General Discussion
Chapter 7

**Disc herniation**

Low back pain (LBP) is a leading cause of disability in our population, affecting most people at some point in life. Chronic LBP decreases quality of life and has significant socio-economic consequences due to absenteeism from work and increased medical consumption [37]. LBP is a multifactorial condition in which muscular, psychological and socioeconomic factors act in concert. Intervertebral disc (IVD) degeneration is also a strong etiological factor, but the exact contribution is still unclear since imaging modalities often do not correlate with symptomatology [6]. IVD degeneration is a complex disease, in which changes to certain extent develop physiologically due to aging, but can become pathologic in severe degeneration with no clear border inbetween. Due to degeneration, several structural changes occur in the IVD, most notably the dehydration of the nucleus pulposus (NP) in association with tears in the annulus fibrosus (AF) and endplates (Schmorl’s nodes) [6]. Damage to the AF may diminish its capability to cope with the local stresses and as a result the NP may herniate through the disrupted AF [6]. Initially, the herniated NP material results in direct mechanical compression of the nerve roots that are located posterior of the IVD. Furthermore, in the absence of vascularity the NP is normally an immune privileged tissue. Herniation of this material provokes an inflammatory response further adding to the irritation of the nerve roots [35]. Disc herniation occurs in up to 2% of the general population and these patients can often recall an episode of (sub)acute LBP in combination with radicular complaints. Whereas the radicular symptoms will resolve spontaneously in over two-thirds of the patients within 6 weeks, the LBP often persists or even progresses. In contrast to other forms of disc degeneration that develop in a slow progressive manner, in IVD herniation there is an acute change in local biomechanics and a disruption of homeostasis. The loss of intradiscal pressure results in decreased disc height and a diminished capability of the NP cells to maintain their ECM. The ECM, rich in waterbinding proteoglycans, is essential for the water maintaining capacity of the IVD. That IVD cells need a certain hydrostatic pressure to function properly was recently demonstrated in a clinical study among astronauts. The absence of gravity during the space flight and subsequent reduced IVD pressure resulted in an increased incidence of IVD herniation in the period after the flight [18]. If the biomechanical changes due to herniation are not reversed, progression to advanced stages of IVD degeneration will usually be inevitable, explaining the persisting LBP in
patients after an episode of IVD herniation. Moreover, if the local effects of the IVD herniation are reversed quickly, homeostasis might be restored, however if treatment is delayed degenerative changes become irreversible. Recently it was shown that if treatment for IVD herniation is delayed over 6 months, outcomes are worse following both operative and non-operative treatment [33]. The aim of this thesis was to develop a tissue engineered strategy to reverse the state after IVD herniation and to restore local homeostasis. To minimize patient discomfort, the therapy should ideally be combined with the neurological decompression surgery. In this thesis, we have translated this strategy into practice, starting with material design and ending up with the in vivo evaluation in a large animal model.

Tissue engineering
Tissue engineering is generally described as “the use of a combination of cells, engineering and materials methods, and suitable biochemical and physio-chemical factors to improve or replace biological functions” [1]. It is also referred to as “regenerative medicine”, underscoring its relation to and among other medical disciplines. Tissue engineering has evolved very quickly as an area of research since the 90s of the past century. Where initial opportunistic research hypothesized that the injection of stem cells would be sufficient for the regeneration of virtually every organ or tissue, scientists now slowly learn all circumstances that are involved [23, 30]. For the herniated intervertebral disc, numerous regenerative strategies have been studied, however broad clinical success is still lacking. In the end, this will rely on clinical treatment outcomes and costs and these will require clinical trials to establish superiority over conventional treatment standards [24]. Figure 1 shows the development pathway for novel regenerative therapies. Even when all the steps in the figure have been passed successfully, commercial success is not guaranteed. Education of the clinicians with the intention to use tissue engineered products for their patients will be crucial. This will require scientific experts, parties involved in the fabrication/development and clinicians to work in concert.
In this thesis, several steps of the development pathway (Figure 1) for IVD engineering have been challenged. The first step was to choose a suitable scaffolds material. **Chapter 2 & 3** focused on the optimization of these scaffold materials and the interaction with cells (Point 2). The cell source selection (point 1) was studied in **chapter 4**. Since we preferred the development of an a-cellular scaffold these cells consisted of the cells from surrounding structures that were expected to invade these scaffolds. In these first chapters other important developmental issues were assessed including sterilization, formulation and method of delivery. The feasibility, safety and efficacy (point 3) were finally studied in goats in **chapter 6**. Every chapter revealed crucial answers and opportunities, but often also serious challenges and drawbacks, findings inherent to a relatively young area of medicine.

**Material development**

Like every tissue in the human body, the IVD is composed of extracellular matrix (ECM) in which native cells reside. The best material, or scaffold, used for replacement of the tissue should ideally mimic several, or ultimately all, functions of native IVD ECM [5]. Four important functions and features of scaffold with respect to native ECM were recently summarized by Chan et al. [5]:

**Figure 1: Development pathway for tissue engineering [24]**
1. **Architecture:** Scaffolds should provide void volume for vascularization and remodeling and the rate of degradation should match to the new ECM formation.

2. **Cyto-compatibility:** The scaffolds should allow (native) cells to attach, grow, proliferate and differentiate.

3. **Bioactivity:** scaffold may include biological cues to influence cell morphology, alignment, migration speed and differentiation.

4. **Mechanical properties:** scaffolds should provide mechanical and shape stability of a tissue defect. Interestingly the mechanical properties of a scaffold are also known to influence the biosynthetic cell response and thus act as a passive cue.

At this moment, these material demands are principally qualitative and quantification is highly desirable with respect to material development. Numerous (bio)polymers and materials have been suggested as a suitable candidate for IVD engineering including collagen, alginate, gelatin, chitosan, poly-L-lactic acid and hyaluronan [5]. All of the materials have their advantages and disadvantages and the optimal material, fulfilling all the desired functions, will probably yet have to be invented. Beside the desired functions described above other concerns are involved in choosing the optimal scaffold, these involve availability, costs and handling [4]. The NP itself exists of Type II collagen, proteoglycans and small fractions of several other (glyco)proteins including elastin, fibronectin, laminins and tenasins [6]. It is not possible to remake the tissue in vitro, and attempts to overcome this problem have proposed. Mercuri et al. for example, harvested porcine NP’s which were completely decellularized using a combination of chemical detergents, before the scaffolds were repopulated with human adipose-derived stem cells [25]. Although their results seem promising the technique is very laborious and time consuming and not necessary since the aim of tissue engineering is to shape the optimal environment in which cells are able to synthesize and maintain the desired ECM [11]. In this thesis, we choose two very promising biomaterials to imitate the visco-elastic properties of the NP: collagen (**chapter 2**) and alginate (**chapter 3**). For this purpose, we first determined the visco-elastic properties of the goat NP and showed that this is comparable to the human NP known from literature.
In Chapter 2, rat-tail derived type I collagen was used to match to the visco-elastic properties of the NP. The rat-tail derived collagen Type I scaffolds used for this study are already used in humans for the treatment of articular cartilage defects of the knee (‘The cartilage regeneration system’, Cares) [34]. Recently, the results of a large prospective multicenter clinical trial showed that the usage was safe and clinically effective after a mean of 30 months follow-up [34]. Unfortunately, it is not possible to retrieve scaffold material for histology in clinical trials. Instead, magnetic resonance imaging of the cartilage defects treated with Cares showed no signs of inflammatory reactions and comparable results to defects treated with Hyalograft C 2 years after implantation [40]. The absence of anti-immunity reactions in vivo was also confirmed in a rat model [21]. The rat, however, is arguably not the optimal species to evaluate inflammatory response of rat-tail derived collagen. In this thesis we showed the absence of anti inflammatory response in vivo in goats (Chapter 7). With respect to IVD regeneration, the collagen scaffolds were studied in vitro in bovine lumbar spinal motions units. In this study, the scaffolds were capable to restore the range of motion to native values after implantation in a discected IVD [41]. These findings show that a collagen type I scaffold is a very promising candidate to restore the biomechanical disturbances after IVD herniation and possibly prevent the degenerative cascade in the spinal motion unit. However, in order to prevent degeneration in the long term, the scaffolds should be remodelled into native ECM by cells.

It has been appreciated that the local biomechanical environment acts as a passive cue for the gene expression and ECM production of native cells [8]. Cells mechanosense the stiffness of the ECM by actively exerting traction forces generated by their internal cytoskeleton. Moreover model studies on 2D substrates suggest that the cells adapt their biosynthetic response to changes in matrix stiffness [8]. Ideally therefore, a scaffold imitates the mechanical properties of NP tissue [5, 28]. An advantage of a collagen type I matrix is that the visco-elastic properties can be adjusted by a rapid filtration process called plastic compression [27]. In chapter 2 we used this process to increase the stiffness of the collagen matrix and found that a stiffness of 23 % w/w collagen agreed with the stiffness of NP tissue of the goat. However, the viscosity at this density of collagen was still lower compared to the NP and therefore a complete biomechanical match could not be found. An interesting finding was that the
swelling capacity of dense collagen scaffolds increases with increasing density. This is a favorable finding with respect to the replacement of NP tissue that has also an impressive capacity to swell. In contrast, free-floating collagen scaffolds, often studied as scaffolds as well, are known to shrink by the contraction of the seeded cells. In Chapter 2, we also showed that γ-sterilization has important effects on the visco-elastic properties of scaffolds. Sterilization techniques, such as γ-sterilization, are necessary steps before materials can be used in vivo and the effects are not always foreseen by scientists involved in the pre-clinical research. In the absence of an exact biomechanical match, type I collagen should still be considered as an attractive scaffold material for IVD engineering. The wide availability (e.g. rat-tail, bovine, transgenic) and known biocompatibility are noteworthy. The handling, however, is perhaps the most interesting feature of type I collagen. Plastic compression allows to produce scaffolds rapidly in virtually every collagen density [27]. Cells survive when added prior to compression resulting in completely seeded scaffolds [27], or the scaffolds can be used as a-cellular scaffolds as in the current studies. Besides the visco-elastic properties of the scaffold, the local hydrostatic pressure also influences cell response [32]. In the IVD space this is dependent on the forces on the motion segment and the capacity of the annulus closure technique to seal the defect.

In chapter 3, we evaluated alginate as a scaffold material for NP replacement. Alginate is a natural polysaccharide used for numerous medical applications due to its non-toxic nature, wide availability, low costs and simple handling and gelling behaviour [10]. In addition, the biosynthetic activity of chondrocytes, like NP cells, cultured in alginate matrices is comparable to the activity in native NP ECM [22, 39]. Alginate beads are therefore widely used as a 3D culture environment for these cell types [38]. Moreover, initial in vitro and in vivo studies on alginate as a scaffold material for NP replacement showed encouraging results [22, 26]. However, inferior biomechanical properties, especially after some time in physiological solutions, have been recognized as important limitations for the usage of alginate as a scaffold material [10]. In our study, alginate was prepared via two different techniques [29] and in different densities to imitate the visco-elastic behavior of the NP. The 2% alginate scaffolds prepared by diffusion gelation closely matched the visco-elastic properties of the NP, even more closely than the collagen scaffolds described in the preceding chapter. The moduli
measured in our study (5kPa for 1% alginate at 10 rad/s) are somewhat lower than in a previous report (16 kPa) [22]. The difference may be due to a completely different gelation protocol in the previous study and is one of several important limitations of the use of alginate revealed in chapter 3: Firstly, there is a wide range in the characteristics of alginate gels due to variations in G/M ratio, gelation temperature and rate, molecular weight, calcium content and type of crosslinker [20]. All these variations strongly affect the final network structure and therefore the reproducibility of scaffold production will be questionable. This concern also underscores the importance of the accurate documentation of exact conditions of preparation in scientific studies. Secondly, incubation of the scaffolds in culture medium has major effects on scaffold stiffness and this was also found after implantation in vivo [29]. Thirdly, and perhaps most importantly, changes in alginate scaffold stiffness do not influence the biosynthetic response of native cells. We showed that the NP cell phenotype was preserved in the alginate matrix, but not changed by altering the scaffold stiffness as would be expected. Explanations include the rapid loss of stiffness during culturing and the absence of integrin receptors on the cells to sense stiffness of the alginate. Both problems are being studied by the substitution of molecules that either stabilize the alginate matrix or promote the mechano-sensitivity of the cells for alginate. Another limitation worth mentioning is the necessity to use a cross linker (currently calcium chloride) and its potential adverse effect on the cell population cultured. Due to these limitations we preferred dense collagen as a scaffold material for the remainder of the studies.

“In Situ” seeding

The classical approach in regenerative medicine is to seed scaffolds with either native or stem cells in order to regenerate the desired tissue. The cells are expected to secrete the appropriate ECM, which finally replace the scaffold material, a process called remodeling. Seeding of a scaffold with cells, however, requires several additional steps that are generally time consuming and expensive. The cells first have to be harvested, cultured and expanded until the desired number of cells is reached. Hereafter the cells have to be seeded into the scaffolds so the surgeon can implant it in the desired location. Many experiments showed favorable results of the former in vitro, but also in vivo in animal models. However, each of the steps mentioned carries risks and drawbacks that are often
overview. Harvesting of (stem) cells requires an additional procedure and thus time, costs and morbidity. Digesting native ECM to obtain the cells demands chemical agents such as collagenases, which if not properly washed out, may interfere with the final therapy. Culturing cells may result in de-differentiation of the cells and infection of the culture. Furthermore, native cells from tissue harvested during discectomy procedures were found to have only a very limited regenerative potential [13]. An alternative for the use of native cells is the use of (adipose) mesenchymal stem cells. This, however, still requires prior (subcutaneous) harvesting procedures and thus possesses a risk on donor side morbidity. Moreover, pre-seeding of the scaffolds may compromise sterilization techniques and may require additional demands of the final form and volume of the implant. The *in vitro* experiments are generally performed by basic scientists and it may be questioned if their enthusiasm will be shared by surgeons who will have to perform the procedures in their patients. Clinicians have become used to deal efficiently with time and costs. Therefore, alternatives are now being studied and including the use of a-cellular scaffolds outlined in this thesis. Since our scaffolds were intended to be used as a-cellular constructs, we did not study scaffolds pre-seeded with cells. When implanted, the scaffolds were expected to be invaded by cells from surrounding tissues, and thus derived from the AF and/or (remnants of the) NP. The term ‘*in situ seeding*’ has been proposed for this concept [15]. *In situ seeding* does not require additional time consuming culturing and seeding techniques necessary for cell seeded scaffolds. In situ seeding therefore allows the implantation of a preformed and sterilized scaffold in a single -one step- surgical procedure in adjunct to a microdiscectomy. If viable, the ‘in situ seeding’ concept is therefore advantageous over seeded scaffold techniques.

A condition sine qua non for the viability of the in situ seeding concept is the invasion of native cells into the scaffolds *in vivo*. An advantage of using dense collagen is the very long half life (~95 year in the healthy IVD, ~215 in the aged IVD [36]) of collagen in the IVD space, which will thus occupy space till migrated cells arrive for remodeling. Interestingly, Cheema et al. recently showed that the oxygen diffusion coefficient of dense collagen scaffolds (11%) falls within the range of native tissue. This finding is crucial for early migrated cells to survive in the absence of any vascularization [7]. In the current thesis (**chapter 4**) we assessed the capability of native IVD cells to invade dense collagens scaffolds *in vitro*. Unfortunately, we could not study densities as high as 23 % (w/w) collagen,
since the time frames (> years) this would require are not compatible with in vitro culturing [27]. We studied densities up to 3% collagen that are still much higher compared to the densities generally studied (0,05% collagen). We showed that both NP and AF cells are capable of invading the scaffolds. As was expected, the migration decreased with increasing collagen densities. Interestingly we found a significantly greater migration capacity of NP cells compared to AF cells. Intuitively we had expected the fibroblast-like AF cells to have a greater migration capacity in dense collagen than the chondrocyte-like NP cells. Unfortunately, no comparable studies that either confirm or reject these findings are published. The chemokine Hst-2, derived from saliva was not found to have any pro-migratory effects on goat IVD cells. Hegewald et al. showed that the chemokine CXCL10 did actively recruit human AF cells [14]. The migration of human NP cells on the other hand was found to be enhanced by human serum [12]. The latter was actually confirmed by our own findings in the scratch assay in chapter 4.

The animals
Having shown that the visco-elastic properties of the NP can be approached with an a-cellular scaffold that also allows the invasion of native cells, our next step was to develop a model to evaluate the scaffold in vivo. Although substantial research is performed on the development and characterization of scaffolds for NP engineering, the number of studies that actually performed in vivo evaluation is very limited. There is only one comparable study which was performed in a small animal model. Huanng and colleagues recently reported the in vivo evaluation of collagenα/hyaluronan/chondroitin-6-sulfate scaffolds seeded with NP cells in a rabbit model [16]. Curiously, no annulus closure technique was described and might perhaps have been not necessary in the small animal model. The authors found maintenance of the disc height and a restoration of the T2 weighted MRI signal after 24 weeks follow-up. Other in vivo studies used scaffolds only as injectable carriers for (stem) cells in disc degeneration. These studies in rabbits [16] or pigs [31] do not deal with the annulus defect typically for disc herniation and are therefore not comparable. One last in vivo study worth mentioning reported the implantation of complete tissue engineered IVD’s consisting of polyglycolic acid and calcium alginate matrices seeded with native cells in mice [26]. Although the ECM production was in line with native tissue up to 12 weeks, the method of delivery of these constructs is perhaps too
complicated to be feasible in humans. The number of in vivo studies for NP scaffolds is remarkably small compared to the massive in vitro work that has been published. Possibly, more in vivo studies have been performed, but not been accepted for publication since the results were negative [24]. A potential explanation for this, and one of the main concerns of NP replacement models in general, is the necessity of a closure technique for the AF [41]. During the planning of the animal studies we soon experienced the problem ourselves and decided to start to summarize all available data from literature on this subject first. This resulted in an extensive review (Chapter 5) from which it can be concluded that, despite numerous attempts, an appropriate AF closing technique is not yet invented. Since our intention was to evaluate dense collagen implants in goats, we needed some AF closure technique and started to test several custom made AF closure devices. These devices were designed to the goat dimensions and should be compatible with our NP replacement model. After thorough in vitro and in vivo (2 weeks) evaluation (Chapter 6) of potential devices we found devices which closed the AF sufficiently. These devices were then used for a next pilot in vivo study (6 weeks) to evaluate the collagen scaffolds. However, after six weeks follow-up the ACD’s showed important signs of dislodgement and destruction. We therefore could still not perform the full in vivo evaluation of the collagen implants that we aimed. However, we did show that the disc height could be preserved in the levels treated with the collagen scaffolds, results that are in line with the findings of Huanng et al. [16]. They also found restored hydration (based on MRI) of the IVD after 24 weeks follow up. We could not make a quantitative analysis of the MR images, since the surgical disturbances were still predominant after 6 weeks follow up. Moreover, the AF closure devices have their own effects on MRI signal, essentially disturbing appropriate quantification. Haunng et al however, used a small animal model, which has a very limited value for translation to humans [24]. Small animal have significantly different spinal biomechanics and the small volume of the disc affects transport [3, 24]. Remaining disc height and an acceptable MRI T2 signal is therefore not very informative. The use of these animals should be preserved for safety studies, but feasibility studies require a large animal model as was used in the current thesis [24]. Although the replacement model was feasible, the surgeries reproducible and the harm to the animals acceptable the results are strongly negatively influenced due to the lack of an appropriate AF closure technique. Therefore no
firm conclusions can be drawn. The experiments deserve further study, accompanied by a proper AF closure technique.

**Future perspectives**

Substantial research in the field of tissue engineering has been based on trial and error rather than on firm scientific knowledge. Not surprisingly, since many factors and circumstances affecting the success of the regenerative medicine still have to be revealed. For example, we studied the visco-elastic properties of a scaffold because this has been recognized as a cue for differentiation and ECM production. However, first studies on this subject have been published only five years ago and involved cells seeded on a flat surfaces [9]. Meanwhile, similar findings were observed in 3D environments, but the exact value, and the relation to other local circumstances such as the hydrostatic pressure, the biochemical environment and scaffold degradation rates remains unclear [32]. During the past decade, *in vitro* research using scaffolds, native cells or stem cells, have detected several individual factors involved in the regeneration of tissues. Probably we still know only a small portion of all the factors and basic research will remain to have a crucial position, before fully scientifically based regenerative strategies can be designed. Even when we are aware of all the factors involved, we still have to study their interactions, counteractions and synergies. This can only be properly studied *in vivo* and studies in relevant animal models are therefore required. Small animal models are useful for screening purposes, but large animal models are required to mimic nutritional and surgical constraints to human application [24]. Early tissue engineering attempts should be accompanied by a vision how the final clinical application will look like and address demands like formulation, delivery and sterilization [4, 19]. To evaluate therapies that are intended to regenerate the herniated IVD, there is a need for standardized magnetic resonance imaging grading and/or scoring systems [2]. The scoring systems should be developed in animal models with histologic confirmation and then translated to the human situation in which histology is not an option [24]. An alternative to the use of animal models, is the use of a bioculture system in which entire IVD’s can be studied under physiological circumstances and with longer time frames [17]. These systems have the advantage to reduce the number of animals needed for research and are currently being developed and their exact value will become clear in the near future. We showed that the lack of AF closure
techniques impedes the *in vivo* evaluation. Moreover, in our goat model only a standardized defect in the lateral region of the AF had to be closed. In the patients suffering from disc herniation the defects are irregular, located posteriorly close to the neurological structures and sometimes even bilaterally. Appropriate closure of the AF defect in these patients will be even more challenging and might further delay tissue engineering development that may have passed the preclinical stage successfully. Several suggestions and requires for annulus closure techniques were extensively described in Chapter 5. An important possibility to prevent the unnecessary repeating of (animal) studies with not publishable negative outcomes is the dissemination of these results between internationally established spinal research sites [24].

In the end, the most important question will be if the patients actually will benefit from the new therapy. Improper patient selecting may place a potential beneficial procedure in disrepute [19]. Clinicians should be instructed how to implement the new types of therapy in their surgical practice. This will demand close cooperation between scientists, industry and medical personnel [24]. Tissue engineering sites should be close to the operating theaters within the same complex. It requires massive efforts to achieve all this but the improvement of health care may be likewise if the promise of tissue engineering is finally fulfilled.
References


Appendices