AG,
Lenzkirch, Germany). Both sensors were placed in the bottom of the aluminum mold in the collagen suspension, where one sensor was placed on the aluminum surface and the other sensor 5 mm above it. The mold containing the collagen suspension was equilibrated at 4 °C, before transferring the mold to the computer-controlled freezing bath, which was set at -20 °C or -73 °C. Temperature measurements at -20 °C or -73 °C were performed for 30 min and 10 min, respectively, and the temperature was recorded with time intervals of 1 min. Cooling rates were calculated from the slope from 0 °C until the end of the measurement. The results are shown as mean ± standard deviation in °C/min.

Scaffold characterization
Magnetic resonance imaging (MRI; 11.7T animal scanner, BioSpec, Bruker, Germany) was used to visualize the hollow structure of the scaffold with the following settings: Turbo-RARE pulse sequence; 37 ms echo time; 1500 ms repetition time; 180° flip angle; 0.195 x 0.195 x 1.5 mm/pixel spatial resolution; 12 min total acquisition time. Using ImageJ (1.47i, Wayne Rasband, National Institutes of Health, USA), the slices were subsequently combined to generate a 3D model.

Pore size quantification
Scanning electron microscopy (SEM, JEOL SEM6340F, Tokyo, Japan) was used to characterize the intrinsic morphology of the scaffolds. Samples were mounted on stubs and sputtered with an ultrathin layer of gold using a Polaron E5100 Coating System. Images were recorded at an accelerating voltage of 10 kV. For both freezing temperatures, three bladder scaffolds were constructed by freezing and lyophilization, and evaluated. Punches were taken from the scaffolds from 14 different locations (bottom: 1; bottom-middle axis: 4; middle axis 4; middle-top axis: 4; top: 1), followed by crosslinking using vapor fixation with 37 % formaldehyde under vacuum for 30 min [19]. From these punches, longitudinal and cross-sections were taken, and the inside and outside were evaluated. The longitudinal orientation was used to assess pore morphology. The cross-sections were used to investigate the pore size. Four images were recorded per cross-section and the lengths of the shortest axis of 40 pores per location were measured using the ImageJ trace tool.

Cell culture
To assess the cytocompatibility of bladder scaffolds, the following cells were used: primary porcine bladder urothelial cells (pbUCs) and smooth muscle cells (pbSMCs), human telomerase reverse transcriptase immortalized normal human urothelial cells (hTERT-NHUCs), further referred to as hbUCs, and primary bladder
smooth muscle cells (hbSMCs). Cells were seeded on scaffolds prepared by freezing at -20 °C because of the larger pore size compared to scaffolds frozen at -73 °C, favoring cell infiltration.

**Primary porcine bladder urothelial and smooth muscle cells**
Mature porcine bladders were obtained from a local slaughterhouse and pbUCs and pbSMCs were isolated as previously described [20, 21]. After isolation, the cells were expanded for no more than 3 passages. Ø35 mm punches were excised from the large hollow spheres, washed with PBS and subsequently pre-incubated overnight with the respective media. pbUCs were cultured in keratinocyte serum-free medium (Life Technologies, Carlsbad, CA, USA) supplemented with 50 µg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor, 30 ng/mL cholera toxin (all Sigma Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies). pbSMCs were cultured in smooth muscle cell medium (ScienCell™ Research Laboratories, Carlsbad, CA, USA) supplemented with 2% fetal bovine serum, smooth muscle cell growth supplement and 100 U/mL penicillin and 100 µg/mL streptomycin (all Life Technologies). Before seeding, the medium-soaked scaffolds were placed on a ~1 cm stack of sterile Whatman paper for about 5 min to remove excess medium, after which the scaffolds were transferred to a fresh stack. Then the cells were seeded on separate scaffolds (2.5 – 5 x 10^6 cells at a concentration of 5x10^6 cells/mL) where the pbSMCs were seeded on the outside of the scaffold and the pbUCs on the luminal sides. The cell suspension was allowed to sit on the scaffold for maximally 5 min after which the scaffold was carefully transferred to a 6-well plate with 2 mL medium. After all scaffolds were seeded, 4 mL medium was added until the scaffold was completely immersed. The seeded scaffolds were harvested after 1 and 7 days of culture. Samples were taken for immunohistochemistry and scanning electron microscopy. As a reference the pbSMCs and pbUCs were also seeded on glass slides and cultured for 7 days.

**Human bladder urothelial and smooth muscle cells**
hbUCs (a kind gift of Dr. M.A. Knowles, Leeds, UK, [22]) were cultured in keratinocyte serum-free medium (Life Technologies, Carlsbad, CA, USA) supplemented with 50 µg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor, 30 ng/mL cholera toxin (all Sigma Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies) up to passage 27. hbSMCs (ScienCell) were cultured in smooth muscle cell medium (ScienCell) supplemented with 2% fetal bovine serum, smooth muscle cell growth supplement and 100 U/mL penicillin and 100 µg/mL streptomycin (all Life Technologies) up to passage 8.
Punches (Ø12 mm) were excised from the large hollow spheres. The punches were dissected in 2 parts (luminal side for hbUCs and outside for hbSMCs). Scaffolds were washed 3 times with PBS and subsequently pre-incubated with the respective media. Before seeding, the medium-soaked scaffolds were placed on sterile Whatman paper to remove excess medium, after which the cells were seeded on separate scaffolds (1 x 10^6 cells at a concentration of 10 x 10^6 cells/mL). hbUCs and hbSMCs were seeded on the luminal side and the outside of the scaffolds, respectively, after which scaffolds were transferred to 12-well plates. After 3 h scaffolds were transferred to new 12-well plates and 2 ml medium was added. The scaffolds were cultured for 7 days. Samples were taken for histology, immunohistochemistry, quantitative polymerase chain reaction (qPCR) and transmission electron microscopy (TEM). As a reference for qPCR analysis of scaffolds cultured for 7 days, cell pellets were prepared from hbUCs and hbSMCs immediately after seeding by centrifugation at 104 g for 7 min.

**Scanning Electron Microscopy**
For scanning electron microscopy, cultured samples were fixed using 2% (w/v) glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (pH 7.4). Hereafter, the samples were washed with 0.1 M phosphate buffer for three times and subsequently dehydrated using ascending series of ethanol solutions (30, 50, 70 and 100%). Samples were dried using a critical point dryer (Polaron, Quorum Technologies, Rignmer, UK) using liquid CO_2 and imaged as described.

**Histology and immunohistochemistry**
The histological samples were fixed overnight in 4% formalin, dehydrated through graded ethanol series, cleared in xylene, embedded in paraffin, and sectioned to yield 5 µm slices, which were mounted on superfrost slides (Thermo Scientific, Menzel GmbH & Co KG, Braunschweig, Germany). Sections were deparaffinized using xylene and graded ethanol series. Sections were stained with toluidine blue to visualize the morphology of the cells on the scaffolds.

Immunohistochemistry was performed to assess the expression of specific UC and SMC markers. For porcine cells, samples were placed in Tissue-Tek (Sakura, Torrance, USA) and frozen in dry-ice cooled 2-methylbutane (Sigma-Aldrich, St. Louis, USA), sectioned (5 µm) using a cryostat microtome (Heidelberg, Heidelberg, Germany), mounted on superfrost slides and stored at -80 °C until use. In general, before staining, the slides were fixed using ice-cold acetone (-20 °C) for 10 min. For human cells, paraffin embedded sections were deparaffinized using xylene and graded ethanol series. Endogenous peroxidase was blocked using a 1% H_2O_2 solution for 30 min and the slides were blocked with 1% (w/v) normal goat/rabbit serum in PBS for 10 min. Next, slides were incubated for 1-2 h with the following
primary antibodies: rabbit anti-bovine type I collagen IgG (1:200, AB746P, Merck Millipore, Watford, UK), mouse anti-human cytokeratin 7 IgG1 kappa (CK7, 1:400, clone OV-TL 12/30, AM255, Biogenex, Fremont, CA, USA) or mouse anti-α smooth muscle actin IgG2a (αSMA, 1:8000, clone 1A4, A2547, Sigma-Aldrich). Then, slides were incubated for 30 min with secondary antibodies. For fluorescent staining, Alexa 488 conjugated goat anti-rabbit IgG (1:200, Invitrogen, Carlsbad, CA, USA) for type I collagen, and Alexa 594 conjugated goat anti-mouse IgG H+L (1:200, Invitrogen) for CK7 and αSMA, were used, followed by a 15 min incubation with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 5 µg/ml, Sigma-Aldrich) to stain nuclei, and mounting using wet mounting medium (Dako). For diaminobenzidine (DAB) staining, slides were incubated with peroxidase-conjugated rabbit anti-mouse IgG (1:100, Dako, Glostrup, Denmark), followed by a 10 min incubation with DAB solution (Sigma-Aldrich) to visualize staining, and mounting using Permount™ mounting medium (Fisher Scientific, Fair Lawn, NJ, USA). As a control, slides were incubated with the secondary antibody only. After each incubation step, the slides were washed three times in PBS for 5 min.

**Real-time polymerase chain reaction**

Total RNA was isolated in TRIzol® (Life Technologies, Carlsbad, CA, USA) using the RNeasy Mini Kit (Qiagen GmbH., Hilden, Germany, 74106) according to the manufacturer’s protocol. The quality of the extracted RNA was evaluated by measuring the A260/280 absorbance ratio using a NanoDrop instrument (Thermo Scientific). TRIzol-extracted total RNA (500 ng) was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and using a T100™ Thermal Cycler (Bio-Rad Laboratories, Inc.) for 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. MilliQ water was used as a no template control (NTC). The reverse transcriptase reaction mixture (20 µL) was diluted 20 times in H2O (MilliQ). Gene expression was determined by SYBR Green qPCR by using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) and 2 µL cDNA as a template, and NTC and MilliQ water controls. The following primer sequences were used in real-time PCR analyses: GAPDH forward TCAAGGCTGAGAACGGGAAG; reverse TGGACTCCACGACTACTCA; CK7 forward GGAGTGGGAGCCGTGAATAC; reverse GGATGGAAATAGCCTCAGGA; and αSMA forward GCATGCAGAAGGAGATCA-CA; reverse GCTGGAAGGTGGACAGAGAG. qPCR was performed on a LightCycler® LC480 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using the following amplification settings: 5 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 20 s at 60°C and 20 s at 72 °C. Crossing-point (Cp) values were determined by using LightCycler® 480 SW 1.5 software (Roche Diagnostics) and are expressed as quantification cycle (Cq). Gene expressions were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results are shown as relative gene expression
levels, expressed as $2^{-\Delta\Delta Cq}$, of CK7 and αSMA for scaffolds seeded with hbUCs and hbSMCs. Control values of hbUCs and hbSMCs pellets were set at 1 for CK7 and αSMA, respectively.

**Transmission electron microscopy**
Samples were harvested and washed three times in PBS, followed by fixation in 2% glutaraldehyde in phosphate buffer for 4 h, followed by washing 2 x 1 h in phosphate buffer and for 1 h in 1% (w/v) osmium tetroxide in phosphate buffer, dehydration using graded ethanol series and embedding in Epon 812. Ultrathin sections (60 nm) were cut and picked up on Formvar-coated grids, post-stained with lead citrate and uranyl acetate, and examined in a JEOL 1010 transmission electron microscope (JEOL, Tokyo, Japan).

**Statistics**
Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., version 5, La Jolla, CA, USA). The effect of the freezing temperature on pore size was assessed by a two-tailed t-test. A one-way ANOVA with Tukey’s posthoc test was performed to determine differences in relative gene expression levels. Results are shown as mean ± standard deviation. P-values < 0.05 were considered to be statistically significant.

**Results**

**Scaffold construction and macroscopic evaluation**
A collagen-based seamless hollow scaffold was constructed by freezing of a collagen suspension in a custom-made mold and lyophilization (Fig. 1A). The freezing process yielded a mechanically stable construct, where the outside was frozen (Fig. 1D). After removing the non-frozen fraction, the frozen part remained and had a spherical lumen (Fig. 1E). In the frozen construct the lumen of the appendices appeared to be clear of any collagen residues. After lyophilization the collagen scaffold could be removed from the mold (Fig. 1F).

The outer diameter of the scaffold was consistent with the inner diameter of the mold. The inside of the spherical part of the scaffold was hollow and the surface had a homogenous structure. Moreover, the lumens of the appendices remained hollow and straight. The appendices were firmly and seamlessly attached to the spherical part of the scaffold.
Scaffold structure characterization and freezing mechanism

Magnetic resonance imaging (MRI) was used to visualize scaffold structure and generate a 3D model of the scaffold. The overall shape of the scaffold, wall, lumen, and appendices with their lumen are easily distinguishable (Fig. 2A and 2B). The volume of the sphere was calculated using the MRI images and was determined to be approximately 380 ml. SEM images of different locations in the scaffold (spherical part and appendices) revealed a unique 3D unidirectional pore structure for scaffolds made at both -20 °C and -73 °C. The lumen showed low porosity with only few open structures whereas the outside of the scaffold was porous (Fig. 2C-I). The radial pore structure has been observed in other collagen scaffold types and is the result of the inward growth of ice crystals during the freezing process [19, 23]. The outer diameter of the collagen scaffold was 8.5 cm (Fig. 2J), while the inner diameter depended on the freezing rate and time. The wall thickness of the spherical part of the scaffold was 11 ± 2 mm and 8 ± 1 mm, and the thickness of

Figure 1 Construction and morphological evaluation of whole bladder scaffold with appendices. A) Custom-made aluminum mold; B and C) schematic representation of the freezing process and strategy to produce a seamless hollow construct with appendices. The mold was completely filled with a collagen suspension and placed in a computer-controlled freezing bath. After 30 min or 12 min freezing at -20 °C and -73 °C, respectively, the non-frozen collagen was removed from the mold; D) frozen hollow collagen construct; E) frozen hollow collagen construct cut in half to display the hollow inside; F) freeze dried scaffold which could easily be removed intact from the mold. Bars represent 1 cm.
the appendices was 6 ± 1 mm and 5 ± 1 mm for freezing at -20 °C and -73 °C, respectively. The inner diameter of the appendices was 5 ± 1 mm and 6 ± 0 mm for freezing at -20 °C and -73 °C, respectively.

The freezing process was evaluated by temperature measurements. For both freezing temperatures, the sensor placed on the aluminum surface always showed a lower temperature compared to the sensor located 5 mm above the aluminum surface. This indicates a temperature gradient inwards; the freezing process started at the aluminum surface and progressed through the collagen suspension. Freezing at -20 °C and -73 °C resulted in cooling rates of 0.45 ± 0.01 and 3.84 ± 0.38 °C/min, respectively (Fig. 3A). Freezing at different temperatures

Figure 2 Overview of the bladder scaffold and its structural characteristics. A) MRI slices of the bladder scaffold; B) 3D model based on MRI slices. C-I) SEM images of the bladder scaffold. C and E) exterior; F and G) cross-section of the wall; H and I) lumen; D) cross-section of the appendix. J) Macroscopical image of the bladder construct cut open. A, B, D, F and H were frozen at -20 °C and C, E and G were frozen at -73 °C. All bars in SEM micrographs represent 200 µm and macroscopical image bar represents 1 cm.
resulted in scaffolds with different pore sizes, the faster freezing protocol resulting in smaller pores (Fig. 3B). Freezing at -20 °C and -73 °C yielded pore sizes of respectively 70 ± 17 µm and 43 ± 10 µm for the spherical part (p < 0.0001) and 74 ± 21 µm and 41 ± 12 µm for the appendices (p < 0.0001).

**In vitro analysis of bladder scaffolds**

**General morphology and immunohistochemistry**

To evaluate the cytocompatibility, porcine (Fig. 4) and human (Fig. 5) urothelial cells (UCs) and smooth muscle cells (SMCs) were separately cultured on the luminal side and the outer side of the bladder construct, respectively, for 7 days. The UCs seeded at the luminal part were mainly located at the outside of the scaffold where they formed a confluent lining over the scaffold surface as indicated by SEM (Fig. 4C) and toluidine blue staining (Fig. 5A). The SMCs were seeded on the porous outside of the scaffold and formed multilayers. The cells

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**Figure 3** Freezing characteristics and pore size quantification. **A)** Temperature of the collagen suspension during freezing at -20 °C and -73 °C. One sensor was placed on the inner surface of the aluminum mold and the other sensor was placed in the collagen suspension 5 mm above the aluminum surface. **B)** Pore sizes of the spherical part and the appendices of the collagen scaffold constructed using freezing temperatures of -20 °C and -73 °C, *p < 0.0001; C and D)** Pore morphology in cross-sections of the spherical part of the scaffold prepared using freezing at -20 °C (C) and -73 °C (D). The scale bars in SEM images represent 100 µm.
were able to penetrate deep into the scaffold, likely due to the unidirectionality of the scaffold (Fig. 5B). A confluent and aligned layer of SMCs was seen on the outside of the scaffolds (SEM (Fig. 4F) and light microscopy (Fig. 5B). The UCs and SMCs were able to attach to the scaffold and maintained their phenotype as

**Figure 4** Bladder scaffolds after 7 days of culture with porcine urothelial and smooth muscle cells and overview of reference staining. **A)** Overview staining of scaffolds seeded with porcine bladder urothelial cells (pbUCs), stained for type I collagen (green) and nuclei using DAPI (blue); **B)** pbUCs show positive staining for cytokeratin 7 (CK7, red); **C)** SEM micrograph of pbUCs showing a typical cobblestone morphology indicative for urothelial cells; **D)** overview staining of scaffolds seeded with porcine bladder smooth muscle cells (pbSMCs), stained for type I collagen (green) and nuclei using DAPI (blue); **E)** pbSMCs show positive staining for α-smooth muscle actin (αSMA, red); **F)** SEM micrograph of pbSMCs showing a stretched and aligned morphology. 5x10⁶ cells were seeded in A, B and D. 2.5x10⁶ cells were seeded in C, E and F. Staining of porcine bladder and subsequently isolated cells with CK7 (G and H) and αSMA (J and K). As a control, scaffolds seeded with either 5x10⁶ pbUCs or pbSMCs were both negative for I) αSMA; or L) CK7, respectively.
indicated by the positive staining for cytokeratin 7 (CK7) and αSMA for UCs and SMCs, respectively (Fig 4). Reference stainings, including staining of bladder tissue, and bladder derived UC’s and SMC’s are given in Fig. 4G-L. Native porcine bladder epithelium was positive for CK7 before and after isolation (Fig. 4G and 4H). The smooth muscle layer in the bladder was positive for αSMA (Fig. 4J and 4K). The scaffolds cultured with pbUCs were positive for cytokeratin 7 (Fig. 4B) but

Figure 5  Human bladder urothelial (hbUCs) and smooth muscle cells (hbSMCs) cultured for 7 days on bladder scaffolds. **A** hbUCs were seeded at the luminal side of the scaffolds. After 7 days the cells mainly resided at the luminal side where they formed a confluent lining covering the surface of the scaffold (toluidine blue staining); **B** hbSMCs seeded at the porous outside of the scaffold and penetrated into the scaffold (toluidine blue staining); **C** hbUCs on the bladder scaffold showed positive staining for cytokeratin 7 (CK7); **D** hbSMCs on the bladder scaffold showed positive staining for α-smooth muscle actin (αSMA). Scale bars represent 100 µm; **E** CK7 expression by hbUCs, increased compared to the hbUCs pellet control. αSMA expression by hbSMCs, decreased compared to the hbSMCs pellet control. *: p < 0.05, ***: p < 0.0001.
**Figure 6** Transmission electron microscopy (TEM) images of primary human bladder urothelial cells (hbUCs) and smooth muscle (hbSMCs) cultured for 7 days on bladder scaffolds. **A**) Cell-cell contacts and presence of an urothelial lining; **B**) Parallel orientation of cells with respect to the scaffold collagen; **C**) Protrusions of the cells reaching into the collagen scaffold; **D**) Adherence of a cell to the collagen scaffold; **E**) Cytokeratin filaments in cells (arrows) as an indication of the urothelial phenotype; **F**) Parallel arranged oblong hbSMCs on the porous outside of the scaffold; **G**) Alignment and adherence of cells to the scaffold, where the cells followed the irregularities of the scaffold; **H**) Collagen scaffold (*) and new collagen matrix (#) deposited by cells; **I**) Presence of actin filaments (arrows) as an indication of the muscle phenotype. The scale bars represent 30 µm (A, F), 10 µm (B, C, G), 5 µm (D) and 2.5 µm (E, H, I). *: collagen scaffold.
negative for αSMA (Fig. 4I). Vice versa, scaffolds cultured with pbSMCs were positive for αSMA (Fig. 4E) and negative for CK7 (Fig. 4L).

**mRNA analysis**

Gene expression of CK7 and αSMA was analyzed in hbUCs and hbSMCs cultured on bladder scaffolds and compared to expression in cell pellets (obtained from confluent cell cultures), respectively (Fig. 5E). CK7 expression was significantly upregulated for hbUCs cultured on bladder scaffolds compared to hbUCs pellets. The expression of αSMA by hbSMCs was lower after 7 days of culture compared to SMC pellets, which may be attributed to the considerable number of cells invading the scaffold (Fig. 5B).

**Transmission electron microscopy**

Ultrastructural morphology of cultured hbUCs (Fig. 6A-E) and hbSMCs (Fig. 6F-I) was evaluated using TEM. The hbUCs resided closely together at the scaffold surface, making cell-cell contact and forming an urothelial lining (Fig. 6A). Cells aligned parallel to the collagen fibrils (Fig. 6B), and filopodia from the cells extended into the collagen scaffold (Fig. 6C). Firm adherence of cells to the scaffolds was indicated by the close contact areas observed (Fig. 6D). The presence of cytokeratin filaments (Fig. 6E, arrows) indicated the urothelial phenotype of the hbUCs. The hbSMCs seeded on the porous outside of the scaffold formed multilayered parallel arrangements and appeared morphologically oblong (Fig. 6F). The cells made contact with the collagen scaffold, oriented along the scaffold template and followed the orientation of the scaffold lamellae (Fig. 6G). The cells deposited new collagen matrix as indicated by the presence of collagen fibrils (Fig. 6H, the collagen scaffold and newly formed collagen are represented with * and #, respectively). Additionally, actin filaments (Fig. 6I, arrows) were observed in the cells, indicating the presence the smooth muscle phenotype.

**Discussion**

RM-based methodologies to improve (re)construction of urinary reservoirs have a long and relatively unfruitful history. The complexity of the envisioned procedures for engineering complete bladders has hampered implementation in general [11, 16]. Taking this into account we have designed a novel, simple, reproducible and adjustable process capable of producing a resorbable seamless hollow scaffolds that mimic the size and shape of a human bladder and include appendices for anastomosis of the ureters and urethra. The casting and freezing method is flexible with respect to scaffold size, volume, wall thickness and pore size, and can
be adjusted by changing mold dimensions and freezing conditions. Pore sizes can be adapted by the freezing protocol that is applied. The size of growing ice crystals decreases with increasing growth velocity [24]. Fast freezing of collagen suspensions results in small ice crystals and therefore small pores. With slow freezing, ice crystals have time to grow, which leads to larger pores in collagen scaffolds [25, 26]. Cell infiltration depth increases by increasing the pore size [27]. In our study, the largest pore size obtained was 70 ± 17 µm using freezing at -20 °C. Previously, we have demonstrated that 0.7% (w/v) collagen scaffolds with comparable pore sizes to the collagen bladder scaffold allowed infiltration of cells in vivo [28]. SEM analysis revealed a closed lumen suited for urothelial lining and a porous exterior, which may, together with the unidirectional structure of the inside of the scaffold’s wall, favor in vivo infiltration of cells and nutrients.

Recent advances in biomaterials for bladder tissue engineering have shown promising results [29-31]. The versatility of the hollow spherical construct described here allows the incorporation of other strategies currently being developed. For instance, other materials may be incorporated into the casing technique to change the material properties (e.g., mechanical strength or degradation rate) [15, 32]. Additional strength can be obtained by increasing the collagen content [26], chemical crosslinking of scaffolds using formaldehyde vapor fixation and/or crosslinking using e.g. carbodiimides [33], and by sterilization using γ-irradiation (with 15 kGy: 40 kPa) and ethylene oxide (42 kPa) [34]. Another methodology to reinforce the collagen bladder scaffold is by the incorporation of polymers. This may be performed by the incorporation of polymer meshes prior to freezing [35], or by using suspensions of collagen and water-soluble polymers [26]. Scaffolds may be seeded with autologous cells from non-bladder sources (e.g., adipose and bone marrow tissue) although this is generally laborious and costly [36-38], but techniques requiring minimal manipulation related to cell harvesting and seeding, that are simple but yet effective, are gaining interest [39].

Although speculative, an acellular approach for bladder regeneration may be probed. By combining the acellular hollow spherical scaffold with existing straightforward techniques such as omental pre-implantation, the scaffold can become vascularized. Omental pre-implantation appears to be an effective way for vascularization, with prevention of fibrosis [14]. Using tubular collagen constructs, omental wrapping proved to be instrumental in providing good vascularization and integration of the scaffold [15]. Additionally, evidence suggests that the construct may be re-epithelialized from urothelial tissue in the ureters or from remaining bladder tissue when an acellular approach is pursued [12, 13]. In addition, the increasing understanding of the bioactive signaling within the bladder ECM may be combined with this approach (e.g. addition of effector...
molecules such as growth factors), and could increase the success-rate of an acellular approach [40, 41]. This approach, emanating from an acellular bladder construct, can now be tested.

Conclusions
In this paper, a novel casting methodology was developed that resulted in a standardized collagen-based bladder scaffold with appendices, which is both easy to produce and customizable. In vitro analysis indicate cytocompatibility for human and porcine urothelial and smooth muscle cells.

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References


Chapter 9

Summary and future perspectives
Samenvatting en toekomstvisie
Summary

Articular cartilage is hyaline tissue which covers the ends of the long bones and provides a low-friction and load-bearing surface for efficient and flexible motion of joints. The structure and function of cartilage is affected by traumatic injuries and osteoarthritis. Since the tissue possesses only minor natural healing response due to its avascularity, it remains a challenge to restore cartilage. Currently, patients may be treated surgically with knee arthroplasty to replace the knee joint. Regenerative approaches to regenerate articular cartilage include microfracture surgery, mosaicplasty and autologous chondrocyte implantation. Microfracture surgery, also known as bone marrow stimulation, may be the treatment of choice since patients can be treated with one-stage minimally invasive surgery and patients will most likely experience less pain, have a shorter recovery period and require shorter hospitalization time. The procedure avoids problems like donor-site morbidity, cell culture costs, off-the-shelf availability and reduces regulatory issues. Although promising short-term clinical results have been described, the newly formed tissue mainly consists of fibrocartilage, which does not resemble the mechanical and biological properties of healthy cartilage. The difficulty for the regeneration of hyaline cartilage by bone marrow stimulation may be explained by the lack of direct control over the chondrogenic process. A template to guide and stimulate the regeneration of more durable cartilage tissue may be a solution to this problem.

Unidirectional collagen scaffolds can be implanted after bone marrow stimulation to guide the formation of cartilage tissue. The anisotropic pore architecture of the scaffolds facilitates the infiltration of autologous bone marrow-derived MSCs in the scaffolds and mimics the micro-environment of the extracellular matrix by acting as a template resembling native articular cartilage. A limitation of porous (unidirectional) collagen scaffolds is the rather weak mechanical strength. Cartilage regeneration guided by the unidirectional scaffold as a template and activated by incorporated biological stimuli has not been investigated. The aim of this thesis was to construct unidirectional collagen scaffolds with improved mechanical strength, incorporated 3D topographical features and biologics, to facilitate long-term implant survival and guided cartilage regeneration. An overview of all novel improvements introduced is described in chapter 1.

In chapter 2 and 3, a systematic review and meta-analysis were performed. The aim was to provide a structured, thorough and transparent overview of literature related to the current evidence for the efficacy of cartilage regeneration by implantation of scaffolds after applying bone marrow stimulation in animal models (chapter 2: cell-free scaffolds versus spontaneous healing, chapter 3: cell-seeded scaffolds versus cell-free scaffolds). In chapter 2, literature data
indicated that cartilage regeneration was improved by 16% by implantation of acellular scaffolds after bone marrow stimulation compared to bone marrow stimulation alone, which was further improved by 8% by the addition of biologics. No differences in cartilage regeneration between various material subgroups, biologics and animal models were detected, which may be explained by the heterogeneity between studies. In chapter 3, literature data indicated that implanting cell-laden scaffolds improved cartilage regeneration by 18.6% compared to acellular scaffolds. No differences were found in cartilage regeneration between the use of stem cells or somatic cells, but there was a difference between cell types. Culture conditions of cells did not affect cartilage regeneration.

In chapter 4, the methodology and adjustability of the directional freezing technique to construct porous collagen scaffolds with aligned unidirectional pores were investigated. Unidirectional collagen scaffolds were developed by directional freezing using a custom-made wedge system. The mechanism of unidirectional ice crystal growth was elucidated, and ice crystal growth was manipulated to develop a wide range of unidirectional collagen scaffolds with distinctive pore structures. In chapter 5, the effect of pore orientation on cell seeding efficacy, cell distribution and tissue formation was researched. Anisotropic scaffolds facilitated superior cell infiltration compared to isotropic scaffold, and may be the scaffolds of choice to develop cellular constructs in vitro. Additionally, the anisotropic pore architecture enabled aligned tissue formation, important for the regeneration of tissues with an anisotropic extracellular matrix. In chapter 6 and 7 various improvements for unidirectional collagen scaffolds were investigated. In chapter 6, specific 3D topographical features were introduced in collagen scaffolds taking into account the use of different diluted acids and freezing and lyophilization. In vitro analysis indicated cytocompatibility of scaffolds. Additionally, myosin staining was stronger and pMHC gene expression levels were upregulated for C2C12 cells cultured on scaffolds with frayed-like micro-morphologies compared to smooth micro-morphologies. Incorporation of 3D micro-morphologies results in differences in cellular differentiation. In chapter 7, bioactive scaffolds were prepared to stimulate chondrogenesis in the prospect of potential in vivo applications. Immobilization of growth factors is required because no biologics can be added after implantation of scaffolds, and to prolong in vivo activity, limit diffusion and reduce the amount of growth factor needed for safe clinical application. The effect of bone morphogenetic protein 2 (BMP2) loaded (heparin-conjugated) unidirectional collagen scaffolds on chondrogenesis of C3H10T1/2 cells, a MSC cell line, was investigated in vitro, and compared to the effect of soluble BMP2. Pre-incubating scaffolds only once with 10 µg BMP2 before cell seeding resembled the results of supplementing the culture medium every
3 days for 2 weeks with 100 ng/ml soluble BMP2 (total 1 µg BMP2). The scaffolds prepared supported *in vitro* differentiation of the pluripotent stem cell line C3H10T1/2 towards the chondrogenic lineage under the influence of BMP2. Unidirectional collagen and heparin-conjugated collagen scaffolds pre-incubated with 10 µg BMP2 may be suitable to induce prolonged cartilage matrix synthesis *in vivo*.

In *chapter 8*, we researched the translation of gained knowledge regarding scaffold construction to prepare a scaffold with unidirectional pores for bladder regeneration purposes. A novel methodology was developed that resulted in a standardized collagen-based bladder scaffold with appendices, which is both easy to produce and customizable. *In vitro* analysis indicated cytocompatibility for human and porcine urothelial and smooth muscle cells.

In conclusion, reinforced instructive bioactive unidirectional collagen scaffolds for cartilage regeneration were constructed and evaluated *in vitro*, which may be pre-clinically tested in future studies.
**Future perspectives**

Novel regenerative medicine and tissue engineering approaches have been developed and widely explored as alternatives and/or additions to clinical strategies that aim to restore damaged cartilage tissue. In this thesis, the construction and evaluation of biomimetic collagen scaffolds for cartilage regeneration was investigated. Various modifications for unidirectional collagen scaffolds previously implanted in osteochondral defects by de Mulder et al. [1] were applied related to: 1) improved scaffold fabrication, 2) increased mechanical strength to provide long-lasting implants, 3) incorporation of 3D topographical features, and 4) enhanced bioactivity by loading of growth factors. Overall, these novel improvements may yield a medical device inducing superior cartilage regeneration compared to previously implanted 'basic' unidirectional collagen scaffolds.

Currently, the development of advanced biomimetic and bioinstructive biomaterials is widely explored. The incorporation of multifunctional and stimuli-responsive properties may result in sophisticated implants for various regenerative medicine and tissue engineering applications. Various adaptations for unidirectional collagen scaffolds, such as morphology, strength and biologics, can be incorporated to mimic nature. Native articular cartilage has a complex zonal organization with unique biomechanical properties for low-friction articulation and optimally resisting joint loading. These zones, the superficial, middle, and deep zone, differ in extracellular matrix (ECM) structure and composition, including variations in collagen fiber diameter and orientation, glycosaminoglycan (GAG) content, and the density and morphology of chondrocytes [2]. The collagen fiber orientation and the number of crosslinks are depth-dependent, which accounts for differences in tensile strength and stiffness among the zones [3]. The collagen fiber diameter varies between zones from approximately 20 nm in the superficial zone to 70-120 nm in the deep zone [4]. In the superficial zone collagen fibers are aligned parallel to the articular surface to optimally resist shear stresses during joint loading. In contrast, the orientation of collagen fibers in the deep zone is perpendicular to the articular surface, providing compressive strength to the tissue. In between, the random orientation of the collagen fibers in the middle zone provides a transition from the superficial to the deep zone [2-4]. Little amounts of proteoglycans and GAGs are present in the superficial zone [5], whereas most proteoglycans, mainly aggrecan, are found in the deep zone [4]. The amount of proteoglycans and GAGs, and as a result the compressive modulus, increases with tissue depth [6]. The highest cell densities are found in the superficial zone and the cell density decreases with tissue depth. To attract cells from the subchondral bone and to facilitate migration throughout the scaffold, scaffolds can be incorporated with stromal cell-derived factor-1 [7]. Specific
loading of stromal cell-derived factor-1 in scaffolds may result in a cell density gradient similar to native cartilage. Currently, by implantation of unidirectional collagen scaffolds with a perpendicular pore orientation relative to the articulation surface, the infiltration of cells is facilitated. Joint loading should ultimately remodel the tissue to resemble the complex organization of native cartilage [4, 6]. The incorporation of a zonal organization in scaffolds may further improve cartilage regeneration. In this study, collagen scaffolds with anisotropic pores were constructed using a custom-made wedge system, resulting in a vertical temperature gradient and controlled solidification. By adapting the temperature gradients applied also other pore orientations may be incorporated. Finite element modeling may offer opportunities to model the effects of variations applied to the wedge system on temperature gradients, such as steepness of the wedge and use of materials with different thermal conductivities, and the use of various insulators.

The addition of cartilage-specific components may create a microenvironment resembling native cartilage. The GAG chondroitin sulfate (CS) is an important ECM component of native articular cartilage [8]. The incorporation of CS in collagen scaffolds resembles the native environment and can be used to bind growth factors to the scaffolds [9-11]. Previously, it has been demonstrated that linkage of CS to type I collagen scaffolds stimulated the bioactivity of chondrocytes and increased the total amount of proteoglycans retained in the matrix [12]. Also the incorporation of other components including hyaluronic acid may be promising since hyaluronic acid also plays physical and biological roles in cartilage tissue [13].

Cartilage regeneration may be further enhanced by the addition of growth factors to scaffolds to stimulate chondrogenesis. These growth factors can be loaded in the scaffolds by their non-covalent interaction with GAGs [20]. Gradients of growth factors can be introduced by GAG gradients in collagen scaffolds [14]. The porous unidirectional scaffold architecture may facilitate gradient formation of (multiple) growth factors over long ranges due to the open porous intrinsic pore architecture (Fig. 1). By placing the scaffolds on Whatman™ chromatography paper to extract fluid from the scaffolds and simultaneously dripping a growth factor solution on top of the scaffolds, the majority of the growth factors may remain at the seeding side of the scaffolds while lower concentrations may be found deeper into the scaffolds.

A limitation of growth factors such as bone morphogenetic proteins (BMPs) is their short half-live and location, resulting in large doses needed to induce the desired response. These non-physiological doses draw concerns related to costs and safety [15]. Therefore, an important consideration for using growth factors is the need of a suitable carrier that allows for controlled release and preservation of their bioactivity. In this thesis, heparin-conjugation in unidirectional collagen
scaffolds was explored to bind and release BMP-2. The use of small molecules and synthetic peptides mimicking the function of growth factors may be promising since small molecules are easier to deliver, more stable and less immunogenic than growth factors. Isoliquiritigenin and 4′-hydroxychalcone are such small molecules mimicking BMP-2 signaling [16]. Moreover, a synthetic peptide consisting of the BMP-2 amino acids 73-92 has been shown to promote chondrogenic differentiation of MSCs [17].

Improving the mechanical strength of unidirectional collagen scaffolds is required to provide long-lasting implants [18]. Scaffolds can be strengthened by increasing the collagen concentration prior to scaffold construction [19], or condensing collagen scaffolds by shrinking scaffolds as a whole. Previously, implanted unidirectional scaffolds were prepared from a 0.7% (w/v) collagen suspension [1]. Unidirectional collagen scaffolds reinforced by using a 2% (w/v) collagen suspension followed by shrinking maintained the unidirectional architecture (Fig. 2). These reinforced unidirectional scaffolds consisted of collagen only, without the need for incorporation of additional (synthetic) materials, which is beneficial for biocompatibility, biodegradability, and
bioactivity of the implants. Additionally, this strategy also prevents a potential mismatch in mechanical properties in case different materials are combined [20].

Besides regeneration of articular cartilage, the underlying subchondral bone may also need to be repaired as it simultaneously undergoes degenerative changes as cartilage tissue. Therefore, scaffolds aiming to restore both cartilage and bone may be the implants of choice to regenerate osteochondral tissues. One of the major components of bone is calcium phosphate (CaP). In native bone, aligned CaP platelets are present in interstices within collagen fibrils and play an important role in the functional properties of bone. CaP can be deposited precisely by applying a method developed by Nijhuis et al. [21]. To deposit CaP in collagen scaffolds, scaffolds can be soaked in simulated body fluid and subsequently the addition of urease from *Canavalia ensiformis* results in the conversion of urea into ammonia and carbon dioxide. As the initial pH increases to 8, the solubility of CaP decreases and subsequently results in deposition of CaP. By placing scaffolds partially in simulated body fluid, the spatial incorporation of CaP in collagen scaffolds may be used to induce a spatial differentiation pattern of MSCs towards bone- and cartilaginous-like tissue [22].

In this thesis, various improvements for unidirectional collagen scaffolds were proposed to enhance cartilage regeneration. Besides *in vitro* research described in this thesis, *in vivo* investigations are required to identify whether these aspects related to mechanical strength, incorporation of intrinsic surface structures and loading of biologics indeed enhance cartilage regeneration.

**Figure 2** Methodologies of reinforcing unidirectional collagen scaffolds based on increasing the collagen concentration and condensing collagen fibrils. The unidirectional pore architecture is still present after all reinforcement procedures. Scale bars represent 100 µm.
Implantation of unidirectional collagen scaffolds with improved mechanical strength, incorporated instructive surface topographies and biologics may result in long-term implant survival and guided cartilage regeneration, and superior cartilage regeneration compared to control unidirectional collagen scaffolds or spontaneous natural healing of defects. To evaluate the effect of scaffolds on cartilage regeneration in animal models, an important consideration is the translational value of animal studies, which depends on the comparability to the clinical situation. Healthy animal models are often used, while osteoarthritic animal models are clinically more relevant. Also, the choice for smaller or larger animal models is important. Smaller animal models are mostly used due to feasibility, while large animal models may more closely resemble humans but are associated with higher costs [23]. Moreover, the experimental design may affect the degree of cartilage regeneration, whereas aspects such as implant location, follow-up period and rehabilitation protocol need to be considered. Overall, results from animal studies could provide insights in strategies for future (pre) clinical research related to biomaterial properties, incorporation of biologics, choice of a suitable animal model, and their effect on cartilage regeneration.

With the improvements described in this thesis and future directions, unidirectional collagen may be used as a novel implant to regenerate cartilage in the future.
Samenvatting

Articulair kraakbeen is hyalien weefsel dat de uiteinden van de lange botten bedekt en zorgt voor lage wrijving en een gewichtsdragend oppervlak, resulterend in efficiënte en flexibele beweging van gewrichten. De structuur en functie van kraakbeen kan worden beschadigd door traumatische letsels en osteoartrrose. Doordat het weefsel van nature weinig zelfregenererend vermogen heeft, door de afwezigheid van bloedvaten, blijft het een uitdaging om beschadigd kraakbeen te herstellen. Momenteel kunnen patiënten chirurgisch worden behandeld door middel van het vervangen van het kniegewricht door een prothese. Daarnaast zijn er ook regeneratieve behandelingen die zich richten op het herstellen van kraakbeen, zoals de microfractuurbehandeling, mozaïekplastiek en autologe chondrocyten implantatie. Het toepassen van de microfractuurbehandeling, ook bekend als beenmergstimulatie, kan de voorkeur hebben omdat patiënten kunnen worden behandeld middels een minimaal invasieve ingreep, met als gevolg dat patiënten waarschijnlijk minder pijn zullen ervaren en een kortere herstelperiode (in het ziekenhuis) nodig zullen hebben. Tevens omzeilt deze behandeling problemen geassocieerd met celbevattende therapieën, zoals morbiditeit op de locatie waar donorweefsel wordt geoogst, celkweekkosten, beperkte ‘off-the-shelf’ beschikbaarheid, en regelgevende kwesties. Ondanks veelbelovende klinische resultaten op korte termijn blijkt dat nieuwgevormd weefsel momenteel voornamelijk bestaat uit fibreus kraakbeen, dat niet dezelfde mechanische en biologische eigenschappen heeft als gezond kraakbeen. Het probleem om hyalien kraakbeen te regenereren middels beenmergstimulatie kan worden toegeschreven aan het gebrek van controle over de chondrogenese. Het gebruik van een template om de regeneratie van duurzamer kraakbeenweefsel te sturen en te stimuleren is een mogelijke oplossing voor dit probleem.

Unidirectionele collageenscaffolds kunnen worden geïmplanteerd na beenmergstimulatie om de groei van kraakbeenweefsel te sturen. De anisotrope architectuur van de poriën in de scaffolds faciliteert de infiltratie van autologe mesenchymale stamcellen vanuit het beenberg in de scaffolds en bootst het micromilieu na van de extracellulaire matrix van natuurlijk kraakbeen. Een beperking van poreuze (unidirectionele) collageenscaffolds is de beperkte mechanische sterkte. Kraakbeenregeneratie middels het gebruik van unidirectionele collageenscaffolds als sjabloon en daarnaast met toegevoegde biologische stimuli is nog niet onderzocht.

Het doel van dit proefschrift was om unidirectionele collageenscaffolds te maken met verbeterde mechanische sterkte en met toegevoegde 3D oppervlaktestructuren en biologische stimuli om langdurig de vorming van kraakbeenweefsel te sturen. Een overzicht van alle nieuwe geïntroduceerde verbeteringen is beschreven in hoofdstuk 1.
In hoofdstuk 2 en 3 staan een systematische review met meta-analyse beschreven. Het doel was om een gestructureerd, volledig en transparant overzicht te verkrijgen van de literatuur met betrekking tot de effectiviteit van kraakbeenregeneratie middels het implanteren van scaffolds na beenmergstimulatie in diermodellen (hoofdstuk 2: celvrije scaffolds in vergelijking met spontane natuurlijke genezing, hoofdstuk 3: scaffolds met cellen in vergelijking met celvrije scaffolds). In hoofdstuk 2 bleek na analyse van de literatuurdata dat kraakbeenregeneratie met 16% verbeterde door het implanteren van celvrije scaffolds na beenmergstimulatie ten opzichte van het alleen uitvoeren van beenmergstimulatie, en dat het toevoegen van biologische stimuli 8% extra verbeterde kraakbeenregeneratie opleverde. Er werden geen verschillen gevonden in kraakbeenregeneratie door gebruik te maken van scaffolds van verschillende materialen, door het toevoegen van verschillende biologische stimuli of door het gebruik maken van verschillende diermodellen, wat kan worden verklaard door de heterogeniteit tussen de studies. In hoofdstuk 3 bleek na analyse van de literatuurdata dat het implanteren van scaffolds met cellen resulteerde in 19% verbeterde kraakbeenregeneratie vergeleken met celvrije scaffolds. Er werden geen verschillen gevonden in kraakbeenregeneratie door het gebruik stamcellen of somatische cellen, maar wel tussen verschillende celtypen. Kweekcondities hadden geen effect op kraakbeenregeneratie.

Hoofdstuk 4 beschrijft de methodologie en veelzijdigheid van een directionele invriestechniek om poreuze collageenscaffolds met unidirectionele poriën te ontwikkelen. Unidirectionele collageenscaffolds werden ontwikkeld middels directioneel invriezen met een speciaal ontworpen systeem bestaande uit wigjes. Het mechanisme achter het systeem, resulterend in de unidirectionele groei van ijskristallen, was doorgroei. Daarnaast was de groei van ijskristallen gemanipuleerd om unidirectionele collageenscaffolds te ontwikkelen met uiteenlopende porie-structuren.

In hoofdstuk 5 is het effect van de oriëntatie van de poriën onderzocht in relatie tot de efficiëntie van het zaaien van cellen, de distributie van cellen in de scaffolds en de vorming van weefsel. Anisotrope scaffolds faciliteerden infiltratie van cellen beter in vergelijking met scaffolds met een willekeurige oriëntatie van de poriën, en daarom lijkt het gebruik van anisotrope scaffolds veelbelovend voor de ontwikkeling van cellulaire scaffolds in vitro. Daarnaast resulteerde de anisotrope architectuur van de poriën in anisotrope weefselvorming, wat belangrijk is voor de regeneratie van weefsels met een anisotrope extracellulaire matrix.

In hoofdstuk 6 en 7 werden verschillende verbeteringen voor unidirectionele collageenscaffolds onderzocht. Hoofdstuk 6 beschrijft het aanbrengen van specifieke 3D structuren in collageenscaffolds middels het gebruik van verschillende verdunne zuren tijdens het productie proces. Uit in vitro analyses bleek dat de scaffolds
cytocompatibel zijn. Daarnaast kleurde myosine sterker aan en was de pMHC genexpresse verhoogd voor C2C12 cellen gekweekt op scaffolds met rafelige structuren vergeleken met gladde structuren. Het toevoegen van 3D structuren kan differentiatie van cellen beïnvloeden. In hoofdstuk 7 werden bioactieve scaffolds gemaakt die chondrogenese stimuleren. Het immobiliseren van groeifactoren in scaffolds is noodzakelijk omdat na implantatie van de scaffolds er geen biologische stimuli meer kunnen worden toegevoegd, om de activiteit in vivo te verlengen, de diffusie te beperken en de hoeveelheid benodigde groeifactor te verminderen voor het veilig kunnen toepassen in de kliniek. In vitro werd het effect onderzocht van (heparine-geconjugeerde) unidirectionele collageenscaffolds waaraan bot morfogenetische eiwit 2 (BMP2) was toegevoegd op de chondrogenese van C3H10T1/2 cellen, een mesenchymale stamcellijn, en vergeleken met het effect van vrij BMP2. Het eenmalig preïncuberen van scaffolds met 10 µg BMP2 voor het zaaien van cellen resulteerde in vergelijkbare resultaten als wanneer 100 ng/ml oplosbaar BMP2 elke 3 dagen gedurende 2 weken werd toegevoegd aan het kweekmedium (totaal 1 µg BMP2). De scaffolds bevorderden de differentiatie van de pluripotente cellijn C3H10T1/2 naar de chondrogene lijn in vitro onder de invloed van BMP2. Unidirectionele collageen en heparine-geconjugeerde collageenscaffolds gepreïncubeert met 10 µg BMP2 kunnen geschikt zijn om in vivo langdurige vorming van kraakbeenweefsel te induceren.

In hoofdstuk 8 hebben we onderzocht of we de verworven kennis met betrekking tot het maken van scaffolds konden inzetten om een scaffold te maken met unidirectionele poriën voor de regeneratie van de blaas. Een nieuwe methode was ontwikkeld die resulteerde in een gestandaardiseerde blaascaffold, inclusief anastomoses, gemaakt van collageen, en die zowel eenvoudig is om te produceren als aanpasbaar is. Uit in vitro analyse is gebleken dat de blaascaffold cytocompatibel is voor urotheel- en gladde spiercellen van zowel de mens als het varken.

Concluderend zijn versterkte instructieve bioactieve unidirectionele collageenscaffolds voor kraakbeenregeneratie gemaakt en in vitro bestudeerd. Deze kunnen in toekomstige studies verder preklinisch worden getest.
Toekomstvisie

Nieuwe methoden voor regeneratieve geneeskunde en weefseltechnologie zijn ontwikkeld en uitgebreid onderzocht als alternatieven en/of aanvullingen op klinische strategieën die zijn gericht op het herstellen van beschadigd kraakbeen-weefsel. In dit proefschrift zijn biomimicerende collageenscaffolds voor kraakbeenregeneratie vervaardigd en geëvalueerd. Verschillende modificaties zijn verricht aan unidirectionele collageenscaffolds die eerder zijn geimplanteerd door de Mulder et al. [1], waaronder: 1) verbeterd productieproces van scaffolds, 2) versterkte mechanische eigenschappen voor het langdurig functioneren van de implantaten, 3) integratie van 3D oppervlaktestructuren, en 4) verhoogde bioactiviteit door het toevoegen van groeifactoren. Deze nieuwe verbeteringen kunnen resulteren in een medisch hulpmiddel die het herstel van kraakbeen beter stimuleert in vergelijking met de eenvoudigere unidirectionele collageenscaffolds die eerder zijn geïmplanteerd.

Momenteel wordt de ontwikkeling van geavanceerde en biomimetische biostructieve biomaterialen uitgebreid onderzocht. Het integreren van multifunctionele en stimuliresponsieve eigenschappen kan resulteren in geavanceerde implantaten voor diverse toepassingen binnen de regeneratieve geneeskunde en weefseltechnologie. Diverse aanpassingen kunnen worden gedaan aan unidirectionele collageenscaffolds, bijvoorbeeld gerelateerd aan morfologie, sterkte en biologische activiteit, om moeder natuur na te bootsen. Articulair kraakbeen heeft een complexe zonale organisatie met unieke biomechanische eigenschappen, wat resulteert in weinig wrijving en het optimaal weerstaan van belasting door het gewricht. De extracellulaire matrix (ECM) in deze zones, de oppervlakkige, middelste en diepe zone, verschillen in structuur en samenstelling waarbij er onder andere variaties zijn in de diameter en oriëntatie van de collageenvezels, de hoeveelheid glycosaminoglycanen (GAGs), en de dichtheid en morfologie van chondrocyten [2]. De oriëntatie van de collageenvezels en de hoeveelheid crosslinks is diepteafhankelijk, wat zorgt voor verschillen in treksterkte en stijfheid tussen de zones [3]. De diameter van de collageenvezels varieert tussen de zones van ongeveer 20 nm in de oppervlakkige zone tot 70-120 nm in de diepe zone [4]. In de oppervlakkige zone hebben de collageenvezels een parallelle oriëntatie ten opzichte van het gewrichtoppervlak om optimaal schuifkrachten op te vangen tijdens belasting van het gewricht. Daarentegen hebben collageenvezels in de diepe zone een loodrechte oriëntatie ten opzichte van het gewrichtsoppervlak waardoor compressiekrachten kunnen worden opvangen. De collageenvezels in de middelste zone hebben een willekeurige oriëntatie en vormen een overgang van de oppervlakkige naar de diepe zone [2-4]. In de oppervlakkige zone zijn lage hoeveelheden proteoglycanen en GAGs aanwezig [5], terwijl in de diepe
zone veel proteoglycanen, waaronder hoofdzakelijk aggrecan, aanwezig zijn [4]. De hoeveelheid proteoglycanen en GAGs, en daarbij de compressiemodulus, neemt toe met de diepte van het weefsel [6]. De hoogste dichtheid aan cellen is aanwezig in de oppervlakkige zone en deze neemt ook af met de diepte van het weefsel. Om cellen vanuit het subchondrale bot aan te trekken en om migratie door de scaffolds te faciliteren kunnen de scaffolds worden opgeladen met stromal cell-derived factor-1 [7]. Het specifiek toevoegen van stromal cell-derived factor-1 aan de scaffolds kan mogelijk resulteren in een gradie¨nt van cel dichtheid, vergelijkbaar met natuurlijk kraakbeen. Momenteel wordt de infiltratie van cellen gefaciliteerd door het implanteren van unidirectionele collageenscaffolds met de oriëntatie van de poriën loodrecht op het gewrichtoppervlak. Door belasting van het gewricht moet het weefsel uiteindelijk remodelleren zodat de complexe organisatie van natuurlijk kraakbeen wordt nagebootst [4, 6]. Door het integreren van een zonale organisatie in scaffolds kan kraakbeenregeneratie mogelijk worden verbeterd. Zoals beschreven in dit proefschrift zijn de anisotrope collageenscaffolds gemaakt middels systeem bestaande uit een koudebron en een wig, wat zorgt voor een verticale temperatuurgradiënt en gecontroleerd invriezen. Door het aanpassen van deze temperatuurgradiënt kunnen mogelijk ook verschillende oriëntaties van de poriën worden geïntroduceerd eindige elementen modellering kan hierbij functioneel zijn om de effecten van aanpassingen verricht aan het wigsysteem op temperatuurgradiënten te onderzoeken, zoals de steilheid van de wig, het gebruik van materialen met verschillende thermische geleidbaarheid en het gebruik van verschillende isolatoren.

Het toevoegen van kraakbeenspecifieke componenten kan de micro-omgeving van natuurlijk kraakbeen nabootsen. De GAG chondroïtinesulfaat (CS) is een belangrijke ECM component in natuurlijk gewrichtskraakbeen [8]. Het toevoegen van CS aan collageenscaffolds bootst hiermee de natuurlijke omgeving na en kan tevens worden gebruikt om groefactoren te binden aan de scaffolds [9-11]. Het is eerder aangetoond dat het koppelen van CS aan type I collageenscaffolds de bioactiviteit van chondrocyten stimuleert en zorgt voor een verhoogde productie van proteoglycanen door de chondrocyten in de ECM [12]. Ook het toevoegen van andere componenten zoals hyaluronzuur kan veelbelovend zijn, omdat hyaluronzuur ook fysische en biologische functies heeft in kraakbeenweefsel [13].

Verder kan kraakbeenregeneratie mogelijk worden verbeterd door het toevoegen van groefactoren aan scaffolds om chondrogenese te stimuleren. Deze groefactoren kunnen worden geïntegreerd in de scaffolds middels hun niet-covalente interactie met GAGs [20]. Door middel van het aanbrengen van GAG gradiënten kunnen vervolgens gradiënten van groefactoren worden aangebracht [14]. De open poreuze intrinsieke porie architectuur van unidirectionele collageenscaffolds kan mogelijk het aanbrengen van gradiënten van
(meerdere) groeifactoren over lange afstanden faciliteren (Fig. 1). Het plaatsen van de scaffolds op Whatman chromatografiepapier maakt het mogelijk om vloeistof uit de scaffolds te extraheren en tegelijkertijd een groeifactorenoplossing op de scaffolds te druppelen, waarbij het merendeel van de groeifactoren zich zal bevinden aan de kant waar de groeifactoren op de scaffolds is gedruppeld, terwijl dieper in de scaffolds lagere concentraties aanwezig zijn.

Een beperking van groeifactoren -zoals bot morfogenetische eiwitten (BMPs)- is hun korte halfwaardetijd en locatie, wat resulteert in hoge benodigde doses om de gewenste effecten te induceren. Het gebruik van niet-fysiologische doses leidt tot discussies over kosten en veiligheid [15]. Daarom is een belangrijke overweging voor het gebruik van groeifactoren of er ook niet een geschikt dragermateriaal moet worden gebruikt dat zorgt voor gecontroleerde afgifte en behoud van de biologische activiteit. In dit proefschrift is de conjugatie van heparine aan unidirectionele collageenscaffolds onderzocht om vervolgens BMP2 te binden en af te geven. Daarnaast kunnen kleine moleculen en synthetische peptiden die de functie van groeifactoren nabootsen veelbelovend zijn, omdat deze gemakkelijker op de juiste locatie te krijgen zijn, stabiel zijn en minder immunogeen zijn dan groeifactoren. Ook zijn de kleine moleculen door hun relatief eenvoudige synthese vaak goedkoper. Isoliquitigenin en 4’-hydroxychalcone zijn voorbeelden van

![Figuur 1](image)

**Figuur 1** Illustratie van aangebrachte groeifactorgradiënten voor de regeneratie van osteochondrale defecten. Transformerende groeifactor beta (TGF-β) en bot morfogenetische eiwit 2 (BMP-2) kunnen worden aangebracht om respectievelijk kraakbeen en bot te regenereren.
moleculen die de werking BMP-2 nabootsen [16]. Daarnaast is aangetoond dat een synthetisch peptide bestaande uit de BMP-2 aminozuren 73-92 chondrogene differentiatie van MSCs bevorderd [17].

Het verbeteren van de mechanische sterkte van unidirectionele collageenscaffolds is een vereiste voor het langdurig functioneren van implantaten [18]. Scaffolds kunnen worden versterkt door het verhogen van de collageenconcentratie in de scaffolds [19], of door het laten krimpen van de scaffolds als geheel met als gevolg een hoge collageendichtheid. Eerder geïmplanteerde unidirectionele scaffolds waren gemaakt van een 0.7% (w/v) collageensuspensie [1]. Het versterken van unidirectionele collageenscaffolds door het gebruik van een 2% (w/v) collageensuspensie, gevolgd door het krimpen van de scaffolds, resulteerde in behoud van de unidirectionele architectuur (Fig. 2). Deze versterkte unidirectionele scaffolds zijn gemaakt van enkel collageen, zonder toevoeging van additionele (synthetische) materialen, wat gunstig is voor de biocompatibiliteit, biodegradeerbaarheid en bioactiviteit van de implantaten. Tevens wordt hierdoor een mogelijke mismatch tussen de mechanische eigenschappen van verschillende materialen voorkomen [20].

Naast de regeneratie van articulair kraakbeen moet wellicht ook het onderliggende subchondrale bot gerepareerd worden, omdat het tegelijkertijd met kraakbeenweefsel degeneratieve veranderingen ondergaat. Om deze osteochondrale defecten te laten regenereren lijken scaffolds die zowel het herstel van kraakbeen

Figuur 2 Methoden om unidirectionele collageenscaffolds te versterken, gebaseerd op het verhogen van de collageenconcentratie en het condenseren van collageenvezels. De unidirectionele porie architectuur blijft behouden na het uitvoeren van alle procedures. Maatstreekjes vertegenwoordigen 100 µm.

Figure 2. Methodologies of reinforcing unidirectional collagen scaffolds based on condensing collagen fibrils: 1) increasing the collagen concentration, and 2) shrinking of the scaffolds by treatment with calcium chloride. Scanning electron microscopy images indicate the preservation of the unidirectional pore architecture in reinforced unidirectional collagen scaffolds.
als dat van bot bevorderen de beste optie. Een van de belangrijkste componenten van bot is calciumfosfaat (CaP). In natuurlijk bot zijn uiteindelijke CaP plaatjes aanwezig in tussenruimten in de collageenvezels, waar ze een belangrijke rol spelen in de functionele eigenschappen van bot. CaP kan nauwkeurig worden neergelegd middels het toepassen van een methode ontwikkeld door Nijhuis et al. [21]. Om CaP in collageenscaffolds te brengen kunnen de scaffolds worden gewassen in zogenaamde gesimuleerde lichaamsvloeistof, waarna het enzym urease van Canavalia ensiformis gebruikt kan worden voor de omzetting van urea in ammonia en koolstofdioxide. Als gevolg van een stijging van de pH naar pH 8 neemt de oplosbaarheid van CaP af, wat resulteert in de depositie van CaP. Door de scaffolds gedeeltelijk in gesimuleerd lichaamsvloeistof te plaatsen kan plaatselijk CaP in collageenscaffolds worden aangebracht. In dit gedeelte van de scaffolds kunnen MSCs differentiëren in de richting van bot, terwijl in het CaP-vrije gedeelten de MSCs kunnen differentiëren richting kraakbeen [22].

In dit proefschrift zijn diverse verbeteringen voor unidirectionele collageen-scaffolds aangedragen om het herstel van kraakbeen te bevorderen. Naast het in vitro onderzoek beschreven in dit proefschrift is ook in vivo onderzoek nodig om vast te stellen of de onderzochte aspecten met betrekking tot mechanische sterkte, incorporatie van intrinsieke oppervlaktestrukuren en het toevoegen van biologische stimuli inderdaad het herstel van kraakbeen verbeteren. Implantatie van unidirectionale collageenscaffolds met verbeterde mechanische sterkte en toegevoegde oppervlaktestrukuren en biologische stimuli, resulteert mogelijk in het langdurig functioneren van implantaten en gestuurde kraakbeenregeneratie en in beter kraakbeenherstel in vergelijking met eenvoudige unidirectionele collageenscaffolds en de spontane genezing van defecten. Voor het evalueren van het effect van de scaffolds op kraakbeenregeneratie in diermodellen is de translationele waarde van dierproeven een belangrijke overweging, die afhankt van de vergelijkbaarheid met de klinische situatie. Vaak worden er gezonde diermodellen gebruikt, terwijl osteoarthrotische diermodellen klinisch relevanter zijn. Ook de keuze voor kleinere of grotere diermodellen is belangrijk. Kleinere diermodellen worden meestal gebruikt vanwege haalbaarheid, terwijl grote diermodellen dichterbij de mens staan, maar ook resulteren in hogere kosten en meer ethische bezwaren [23]. Daarnaast kan de experimentele opzet een belangrijke rol spelen bij de mate waarin kraakbeen wordt geregeneerd, waarbij aspecten zoals de locatie van het implantaat, follow-up periode en revalidatieprotocol moeten worden overwogen. Tezamen kunnen de resultaten van dierproeven inzicht geven in strategieën voor toekomstig (pre)klinisch onderzoek met betrekking tot de eigenschappen van biomaterialen, integratie van biologische factoren, de keuze van een geschikt diermodel, en hun effect op het herstel van kraakbeen.
Met de verbeteringen beschreven in dit proefschrift en toekomstvisie kunnen unidirectionele collageenscaffolds in de toekomst wellicht worden gebruikt als nieuwe implantaten voor de regeneratie van kraakbeen.
References


Curriculum vitae (English)

Michiel Pot was born September 24th, 1987 in Zuidlaren, The Netherlands. In 2005 he obtained his VWO diploma from the Zernike College in Haren. The same year he started the bachelor study Technical Medicine at the University of Twente in Enschede, followed by the Technical Medicine master Reconstructive Medicine in 2008. Part of his master education were clinical internships in Cardiothoracic Surgery at MST Twente, Enschede, Pathology at Radboud University Medical Center, Nijmegen, Orthopedics at UMC Utrecht, Utrecht, and in Biomedical Engineering at Case Western Reserve University, Cleveland, OH, USA. Michiel received a travel grant for his internship abroad. He performed his graduation internship at the department of Orthopedics of the UMC Utrecht, Utrecht, during which he also was awarded the 1st prize for his poster presentation at the SEOHS 2011. He obtained his master degree in January 2012.

In February 2012 he started his PhD research at the Radboud university medical center, Radboud Institute for Molecular Life Sciences (RIMLS), under supervision of dr. Toin van Kuppevelt and dr. ir. Willeke Daamen. The results obtained during his PhD training are described in this thesis. During his Ph.D. research, Michiel was involved in several educational responsibilities and supervised multiple students. He presented his results at various national and international conferences, including the Gordon Research Conference Cartilage Biology and Pathology in Le Diablerets, Switzerland, TERMIS World Congress 2015 in Boston, MA, USA, Netherlands Society for Biomaterials and Tissue Engineering, Dutch Society of Matrix Biology, and the Dutch Society for Technical Medicine. Michiel obtained 4 travel grants to present his research on international conferences. Moreover, he was active as a board member within the Radboudumc research theme 'Reconstructive and regenerative medicine' and RIMLS PhD Program Committee, representing interests of PhD candidates within the graduate school.

In November 2016 Michiel started his residency in clinical chemistry at Rijnstate Hospital, Arnhem.
Curriculum Vitae (Nederlands)


Sinds november 2016 is Michiel in opleiding tot klinisch chemicus in het Rijnstate te Arnhem.
List of publications

This thesis was based on the following publications

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Improved cartilage regeneration by implantation of acellular biomaterials after bone marrow stimulation: a systematic review and meta-analysis of animal studies.
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Pot MW, Mihaila SM, Oosterwijk E, van Kuppevelt TH, Daamen WF
A comparison of cell distribution in anisotropic versus isotropic collagen scaffolds.
*Submitted for publication*

Pot MW, van Kuppevelt TH, Gonzales VK, Buma P, IntHout J, de Vries RBM, Daamen WF
Augmented cartilage regeneration by implantation of cellular over acellular implants after bone marrow stimulation: a systematic review and meta-analysis of animal studies.
*Submitted for publication*

Pot MW, Mihaila SM, te Brinke D, van der Borg G, Oosterwijk E, Daamen WF, van Kuppevelt TH
Introduction of specific 3D micro-morphologies in collagen scaffolds using even/uneven dicarboxylic acids.
*In preparation*
Other publications


Versteegden LR, Pot MW, Sloff M, Hoogenkamp HR, Pang J, Smit TH, de Jong T, Leeuwenburg S, Feitz WF, Oosterwijk E, van Kuppevelt TH, Daamen WF. Shrinking collagen scaffolds to modulate mechanical properties. *In preparation*
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