Final Report Summary - NPMIMETIC (Biomimetic nano-fiber-based nucleus pulposus regeneration for the treatment of degenerative disc disease)

Executive Summary:

Low back pain has been the worldwide leading cause of lost work time and years lived with disability for several decades. The main cause for back pain is attributed to degenerative changes in the intervertebral disc (IVD). The IVD is a composite structure formed of the Annulus Fibrosus (AF), a tough outer layer of lamellar fibrocartilage providing strong capacity to withstand peripheral load transfer, which surrounds a soft, visco-elastic and gelatinous core, the Nucleus Pulposus (NP).

Current clinical practice faces a big gap between conservative treatment (e.g. physiotherapy, pain killers) and clinically successful but highly invasive interbody spinal fusion, in which a rigid bony bridge is created between two vertebrae. The NPmimetic project aimed to provide novel concepts for both mild and severe disc degeneration (i.e. biological regeneration and mechanical reconstruction, respectively) which are less or minimally invasive and restore disc functionality rather than eradicating its primary function. In addition, a virtual model of the IVD was developed, both to describe the mechanical characteristics required for the NP restoration modalities, and to evaluate possible effects of that implant on the IVD mechanical performance. Last but not least, NPmimetic developed novel delivery tools and IVD imaging techniques to facilitate these treatment concepts.

In a first (“reference establishment”) step, the native properties of healthy and degenerated IVDs were assessed. It was found that not the rheological properties of the NP, but instead the intradiscal pressure strongly determined the mechanical behaviour of the total IVD, comparable to pressure in a car tire.

For the biological regeneration approach, extensive efforts were made to GMP-conform synthesize, characterize, validate, innovative fibrinogen-hyaluronic acid (FBG-HA) nanobiopolymer conjugates for treatment of the mildly degenerated IVD. Successful covalent coupling of recombinant chondrogenic growth factors (GFs) to the FBG-HA hydrogel conjugate was proven, with maintenance of biological activity upon release by plasmin cleavage. Moreover, stronger biological activity was found for a newly developed BMP-2/7 heterodimer compared to the commonly used BMP-2 homodimer. Exposure of cultured NP cells to the conjugates (+/- the GFs) or injection of these modalities into the NP of complete IVDs was shown to be feasible and safe, both in vitro and in vivo. Efficacy of the GFs to enhance the NP cell phenotype (measured as an anabolic response) was demonstrated in in vitro studies and ex vivo organ cultures, but as yet not shown upon injection in mildly degenerated goat IVDs. Further studies are needed to evaluate this in vitro / in vivo discrepancy, focusing on (i) increasing dosages of the GFs in goat studies; (ii) the level (or absence) of plasmin activity within the NP of the IVD; and (iii) the evaluation of the absolute necessity of this tightly controlled release system, given the strong containment and thus leakage preventing function of the IVD. A pilot in vivo study with supplementation with freshly isolated stem cells from adipose tissue (ASCs) turned out negative, and ASCs were not further pursued within the course of the project.

For the mechanical reconstruction approach, NPmimetic developed and established a highly promising NP replacement strategy. It encompasses a strongly swelling and load-resistant NP implant (“ravioli”), that meets the many requirements identified for NP replacement: (i) polymeric building blocks and raw materials complying with regulatory requirements; (ii) a core material exerting strong swelling pressure; (iii) an envelope showing suitable mechanical strength and efficient wettability; (iv) biocompatibility and absence of cytotoxicity of the ravioli, and cell adhesion and proliferation on the envelope material; (v) a proof of concept for drug containment potential and its controlled release; (vi) mechanical properties, tested
under confined and non-confined conditions, enabling ravioli implants to resist forces well above physiological and peak loading without rupturing. In organ culture models and in an in vivo pilot study, the ravioli implants were delivered via a novel transpedicular endplate approach, in order to avoid damage to the AF and potential subsequent herniation. Although the transpedicular approach was shown to be feasible, swelling pressure buildup as well as implant retention in the NP space in time were yet insufficient. In a pilot study, ravioli’s were found in the drill holes in the goat vertebral bodies at 1 month follow-up, despite filling these with polyurethane plugs and PMMA cement.

Conclusion. NPmimetic generated and evaluated important new technologies and treatment modalities to bridge the big gap between conservative treatment and the well-established but highly invasive interbody spinal fusion. Although further studies are certainly warranted, these new concepts will be less or minimally invasive and restore disc functionality, and may enable prolonged maintenance of Quality of Life and working capacity for patients with, or at risk for low back pain.

Project Context and Objectives:
Summary description of project context and objectives

Introduction
Low back pain has been the worldwide leading cause of lost work time and years lived with disability for several decades according to the Global Burden of Disease Study 2010. The main cause for back pain is attributed to degenerative changes in the intervertebral disc (IVD). The IVD is a composite structure formed of the Annulus Fibrosus (AF), a tough outer layer of lamellar fibrocartilage providing strong capacity to withstand peripheral load transfer (confining phenomenon), which surrounds a soft, visco-elastic and gelatinous core, the Nucleus Pulposus (NP). The NP is rich in sulfated proteoglycans (glycosaminoglycans; GAGs), resulting in high water-binding capacity and high osmotic pressure. With age, the proteoglycan (water) and cellular content of the NP decrease, resulting in a drop of internal pressure, and thus gradual loss of the cushion function of the disc.

The NPmimetic project realised that clinical treatment is different for patients with mild and severe disc degeneration. In mild degeneration the surrounding AF is more or less intact, which means that it suffices to “re-inflate” the NP with a gel, possibly enriched with cells and/or growth factors (biological reconstruction/ regeneration). In severe degeneration, on the other hand, both NP and AF are damaged and need to be replaced (mechanical reconstruction). This consortium concentrated on the development of both types of NP mimetic substitutes for restoration of IVD functionality, with a special focus on nanotechnology based strategies.

This report summarizes the main achievements of the NPmimetic project along the following topics:
1. Determination of native rheological properties of the healthy and degenerated nucleus pulposus
2. Development and in vitro/ex vivo evaluation of novel nano-biopolymers for biological regeneration
3. Development and in vitro/ex vivo evaluation of novel nanotechnology-based, biomimetic NP replacement implants for mechanical reconstruction
4. Development of a virtual model of the IVD, and in silico integration of NPmimetic consortium findings
5. Validation (animal models/clinical view) of the proposed developments for medical application

1. Determination of native rheological properties of the healthy and degenerated nucleus pulposus
Before designing mimetic substitutes for the NP (the main goal of the NPmimetic program), it was necessary to determine the rheological properties of the healthy and degenerated NP itself, so that we knew what to aim for: the specifications of the substitute. In order to obtain reproducible data, we degenerated healthy goat discs in a controlled manner by injecting an enzyme that degrades the water-attracting proteoglycans. Mild degeneration in goat IVDs could reproducibly be achieved as established before. Rheological characterisation of NP material showed a large inter-animal variation (more than factor 2 difference), but degeneration only resulted in a small decrease in rheological properties (about 10% lower moduli) . In contrast to that, physicochemical analysis revealed a consistent change in composition following degeneration as well as a difference in rehydration and fibre dimensions. Furthermore, FTIR (infrared spectroscopy) revealed a difference in water binding between healthy and degenerated IVDs, namely in degenerated discs, there appears to be less water present, but more tightly bound to the proteoglycans. Mechanical behaviour of the whole spinal motion segment (SMS) changed consistently with mild
degeneration (decreased neutral zone stiffness and increased range of motion). Thus, we found that the mechanical behaviour of the SMS is hardly sensitive to the rheological properties of the NP. Intradiscal pressure, on the other hand, strongly determines the load bearing capacity of the NP and tension in the AF and thereby the mechanical behavior of a SMS, comparable to pressure in a car tire. This was confirmed in a matching project on IVD behavior as a function of water content. Thus, tissue-engineering strategies should focus on the restoration of intradiscal pressure rather than on mimicking the rheological properties of the NP.

2. Development and in vitro/ex vivo evaluation of biomimetic injectable hydrogels for NP regeneration

The objective of this section was to develop and evaluate novel nano-biopolymers to support replacement and regeneration of the NP tissue of the IVD. Various formulations of Fibrinogen (FBG)-Hyaluronan (HA) conjugate hydrogels were produced with different types of HA and different ratios of FBG to HA. The FBG-HA conjugates were characterized using a set of quality control assays, including chromatography, clottable protein assay to measure the FBG content, gel transparency, and long-term stability studies. Cytocompatibility was confirmed for different human cell types. The production process was scaled-up in order to enable reproducible manufacturing of stable hydrogels for in vitro, organ culture and in vivo experiments. The potential of FBG-HA conjugate hydrogels to support growth and activity of NP cells was investigated using in vitro cell culture assays. Conjugation of HA to FBG enhanced gel stability and resulted in improved rheological properties and cell retention. Extracellular matrix synthesis and retention was improved in cell-seeded FBG-HA conjugates, and the hydrogels integrated well in the native tissue when injected in organ cultured IVDs. Injection of cell-hydrogel suspensions was feasible and maintained high cell viability, suggesting that FBG-HA conjugate is suitable as an injectable biodegradable hydrogel for biological NP regeneration.

An additional objective was to further functionalize these FBG-HA conjugates by incorporation of growth factors. Transglutaminase (TG) enzymatic crosslinking site containing bone morphogenetic protein (BMP) molecules were synthesized and integrated into FBG-HA conjugate hydrogels. Covalent binding of BMP to the FBG in the gel was demonstrated. Upon enzymatic release of BMP2 from hydrogels, the BMP activity was preserved, as demonstrated in cell-based assays and in an in vivo model. TG-BMP2/BMP7 heterodimers were produced, which demonstrated a higher activity compared to TG-BMP2 homodimers. Incorporation of TG-BMP2/BMP7 in the FBG-HA conjugate hydrogel increased the glycosaminoglycan synthesis of encapsulated NP cells in vitro.

In organ culture experiments using whole IVDs from bovine tail, it was assessed whether addition of the FBG-HA conjugate hydrogel with/without TG-BMP2/BMP7 in conjunction with the NP reconstruction implants (“ravioli’s; see below) had an added value to functional outcome. For this purpose, bovine IVDs were nucleotomized and reconstructed with combinations of materials for biological and mechanical reconstruction, followed by culturing in an IVD bioreactor under dynamic load. The ravioli was able to swell in situ and to restore the height and mechanical function of nucleotomized discs. Additional injection of FBG-HA hydrogel did not affect the swelling capacity of the ravioli in situ. To conclude, ravioli’s may be suitable for NP replacement, while FBG-HA hydrogel and TG-BMP2/BMP7 may further support biological repair of NP tissue.

3. Development and in vitro/ex vivo evaluation of novel nanotechnology-based, biomimetic NP replacement implants for mechanical reconstruction

Two prototype scaffold structures were developed: Flat discoid (“ravioli”) and cylindrical shape (“sausages”). The ravioli shape was selected for function, mechanical, in-vitro and in-vivo studies. The ravioli is composed of envelope (E) and core-a (Ca) to form ECa construct and it is optional to include in it also core-b, drug delivery core, to form ECab. Core-a is the swelling core which was designed to mimic NP characteristics. It is made of a polymeric film and swells to a hydrogel following contact with an aqueous medium. The envelope is made from electrospun polycarbonate urethane and shown to have a homogenous fibrous mesh like structure. It supplies mechanical support, protects the gel from leaking and serves as a scaffold for tissue integration. Formulations and manufacturing methods for Core-a and envelope were developed, defined and finalized. NP mimetic polymeric building blocks and raw materials selected for manufacturing the ECab construct were demonstrated to comply with regulatory requirements. Ravioli’s assembly and manufacturing procedures were established. Ravioli’s at sizes of 6, 8, 9, and 14 mm are available in various core-a’s contents. Core-b manufacturing, using electrospinning technology, was designed as stand-alone process that can be integrated post manufacturing with ECa. A proof of concept for encapsulation of a
model NSAID, diclofenac sodium, was demonstrated.

Scaffold characteristics:

Function and cytotoxicity: The swelling capacity and swelling kinetics of the ravioli in aqueous solutions was demonstrated using several Core-a’s contents. Scaffold volume and weight increased with an increase in core-a content. Fast swelling kinetics, minutes to hrs depend in the core-a content, was observed. Final Core-a composition was shown to be cell friendly: It stimulated cell proliferation of hMSCs and did not hamper bNPCs growth. The envelope formulation was found non-toxic for bNPCs and hMSCs. High cells adhesion ratio and significant cell proliferation was observed following seeding of bNPCs on the envelope, suggesting that it is capable of supporting NPCs attachment and growth.

Chemical analysis: The chemical composition of the envelope and core-a constructs was validated using ATR-FTIR spectroscopy. The material behavior following hydration was demonstrated. The thermo-mechanical properties of the novel scaffold components were defined using dynamic mechanical spectroscopy. Shelf life studies showed long term stability of the scaffold components.

Mechanical properties: Swelling of the ravioli strongly depended on the osmolarity of the medium; when in medium with IVD-comparable values, swelling reduced 2-3 fold. It was found that material could bear the applied pressures in confined compression very well, but pressures were less than we measured with NP material. Thus, the swelling and load-bearing capacity of the NP substitute is close but does not fully match the properties of the natural NP. In non-confined compression, the scaffold lead with a stress of 5 MPa, which is highly superior to normal stress acting on IVD during a moderate effort. No rupture was observed for the range of physiological values.

Scaffold clinical aspects:

• Addition of an anchor to the ravioli for optimal clinical employment was designed and a prototype was demonstrated.
• A special assembly procedure was established to enable insertion of 6 and 9mm raviolis through 2.8mm ID needle. Similar methodology can be applied for 14mm ravioli through 5mm ID needle.
• A method for incorporation of barium sulphate, as a radiopaque agent, was developed. Ravioli containing BaSO4 were manufactured at several doses. It was demonstrated that BaSO4 embedded in scaffold enabled its imaging and positioning.

4.Development of a virtual model of the IVD, and in silico integration of NPmimetic consortium findings

The development of a virtual model of the human intervertebral disc (IVD) had, as main objectives, firstly to describe the mechanical characteristics required to the design of a NP restoration implant and, secondly, to evaluate the possible effects of that implant on the mechanical performance of a restored IVD.

To achieve those goals a series of steps was taken that can be summarized as follows:

i) Mechanical characterization of the ‘natural’ IVD behaviour, bearing in mind the different structures it is composed of, and the role performed by each structure on the load carrying capacity of the IVD unit.

A model of the IVD was built over a ‘standardized’ geometry of a lumbar spine L1-L2 motion segment (MS), with mechanical characteristics taken from experimental results – performed within the consortium and/or gathered, carefully filtered and selected from published data – and taking into special consideration the geometric complexity of AF fibers structure. The model was then extensively validated, against available test data, followed by a parametric analysis of the behavior and contribution of each of its components. It was shown that the specific load carrying capacity of the IVD is neither directly dependent on NP material nor on its viscoelastic characteristics. Instead, the ‘cushion’ load/shock absorber role of the IVD may be mainly attributed to the mechanical constraint exerted by the AF fibers to the hydrostatic pressure of the NP.

ii) Identification and quantification of other direct mechanical influences, exogenous to the IVD itself, that can potentially interfere with, and modify its behavior.

The motion segment (MS) is co-stabilized by several ligaments that interconnect the vertebrae, a.o. the anterior and posterior longitudinal ligaments (ALL, PLL). Analysis of Finite Element Modeling (FEM) results for AF dynamics, when the MS is subjected to frontal flexion/extension rotation, clearly showed that those ligaments have a noticeable influence on the concave/convexity shape of the AF external wall and, as a direct consequence, on the overall behavior of the MS. Furthermore, all the ligaments are in a pre-stretched state, imposing a given state of compression to the MS. This effect, along with the osmotic properties of the IVD components, is responsible for maintenance of the IVD’s internal pressure, even in steady-state conditions. It
appeared important to consider this in the IVD model, in particular since it was shown to also strongly influence the FEM predictions of the overall behavior of the MS.

iii) Characterization of the effective loading states that can be produced, and must be withstand, by the IVD.

FEM models for standard IVD geometrical characteristics from L1-L2 to L5-S1 where produced, and their mobility results (i.e. force/displacement and torque/rotation, in the three coordinated axes) where used to build a MultiBody System (MBS) model of the all lumbar column, that also incorporates the vertebrate body geometries and masses, the ligaments non-linear springs and the articular spinous facets contacts. This model is able to emulate the spine (static or dynamic) behaviour and to predict the reaction forces/torques developed on each individual level, produced by any kind of action performed on the all segment, thus providing the specific load state at each IVD for a given set of conditions. Besides the access to the complex loading state of the IVD – which can be feedback to the FEM model, for analysis of the individual components behavior and, specifically, to the working performance of the NP and any implant within it – it also can be used in the ‘reverse’ way, i.e. for prediction of the spine behaviour to a ‘non-conformable’ IVD (i.e. for instance, a damaged or a prosthetic implanted NP) whose characteristics where previously determined by a FEM approach.

5. Validation (animal models/clinical view) of the proposed developments for medical application

From the above, it may be clear that NPMimetic employed a thorough step-by-step approach with extensive in vitro testing for a large number of relevant parameters, to ensure that the consortium would deliver safe and biocompatible solutions for in vivo animal and clinical application.

Biological reconstruction/regeneration. Several studies were performed to evaluate the safety and efficacy of the nanobiopolymeric fibrin-hyaluronic acid (FBG-HA) conjugated hydrogels, either or not combined with transglutaminase (TG)-coupled BMP-2 or BMP2/7 osteo/chondrogenic growth factors and/or freshly isolated adipose stem cells (ASCs). In brief, mild IVD degeneration was induced chemically with Chondroitinase ABC, after which intradiscal injections of the various (mixtures of) compounds was performed. After a follow-up time of twelve weeks, safety and efficacy were assessed using multiple outcome parameters (radiography, MRI T2* mapping, and biochemical and histological analyses). From these studies, it was concluded that (i) fibrin-hyaluronate hydrogels, both alone as well as conjugated with 1 or 5 µg/ml BMP-2 homodimers or BMP-2/7 heterodimers appeared safe; (ii) a mild (pilot study) or no (study 2) regenerative effect of the BMP-supplemented FBG-HA gels was observed after 3 months follow-up; (iii) Freshly isolated adipose stem cell preparations caused adverse events, and are therefore discouraged for IVD regeneration (cultured stem cells may still work); (iv) T2* MRI mapping, allowing degenerative measurements on a continuous scale, showed good and linear correlation between T2* relaxation time and accepted parameters of disc degeneration, and as such is a promising tool to assess disc degeneration in clinical practice.

Mechanical reconstruction/NP replacement. In an in vivo pilot study, the consortium employed delivery of the implants via a transpedicular endplate approach, in order to avoid damage to the AF and potential subsequent herniation via the AF. Although the transpedicular approach was shown to be feasible, swelling pressure buildup as well as implant retention in the NP space in time were yet insufficient. In the goat pilot study, the ravioli’s were found to be located in the drill holes in the vertebral bodies at 1 month follow-up, despite filling these holes directly after ravioli delivery in the NP space with polyurethane plugs and PMMA cement.

Clinical perspective. A co-developed 3-needle minimally invasive delivery system, enabling of mixing and injecting the components of the hydrogel and bioactive moieties at the same time while avoiding iatrogenic AF damage, is an important additional asset. With the ravioli NP replacement, made clinically visible with CT imaging by Barium sulphate incorporation, the project has generated a novel NPMimetic implant which will allow restoration by restoring the intradiscal pressure exerted by the NP, pivotal for the primary function of the IVD. The smaller in vivo dimensions of goat IVDs hampered the evaluation of the endplate approach considerably, and this may be significantly improved when applying a clinically available technology for the human spine.

Conclusion. NPMimetic generated and evaluated important new technologies and treatment modalities to bridge the big gap between conservative treatment and the well-established but highly invasive interbody spinal fusion. Although further studies are certainly warranted, these new concepts will be less or minimally invasive and restore disc functionality, and may enable prolonged maintenance of Quality of Life and working capacity for patients with, or at risk for low back pain.
1. Determination of native rheological properties of the healthy and degenerated nucleus pulposus

When designing a substitute for degenerated or damaged tissues, it is important to have an idea of the properties of the healthy tissue, so that design specifications can be defined. In the case of the intervertebral disc, the main tissue of interest is the core of the disc, the nucleus pulposus. The nucleus is a homogeneous, gel-type structure which consists for more than 70% of water. We are thus dealing with a poro-elastic structure, which typically is characterised by rheology. Rheology determines both the viscous and the elastic properties of a material by dynamic loading tests: the solid part responds elastically (directly) to the applied load, while the viscous part responds to the speed of loading. To obtain reliable rheological values for a healthy nucleus pulposus and for degenerated discs, we used the spines of goats from the slaughterhouse. These goats were young enough to have non-degenerated discs, and we are able to degrade them in a controlled way by injecting an enzyme that breaks down the water-binding proteoglycans within the nucleus. As a result, the nucleus loses water and thus hydrostatic pressure, comparable to deflating a tire. The effect of injecting the enzyme could be confirmed at the macroscopic scale: the disc height decreased, the MRI score decreased, the stiffness under bending and rotation decreased and the range of motion increased (Figs. 1.1, 1.2).

Fig. 1.1: degeneration of the intervertebral goat discs by injection of the enzyme Chondroitinase ABC. Left a decrease in disc height, right a decreased value for MRI. The changes indicate mild degeneration.

Fig. 1.2: Changes in the mechanical behaviour of the spinal segments due to induced degeneration: the specimens are less stiff and have a larger range of motion, indicating more instability of the segments.

While the degeneration of the intervertebral discs with the enzyme were successful and realistic, the rheological data were somewhat surprising: The elastic and viscous moduli of the nucleus pulposus in the different goats differed tremendously, while the changes due to induced degeneration were rather marginal (Fig. 1.3).

Fig. 1.3: elastic and viscous moduli in different goats. Note the large variations (more than factor 2) between goats, and the rather low but consistent decrease of moduli with degeneration.

We conclude from this study that we can reproducibly induce a realistic and mild disc degeneration in terms of disc height and biomechanical behaviour. The absolute numbers of the elastic and viscous moduli give an indication of what is normal, but their values are not very sensitive for disc behaviour, since we found that the mechanical behaviour of the SMS is hardly sensitive to the rheological properties of the NP. Intradiscal pressure, on the other hand, strongly determines the load bearing capacity of the NP and tension in the AF and thereby the mechanical behavior of a SMS, comparable to pressure in a car tire. This was confirmed in a matching project on IVD behavior as a function of water content (Vergroesen et al, subm). Thus, tissue-engineering strategies should focus on the restoration of intradiscal pressure rather than on mimicking the rheological properties of the NP.

2. Development and in vitro/ex vivo evaluation of biomimetic injectable hydrogels for NP regeneration

Within NP mimetic novel nano-biopolymers were developed for supporting nucleus pulposus cells (NPCs) growth and metabolic function. Specifically, an injectable biodegradable hydrogel, based on Fibrinogen (FBG)- Hyaluronic acid (HA) conjugate, was developed to mimic native extracellular matrix for minimally invasive intervertebral disc (IVD) regeneration. The main objective was to manufacture and characterize FBG-HA conjugates of different FBG:HA ratios (from 40:1 to 5:1) and HA molecular weight (from 17KDa to 3MDa).

The biopolymers are composed of two natural macromolecules: 1. Plasma derived human fibrinogen (FBG); 2. The polysaccharide hyaluronic acid (HA). A modified HA is prepared by transient activation of an intrinsic carboxylic group in HA under defined conditions. The activated HA is subsequently mixed with the FBG protein to yield a defined polymeric conjugate of HA and fibrinogen (Scheme 2.1). The water soluble HA-hFBG product is free of any residual chemical entities due to the unique mode of pre-activation and isolation of the active species of HA. The soluble FBG-HA conjugates form biocompatible
hydrogels by virtue of the addition of thrombin; the final hydrogel was also termed Core-c in the context of NPMimetic. The co-
polymers yield clear hydrogels which are transparent, stable, mechanically versatile and biocompatible for a variety of cells. Various combinations with different FBG:HA ratios (“A-D”) and HA molecular weights (“1-4”) were successfully produced and characterized. An effort was devoted to scale-up their production and adapt it towards GMP processing.

Scheme 2.1. General description of Fibrinogen (FBG)-Hyaluronic acid (HA) conjugate production.

Fig. 2.1. Microscopic view of hydrogels overhanging a spatula after 20 min at 37°C.

In vitro evaluations of biomimetic hydrogels
In vitro studies of bovine NPCs cultured in several Core-c formulations of FBG-HA conjugates, FBG-HA mixtures, and FBG were performed. Bovine NPCs were cultured in FBG-HA conjugate based 3D beads in vitro and in a nucleotomized organ culture model. FBG-HA conjugate based hydrogels prepared with 235K Da HA at FBG/HA w/w ratio of 17:1 showed superior gel stability and mechanical properties and markedly increased glycosaminoglycan (GAG) synthesis compared with FBG/HA mixture based hydrogels or fibrin gels (Fig. 2.1 2.2). Gene expression levels of NP markers were maintained in the beads in vitro. In organ culture, NP cells seeded in FBG-HA conjugate based hydrogels showed improved integration with native NP tissue compared with fibrin gels (Fig. 2.3). Moreover, FBG-HA conjugate based hydrogels restored compressive stiffness and disc height after nucleotomy under dynamic load. Injectability tests of cell suspensions in FBG-HA conjugate through a 30G needle showed cell viability of higher than 85%, suggesting that it can be injected into the IVD and may be suitable as an injectable hydrogel for biological NP regeneration.

Fig. 2.2. GAG/DNA ratio of hydrogels cultured for 3, 7 or 14 days. FBG-HA conjugate showed significantly higher GAG/DNA ratios compared with the FBG-HA mixture and FBG only. This indicates that FBG-HA conjugate is superior for GAG synthesis and retention. Mean±SEM, n=9, *p<0.05 **p<0.01 ***p<0.001 #p<0.05 vs day 3.

Fig. 2.3. Sections of bovine caudal discs stained with Safranin O/Fast Green, 14 days after nucleotomy. (A, G) nucleotomized disc refilled with fibrin gel, (D, J) nucleotomized disc refilled with fibrin gel with embedded NPCs, (B, H) nucleotomized disc refilled with FBG-HA conjugate based hydrogel, (E, K) nucleotomized disc refilled with FBG-HA conjugate based hydrogel with embedded NPCs, (C, I) nucleotomized disc as negative control, (F, L) intact disc as positive control. t –disc native tissue, h –implanted hydrogel. Scale bar 2 mm (A-F), 500 µm (G-L).

Growth factor integration into biomimetic hydrogels
To further functionalize the Core-c product, growth factors were integrated into the biopolymer. The basis for the integration of any factor is the cloning, expression, refolding, and purification of those factors. First, Transglutaminase enzymatic crosslinking site containing (TG)-BMP2 were expressed, refolded and purified as model to study the integration into Core-c materials. The results showed that TG-BMP2 was covalently integrated into the Core-c materials and could be released by plasmin or trypsin. TG-BMP2 upon enzymatic release was still active. Since heterodimers are known to be more active than the respective homodimers TG-BMP2/BMP7 heterodimers were produced to further improve the effectiveness. Sufficient quantities of highly purified TG-BMP2/BMP7 heterodimers be used for in vitro and in vivo studies could be synthesized (Fig. 2.4).

In vitro cell culture results confirmed that these heterodimers were more active than TG-BMP-2 homodimers (Fig. 2.5). Studies on bone defects in the calvarial bone of rats confirmed the bioactivity of these proteins in conjunction with fibrin and different Core-C biopolymers. Moreover, incorporation of TG-BMP2/BMP7 in the FBG-HA (Core-c) conjugate hydrogel increased the glycosaminoglycan synthesis of encapsulated NPCs in 3D bead cultures in vitro.

Fig. 2.4. TG-BMP2/BMP7 heterodimer characterization. Western blot of the TG-BMP2/BMP7 heterodimer compared to TG-BMP2, R&D Systems BMP7 and R&D Systems BMP2/BMP7. a) BMP2 detection, b) BMP7 detection and c) overlay; d) PAGE of TG-BMP2/BMP7 shown next to molecular weight marker. e) The ALP activity of TG-BMP2/BMP7 is almost 2.5 times more active
Fig. 2.5. Alkaline phosphatase (ALP) activity induced by TG-BMP-2 and TG-BMP-2/BMP7. Pluripotent C2C12 cells were stimulated with 500 ng/mL TG-BMP2/BMP7 and the induction of ALP was compared to different concentrations of TG-BMP2. The results indicate that 4 times more TG-BMP2 is needed for the same level of induction than with TG-BMP2/BMP7.

Assessment of the nano-fibrous polyurethane (PU) scaffold, biomimetic conjugate hydrogel and TG-BMP 2/BMP7 in organ culture bioreactor system

A bovine disc nucleotomy model created mechanically by incision through the endplate was used. Nano-fibrous PU scaffolds, with swelling capacity, of 6 mm and 9 mm diameter were implanted into NP defects with 4 mm and 6 mm diameter, respectively. The effect of PU scaffold, FBG-HA hydrogel, and TG-BMP2/BMP7 was investigated in discs implanted with the following biomaterials: (1) PU scaffold; (2) PU scaffold surrounded by FBG-HA hydrogel; (3) PU scaffold surrounded by FBG-HA hydrogel containing TG-BMP2/BMP7. After implantation of biomaterials, the removed endplate stopper was re-inserted, and the crack in the endplate was sealed by polymethyl methacrylate. Discs were loaded dynamically for 3 hours/day (0.1 Hz) during 14 days.

Addition of FBG-HA (Core-c) biomimetic hydrogel did not affect the swelling capacity of the nano-fibrous scaffold in the disc in situ. Implantation of nano-fibrous polyurethane scaffold, with or without surrounding biomimetic hydrogel, maintained the disc height after repeated cycles of mechanical load (Fig. 2.6). Histology confirmed that the swollen PU scaffold completely filled the nucleotomized region in the disc. The remaining NP tissue was still intensely stained after 14 days of dynamic load. When FBG-HA hydrogel was delivered into NP cavity surrounding the PU scaffold, the scaffold showed more intense staining.

Fig. 2.6. Disc height change of partial nucleotomized discs implanted with different combinations of biomaterials and growth factor under long-term dynamic load. All implants maintained the disc height compared to the empty nucleotomy control. Mean ± SEM, n=6, *p<0.05.

3. Nano-fibers based gel composites providing NP mechanical properties and drug release capabilities

NP mimetic Scaffold/ECab construct

Two prototype implant structures were developed (See Figs 3.1 and 3.2): Flat discoid ("ravioli") and cylindrical shape ("sausages"). The ravioli shape was found more suitable for clinical applications and therefore was selected for in-vitro and in-vivo studies.

Fig. 3.1: Cylindrical "sausages" shape (left panel) and flat discoid "ravioli" shape prototypes (6, 9, and 14 mm, left to right, respectively) before and after swelling

Fig. 3.2: Schematic illustration of the assembly of flat discoid, "ravioli" shape

NP mimetic polymeric building blocks and raw materials selected for manufacturing the scaffold were demonstrated to comply with regulatory requirements

Envelope – Structure

The envelope has a homogenous fibrous mesh like structure with fibers size of 0.5-1.5 µm. Pore size of the fibrous mat is in the range of 3-5 µm with an average of 3.7 µm. Bubble point measurement indicates the largest pore (See Table 3.1). SEM image of the envelope is shown in Fig. 3.3. It demonstrated sufficient mechanical strength and efficient wettability.

Table 3.1: Results of envelope’s pore size analysis Fig. 3.3: SEM micrograph of an envelope.

Core-a Structure

Core-a is the swelling core designed to mimic NP characteristics. It is made of a polymeric film that swells to a hydrogel following contact with an aqueous medium. At a defined envelope dimension, Core-a’s content can vary in a range of one fold.
SEM image of core-a in dry and hydrated stage is shown in Fig. 3.4.

Fig. 3.4: SEM micrograph of core-a before (right) and after (middle and left) hydration.

Core-b, the Drug Delivery Core
Core-b manufacturing, using electrospinning technology, was designed as stand-alone process that can be integrated post manufacturing with ECa (See Fig. 3.5). Biocompatible and biodegradable polyesters (PCL, PLGA 50:50 and PLGA 75:25) were used as the potential drug carriers. A proof of concept for encapsulation of a model drug, the NSAID diclofenac sodium, in core-b was demonstrated. SEM micrograph of drug candidate loaded core-b is shown in Fig. 3.5. The feasibility of incorporating drug in core-a and/or in the envelope was shown as well.

Fig. 3.5: Core-b. Left panel: Due to its structural design, core-b can be assembled with core-a between two envelope sheets to form a ravioli. Right panel: SEM micrograph of a representative sample of PLGA based core-b loaded with diclofenac.

Scaffold Function
Swelling capacity of the ravioli scaffold in aqueous media was demonstrated (Table 3.2). Sizes of 6, 8, 9 and 14 mm were tested with range of core-a range tailored to each size. The swelling increased with an increase in core-a content (see Fig. 3.6). Fast swelling rate was observed (see Fig. 3.7). The rate increased with the decrease in Core-a’s content. However, when the osmolality of the medium was increased, the swelling diminished to 200-400%, depending on the size of the implant.

Table 3.2: Swelling capacity (at saturation) of PU scaffolds with various sizes in aqueous media. Mean ± SD.

Fig. 3.6: Left panel: Ravioli’s swelling after 24h incubation in aqueous media. 14 mm scaffold with various core-a contents. Right panel: swelling dependency on the osmolarity of the medium.

Fig. 3.7: Swelling kinetics of NPmimetic scaffold (ravioli). Mean ± SD (n=3 for each time point).

Scaffold – Cytotoxicity
The cytotoxicity of the scaffold and its components was tested with hMSCs and bNPCs. Results are shown in Fig. 3.8. The scaffold was found to be non-cytotoxic. The envelope formulation was found non-toxic for bNPCs and hMSCs. Final Core-a composition was shown to be cell friendly: It stimulated cell proliferation of hMSCs and did not hamper bNPCs growth.

Fig. 3.8: Cytotoxicity study results. Absorbance value of WST-1 assay normalized to positive control. Bovine NPCs and Human MSCs cultured in conditioned medium of respective material for 24 and 72 hours. Mean + SD, n=3.

Scaffold – Cell Adhesion
High cells adhesion ratio and significant cell proliferation was observed following seeding of bNPCs on the envelope. Results suggesting that the envelope is capable of supporting NPCs attachment and growth (See Fig. 3.9)

Fig. 3.9: Left panel: SEM images of cell-envelope constructs after 1 or 7 days of culture. Scale bar: 500 µm. Middle panel: toluidine blue stained cross sections of cell-envelope constructs cultured for 1 or 7 days. Scale bar; 100 µm. Right panel: DNA content of cell-envelope constructs cultured for 1 or 7 days. Mean ± SEM, n=3.

Scaffold – Chemical Characteristics
Representative results of chemical characterization are shown in Fig. 3.15. The chemical composition of envelope and core-a constructs was validated using ATR-FTIR spectroscopy. The hydration properties were demonstrated. The thermo-mechanical properties of the novel scaffold components were defined using dynamic mechanical analysis. Shelf life studies were designed according to ISO10993:13 for ageing studies. Three data sets were evaluated for stability: components as produced, following ETO sterilization and following 25kGy g-irradiation. The storage conditions tested were 4, 25 and 40oC. Changes in appearance, morphology, chemical identification and mechanical characteristics were monitored. Results showed long term
stability of the scaffold components.

No treatment gamma ethylene oxide

Fig. 3.15: (top left) FTIR-ATR spectra of envelope and core materials, (top right) typical output from FTIR-ATR hydration experiment of Hydromed, (middle left) DMA analyses for envelope materials during shelf life study, (middle right) SEM images of electrospun envelope samples during shelf life study, (bottom left) GPC output for envelope and core materials post sterilisation, (bottom right) statistical analysis of fibre dimension study.

Scaffold – Mechanical Properties

Mechanical properties were evaluated using confined and non-confined mechanical measurements. The swelling behaviour of raviolis with respect to their ability to build up an internal pressure inside a confined volume was characterized. The first mechanical test with the implants was whether the swollen gel could bear mechanical loading. We tested the material in confined compression and compared with natural NP material (see Fig. 3.10). It was found that material could bear the applied pressures very well, but the pressures were an order of magnitude less than what we measured with the natural NP. Thus, there is swelling and load-bearing capacity of the NP substitute, but it does not match the properties of the natural NP.

Figure 3.10: Pressure built up in the swelling nucleus pulposus (left) and implant (right) under confined compression conditions. The implant could swell and bear external mechanical loading very well, but the pressure is 3-4 fold lower than found in a natural nucleus pulposus. Swelling pressure thus is yet below what would be desirable for the implant.

The non-confined tests were conducted in two configurations, both with placing the compression plates in parallel with the ravioli ring and with the ravioli ring perpendicular to the compression plate – see Fig. 3.11.

Fig. 3.11: Direction of load for the two different ravioli’s load tests: Left: ‘Parallel’ test. Right: ‘Perpendicular’ test. The results indicate that in non-confined compression, the scaffold lead with a stress of 5 MPa, which is highly superior to normal stress acting on IVD during a moderate effort. No rupture was observed for the range of physiological values. Biaxial tensile strength test to the envelope demonstrated that, for an engineering strain of 1.5 there was no rupture on envelope samples (see Fig. 3.12)

Fig. 3.12: Biaxial tensile tests of envelope material. Left: Test rig with envelope sample. Right: Graphical representation of the test results.

Ex vivo testing of ravioli implants

In recent years, many attempts have been made to access the NP through an artificially created opening and consecutive closure of the AF. However, the lack of adequate closing techniques allows implants to migrate, and accelerates disc herniation, degeneration, and the development of reactive changes to the endplate. In order to bypass the need to make a hole in the AF, an alternative, transpedicular route has been developed, accessing the NP through the vertebral body without damaging the AF (Fig. 3.13). We used this novel surgical approach for implantation of the NP substitute in goat lumbar spines.

Figure 3.13: schematic illustration of the applied surgical approach (according to Vadalà et al, Spine (Phila Pa 1976) 2013;38(6):E319-24): a transpedicular approach into the NP is feasible for sheep and goats.

Fig. 3.14: Biomechanical behaviour of healthy, damaged and repaired discs. In most loading conditions, a slight and sometimes significant repair of stiffness and range of motion was observed, but the implants did not result to a normalisation of disc behaviour. This we attribute to the insufficient swelling of the implants as discussed above.

Segments were serially tested in three testing conditions: 1. native IVD; 2. after a transpedicular-approach nucleotomy; 3. after implantation of the NP substitute and closure. Per testing condition, specimens were tested in three directions: flexion-extension, right and left lateral bending, and right and left axial rotation. It was shown that it is surgically feasible to place the
implant via the endplates, but the implants did not swell sufficiently to build up proper pressure. Consequently, the biomechanical properties of spinal segments were not properly restored, although improved compared to the punctured degenerated IVDs (Fig. 3.14).

Scaffold clinical aspects

Addition of an anchor to the ravioli scaffold was suggested for easier handling during implantation and optimal use. Two prototypes were presented and shown to be feasible for clinical employment (See Fig. 3.16)

Fig. 3.16: Two prototypes of ravioli shaped scaffold with anchor.

A delivery system to the ravioli/NP mimetic scaffold was developed. Special assembly procedure was established to enable insertion of 6 and 9mm raviolis through 2.8mm ID needle. A special needle with insertion guide was developed according to the product specifications. A demonstration of the insertion and scaffold function (measured by swelling capacity) is shown in Fig. 3.17. The same assembly methodology can be applied for insertion of 14mm raviolis in 5 mm Ø needle.

Successful clinical application of the biomimetic scaffold requires the option to control its position following implantation and ensuring its targeting to the NP space. Therefore, the feasibility of incorporating in the raviolis an imaging material was evaluated. Barium sulfate was selected as the radiopaque agent. Relatively high amounts of barium (20-50% by weight), were incorporated using core-a as the carrier component. Barium sulfate containing raviolis were demonstrated to enable imaging and positioning of the scaffold in the target site (see Fig. 3.17). The optimal amount for imaging was shown to be 50% (w/w).

Fig. 3.17: Left panel: Ravioli delivery system: A demonstration of the scaffold insertion and swelling function. Middle panel: representative X-ray images of barium sulfate containing ravioli shaped scaffold before (top) and after hydration (bottom). Right: after placement in the lumbar spine. Arrows indicate ravioli implantation positions in the intervertebral discs.

4. Development of a virtual model of the IVD, and in silico integration of NP mimetic consortium findings

Image-based tool for 3D parameterization

Within the intervertebral disc (IVD) high-resolution medical image-based tool for 3D parameterization, a sound ‘patterns recognition’ process was developed, able to identify, smooth and sample the soft/hard bio-structures (i.e. bone, annulus fibrosus and nucleus pulposus) followed by the modeling of boundaries and interfaces, through a 3D voxel/pixel data processing, in order to produce a coherent geometrical description and an optimized finite elements meshing set of the various ‘regions’ previously identified – Fig 4.1.

In spite of been the most straightforward procedure to obtain a ‘tailored’ model for further high level analysis, the method is strongly dependent on technical constraints, namely in what concerns to in vivo accessibility and the native resolution of the source medical imagery for soft biological tissues.

Contact model for hyperelastic surface interaction

a) The ‘natural’ lumbar spinal motion segment

On the virtual simulation of the IVD, partial differential subroutines based on the development of non-linear equations (and subsequent analysis and quantification of the derivative parameters, for a sound description of the hyper-viscoelastic characteristics of the bio-materials involved) where implemented on a specifically built finite elements method (FEM) iterative framework, in Fortran® programming language.

The parameterization of the viscoelasticity characteristics of both nucleus and annulus was validated using VUmc experimental data, obtained via DMA tests (for goat nucleus), and several others available published human in vivo data. A major assumption, based on the similarity between NP bulk and AF matrix materials was extensively confirmed, as well as a precise valorization of each one of the materials’ viscous contributions to the overall dynamic response of the motion segment – Fig. 4.2 – through the FEM model.

Furthermore, a standard L1-L2 motion segment was built with special care into retrieving and emulating viscoelastic behavior of both NP and AF matrix materials and the osmotic pressure development due to NP swelling phenomena, as well as the fibers mechanical characteristics and their three-dimensional 3D complex structure within the AF – Fig2..3 (making predictable the
b) The prosthetic implant for NP pressure restoration

The FEM modeling of the voxel-based geometrical model of the prosthetic implant (a discoid shape, composed of a swelling core and a wrapping envelope, circumferentially closed by thermal welding) was based on experimental mechanical data in what concerns to the ‘envelope’, the core and the all set together, by a re-engineering procedure that implied the tuning up of the different parameters involved in the swelling/internal pressure development, to fully parameterization and suitably emulation of both implant’s free and confined behaviors – Fig.4.4.

c) The ‘IVD with prosthetic implant’ model

Finally, the “IVD with implant” model was created, by replacement of a given original NP volume by the ravioli’s model developed, in an osmotic pressure swelled state – Fig. 4.5 – suitable for a comparative study of the influence on the mechanical behavior and performance of the set (as well as to access the loading and stress demands over the implant), for a given number and/or location of the implants within the nucleus.

Three - stages (FEM, MBS & Hybrid) virtual simulation

a) The FEM numerical analysis

An extensive parametric study was developed, in order to access and evaluate the sensitivity of the IVD to geometric variability (such as the sagittal wedge angle between the vertebrae, axial average thickness, coronal length/width ratio and NP/AF relative position), so that the model can suitably emulate the relevant geometrical/mechanical variations correspondent to the different levels of the lumbar spine.

Also a throughout search on published experimental data of the main ligaments on the lumbar spine was carried out, to select pertinent data and use it in the evaluation of their interference on IVD’s performance. This implied a deep search of all available data on the viscoelastic properties of the main ligaments of the motion segment, to qualify and quantify the most relevant parameters of these structures.

In that process it was possible to conclude that at least ALL and PLL could not be discarded from an accurate IVD analysis, due to their anatomical location that implies a restraining effect on the external walls of the AF – Fig.2.6 – and the pre-tensioning (pre-loading) induced in the motion segment, which deeply influence the performance of the all set.

The mechanical characterization and parameterization of the ligaments was extended to all the structures, in order to complement the MBS model, in which they were incorporated. In due course, the AF restraining effect is carried out by the FEM model, while the MBS model takes into account the pre-stretching and stabilization effects between motion segments (it worth noting that this process showed to be substantially profitable in terms of computer processing time).

The motion segment models were then subjected to an extensive sensitivity analysis, in order to evaluate the influence of the internal pressure development – induced by the ligaments stretching and their pre-loading effect over the motion segment, but also by the osmotic peculiar behavior of the NP and the AF, as these regions present different swelling gradients even for static states – and the overall ‘macro’ mobility behavior, for any of the 3D/6DOF possible loading cases.

Finally, a validation procedure against experimental data revealed that the model can be considered a reliable solution – an example, for plain flexion, is shown in Fig 2.7.

b) Development of the MBS dynamic model

The multibody systems (MBS) dynamic model consists of the entire lumbar spine segment, involving 6 solid mass bodies (L1 to L5 and sacrum) interconnected by specially designed non-linear spring/damper sets (emulating the IVD 3 translational and 3 rotational degrees of freedom), the anterior and posterior longitudinal, flavum, inter and supraspinous ligaments (5 translational non-linear spring joints) plus the articular spinous processes (2 null distance separators), representing a total of 30 DOFs and 65 constrains – Fig. 4.8.

The model was developed with a commercial package, introducing the IVDs emulation response (taken from FEM results for solicitations in the 6DOF, tuned up for each IVD level along the column segment), the ‘ligaments’ mechanical characteristics, gathered from a carefully curated selection among experimental published data, and the facets, located in position and angle from an extensive set of medical imagery.
It showed to be a fully operational tool, cinematically stable and computationally light, suitable to access the overall motion kinetics analysis. Its validation, against several published experimental works, displayed a very good correlation within the normal physiological ranges of motion.

c) Development of an hybrid FEM/MBS model

Evaluation of the mechanical behavior, at each IVD level and at the all the lumbar segment has been performed, in order to validate the models data interchange and mechanical stability, prior to validation against available in vitro and in vivo experimental data.

Sample tests demonstrated the potential of using this MBS/FEM interconnection, namely by the use of the MBS to produce a given global situation and, then, to introduce the individual (i.e. at a given level) force/torque data into the FEM, to access the performance conditions of the NP ‘under work’ (i.e. emulating common daily activities) and analyze the behavior of the ‘internal’ IVD structures.

From these simulations it was possible to verify that the model gives sound data on the loading conditions of each motion segment, showing that remarkable variations can occur not only in the values reached at each one of the levels, by themselves, but also in the various forces and torques that can be generated, even for simple loading cases, as illustrated in the examples of Fig. 4.9.

5. Validation (animal models/clinical view) of the proposed developments for medical application

As indicated above, the NPmimetic project realised that clinical treatment is different for patients with mild and severe disc degeneration. In mild degeneration the surrounding AF is more or less intact, which means that it suffices to “re-inflate” the NP with a gel, possibly enriched with cells and/or growth factors (biological reconstruction/ regeneration). In severe degeneration, on the other hand, both NP and AF are damaged and need to be replaced (mechanical reconstruction). In the previous sections, the development of both types of NP mimetic substitutes for restoration of IVD functionality in in vitro and ex vivo studies were described, as well as the technologies and tools to deliver these to the NP. Here, we will describe the in vivo assessments of both treatment modalities in large animal studies, i.e. in goat IVDs.

a) Biological reconstruction/regeneration of the NP (mildly degenerated IVDs)

Using our previously validated goat IVD degeneration model, several studies were performed to evaluate the safety and efficacy of the nanobiopolymeric fibrin-hyaluronic acid (FBG-HA) conjugated hydrogels, either or not combined with transglutaminase (TG)-coupled BMP-2 or BMP2/7 osteo/chondrogenic growth factors and/or freshly isolated adipose stem cells (ASCs). In brief, mild IVD degeneration was induced chemically with Chondroitinase ABC, after which intradiscal injections of the various (mixtures of) compounds was performed. After a follow-up time of twelve weeks, safety and efficacy were assessed using multiple outcome parameters (radiography, MRI T2* mapping, and biochemical and histological analyses).

Animal study 1 (Pilot study with FBG-HA hydrogels +/- ASCs +/- BMPs)

In a first study (7 goats), mildly degenerated lumbar IVDs were randomised over five intervention groups: (i) untreated (negative control); (ii) hydrogel alone; (iii) hydrogel with 200 ng/ml covalently conjugated bone morphogenetic protein (BMP) 2; (iv) hydrogel with a fresh adipose stem cell (ASC) isolate containing 106 nucleated cells; and (v) hydrogel with both the BMP-2 and the ASCs. Another lumbar level which was non-degenerated served as a positive control. After sacrifice, at 3 months follow-up, lumbar spines were harvested and analysed as described above.

It was found that in the ASC treatment groups (iv and v), an inflammatory response was observed in 83% of cases, associated with severe degenerative changes on all parameters. Radiographs showed holes in the involved endplates; on MRI the NPs could not be distinguished from the surrounding AFs. Macroscopically, massive scarring was observed, including endplate destruction and osteophyte formation (figure 5.1). Histological scores deteriorated from 1.7 with hydrogel only and 2.1 in the no intervention group, to 4.1 in the ASC groups (scale 0-6).

Fig. 5.1. Two IVDs after injection with Core C and SVF (top) and two IVDs after injection with Core C, SVF and BMP-2. Endplate destruction and osteophytes (top right) can be observed.

In the groups without the ASCs, the MRI index was significantly higher in IVDs treated with BMP-2 when compared to hydrogel alone (figure 5.2A) and significantly lower in the hydrogel group than in control IVDs. Glycosaminoglycan (GAG) content analysis showed the same tendency throughout the groups, yet these differences did not reach statistical significance (figure
5.2B). Hydroxyproline content remained relatively constant between the groups, representing an unchanged amount of collagen in the NP (figure 5.2C).

Fig. 5.2: MRI index in % of healthy level (A), GAG (B) and hydroxyproline content (C) in μg / mg dry weight plotted per intervention group (n = 7). Data plotted as mean and SD (*p < 0.05; **p < 0.01).

The broad range of degenerative states within the various treatment groups allowed us to evaluate a recent MRI mapping technique, T2*, reported to be a sensitive diagnostic tool for monitoring early IVD degeneration. Moreover, we correlated T2* mapping with GAG content and histological features, which was not performed before. Briefly, the IVDs from the study described above, supplemented with the lumbar IVDs from two healthy goats, were examined using sagittal standard T2-weighted and T2* mapping MRI protocols at 1.5 Tesla. Regions of interest (ROIs) were drawn on the T2* maps, covering the IVD. Based on T2 weighted MRI, discs were morphologically classified using the Pfirrmann score. The other analyses were as described above.

Fig. 5.3. Scatter plots and linear regression lines of T2* (ms) vs. GAG content (μg/mg dry weight; A), macroscopic (B), histological (C) and Pfirrmann grade (D)

The mean GAG content in the NP was 450 μg/mg dry weight (range 20-730 μg/mg dry weight) and the mean histological grade was 2.2 (range 0-6), corresponding with relatively mild disc degeneration. A linear positive correlation was observed between T2* and NP GAG content (r = 0.65 p < 0.001; figure 5.3A). T2* in the NP decreased linearly with increasing degeneration as assessed with macroscopic (p = 0.33 p < 0.05; figure 5.3B) and histological (ρ = -0.45 p < 0.05; figure 5.3C) grading, as well as with the Pfirrmann scoring system (p = -0.67 p < 0.001; figure 5.3D).

From this study, we conclude that:
• Fibrin-hyaluronate hydrogels, both alone as well as conjugated with 200 ng/ml BMP-2 appeared safe
• Our results suggest a regenerative effect of covalently conjugated BMP-2 compared to hydrogel alone
• Possibly, increasing the dose of BMP-2 could enhance this regenerative potential.
• We attribute the observed negative effect of injected hydrogels compared to controls to either a lower water-binding capacity of the hydrogel than native NP, affecting the MRI index, or decreased damaging effects due to lesser AF injection in the control IVDs
• The observed inflammatory response in the IVDs injected with freshly isolated ASCs may be caused by the combination of different chemical agents and/or cells (e.g. monocytes and erythrocytes) in an avascular, cell-hostile environment. ASCs alone and residual enzyme levels are less likely the cause of inflammation, as tested and proven safe in previous studies. Freshly isolated ASC preparations were applied successfully before in articular cartilage and bone regeneration studies; however, we discourage freshly isolated ASCs for IVD regeneration as long as the cause of the adverse effects reported here remain to be elucidated.
• T2* mapping, an MRI technique for IVD evaluation allowing measurements on a continuous scale, showed a relatively good and linear correlation between T2* relaxation time and accepted parameters of disc degeneration. We suggest that T2* mapping is a promising tool to assess disc degeneration in clinical practice.

Animal study 2 (Dose-response study for IVD regeneration using BMP-2 and heterodimer BMP-2/7)

Based on the pilot study, it was decided not to proceed with stem cell supplementation, and to increase the growth factor dosages. Moreover, based on our in vitro evaluation in section 2 of this report and a recent rat calvarial defect study from our group, we also included recombinantly produced TG-BMP-2/7 heterodimers in this study, since the latter appeared more efficacious compared to the BMP-2 homodimers. The experimental setup was similar to the pilot study, however, treatment groups consisted of either FB/HA-hydrogel only, or supplementation of the hydrogels with 1 or 5 μg/ml of either BMP-2 or BMP-2/7.

In line with the pilot study, no adverse effects were observed, and significant disc height loss upon degeneration induction was found (figure 5.4). MRI T2* mapping showed strong and significant correlations with biochemistry and histology (figure 5.5). Surprisingly, no differences could be demonstrated in any parameter between interventions groups (table 5.1).

Fig. 5.4: Disc height index of IVD before and after injection with CABC. Data plotted as mean ± SD, ** p =0.002.
Fig. 5.5: Correlations between a) T2* and GAG (ρ = 0.76 p < 0.0001); b) T2* collagen (HYP), (ρ = -0.61 p < 0.0001); and c) T2*and histology (ρ = -0.53 p = 0.0002)

Table 5.1: Overview of analyzed parameters for each experimental intervention for the NP.

From this study, we conclude:
• Fibrin-hyaluronate hydrogels, both alone as well as conjugated with 1 or 5 µg/ml BMP-2 homodimers or BMP-2/7 heterodimers appeared safe
• No difference could be demonstrated in any parameter between intervention groups, and no disc regeneration was observed, while MRI T2* mapping showed strong and significant correlations with biochemistry and histology.
• Possible explanations are too low dosages, absence of notochordal cells (present in all currently used models but not in goats and humans), short follow-up time, and/or insufficient release of the conjugated BMPs.
• These aspects should be addressed in future studies.

b) Mechanical reconstruction/NP replacement (severely degenerated IVDs)
In order to investigate the surgical feasibility and the in vivo performance of the ravioli NP substitute, two of them were placed in the lumbar discs of an adult female Dutch milk goat. They were implanted via a transpedicular approach (figure 3.13). Under fluoroscopic guidance, five lumbar levels were approached; three received NP substitutes containing 25% barium sulphate after which the vertebral tunnels were closed using polyurethane plugs (4mm diameter, 1cm length) and bone cement (PMMA). Negative controls consisted of implanting either an isolated polyurethane plug, or a plug supplemented with PMMA, respectively.

The transpedicular approach and subsequent implantation of the NP substitute appeared feasible; correct placement of the implant was radiographically confirmed during the procedure (figure 5.6; left panel). The goat regained normal ambulatory activities on the second post-operative day and no adverse events were recorded during follow-up. After sacrifice, however, the MRI of the lumbar spine showed migrated NP substitutes, into the caudal vertebral body where the plug and bone cement were placed to close the hole (figure 5.6; middle and right panel). Therefore, the specimens were not amenable to ex vivo mechanical testing.

Fig. 5.6: Left panel: Fluoroscopic image of the goat lumbar spine showing proper ravioli placement (dark spots within the IVDs, indicated by white arrows) during surgery, and two MRI images one month after implanting the nucleus pulposus substitute. Middle panel: Ravioli receded into the vertebral tunnel, well below the disc space above. Right panel: transverse section at the level of the green line, showing the implant (white) within the contours of the vertebral body.

From this pilot study, we conclude:
• The transpedicular approach appears feasible
• However, the closure of the vertebral tunnel with the plug/PMMA combination appeared insufficient, since the ravioli’s receded in the entrance tunnel, i.e. penetrated the vertebral endplates (figure 5.6).
• We conclude that the swelling capacity of the NP substitute was not sufficient to build up the natural pressure of the nucleus pulposus, and that the placement of the implant inside the intervertebral disc is quite challenging. These challenges have now been identified and will help to enter the next phase of development towards a second generation of the NP mimetic.

c) Summary, and clinical perspective
The degenerating IVD is the economical and clinical concern of this project. The current clinical practice faces a big gap between conservative treatment (e.g. physiotherapy, pain killers) and the well-established but highly invasive interbody spinal fusion, in which a bony bridge is created between two vertebrae. The NP mimetic aimed to provide novel concepts for mild and severe disc degeneration which are less or minimally invasive and restore disc functionality rather than replace the disk with a rigid solution that eradicates its primary function.
The NPmimetic project realised that clinical treatment is different for patients with mild and severe disc degeneration. In mild degeneration the surrounding AF is more or less intact, which means that it suffices to “re-inflate” the NP with a gel, possibly enriched with cells and/or growth factors (biological reconstruction/ regeneration). In severe degeneration, on the other hand, both NP and AF are damaged and need to be replaced (mechanical reconstruction). This consortium concentrated on both types of NP mimetic substitutes for restoration of IVD functionality, with a special focus on nanotechnology based strategies. Moreover, we also focused on the proper delivery of these new treatment modalities, developing novel administration routes and delivery tools, and on evaluation of innovative, more accurate ways to assess the degenerative state of the disc.

The current report describes the great amount of sound research work that has been performed to achieve these goals. Rather than empirical “try and error”, the NPmimetic project employed a thorough step-by-step approach with extensive in vitro testing for a large number of relevant parameters, to ensure that the consortium would deliver safe and biocompatible solutions for in vivo animal and clinical application.

Biological reconstruction/regeneration

Extensive efforts were made to synthesize, characterize, and validate the innovative fibrinogen-hyaluronic acid (FBG-HA) nanobiopolymer conjugates for treatment of the mildly degenerated IVD. Moreover, the production of the hydrogel was upgraded to a GMP-conform level within the project. Successful covalent coupling of recombinant chondrogenic growth factors to the FBG-HA hydrogel conjugate was proven, with maintenance of biological activity upon release by plasmin cleavage. In this regard, stronger biological activity was found for a newly developed BMP-2/7 heterodimer compared to the commonly used BMP-2 homodimer. Exposure of cultured NP cells to the conjugates (+/- the growth factors) or injection of these modalities into the NP of complete IVDs was shown to be a feasible and safe approach, both in vitro as in vivo. Efficacy of these growth factors to enhance the NP cell phenotype, measured as an anabolic response, was well demonstrated in in vitro studies and ex vivo organ cultures, but as yet not shown upon injection in mildly degenerated goat IVDs. Further studies are needed to evaluate the discrepancy between the in vitro and in vivo data, focusing on (i) increasing the dosages of the growth factors in the goat studies; (ii) the level of (or absence of) plasmin activity within the NP of the IVD, necessary to release the growth factors from the hydrogel; and (iii) the absolute necessity of this tightly controlled release system for growth factor delivery to the NP, given the strong containment and thus leakage preventing function of the IVD. An in vivo study in which freshly isolated mesenchymal stem cells from adipose tissue (ASCs) were evaluated for additive regenerative potential turned out negative, likely due to inflammatory factors and/or haematopoietic cell types present in the fresh isolate not normally encountered by the avascular IVD. The consortium decided not to pursue this further within the time course of the project, but we are confident, based on other studies both from some of our consortium partners as well as other published studies, that cultured instead of freshly isolated mesenchymal stem cells may well be combined and effective with the conjugates, either or not in combination with growth factors.

Mechanical reconstruction/NP replacement

Using a systematic and sophisticated nanotechnological approach, the NPmimetic consortium has developed and established a highly promising NP replacement strategy. The concept encompasses a strongly swelling and load-resistant NP implant (“ravioli”), that meets the many requirements identified for NP replacement: (i) polymeric building blocks and raw materials comply with regulatory requirements; (ii) the core material exerts strong swelling pressure; (iii) the envelope shows suitable mechanical strength and efficient wettability; (iv) absence of cytotoxicity and biocompatibility of the ravioli, and cell adhesion and proliferation on the envelope material were shown; (v) a proof of concept for drug containment potential and its controlled release was shown; (vi) mechanical properties, tested under confined and non-confined conditions, showed that the ravioli implants could resist forces well above physiological and peak loading without rupturing.

In organ culture models and in an in vivo pilot study, the consortium employed delivery of the implants via a transpedicular endplate approach, in order to avoid damage to the AF and potential subsequent herniation via the AF as much as possible. Although the transpedicular approach was shown to be feasible, swelling pressure buildup as well as implant retention in the NP space in time were yet insufficient. In the goat pilot study, the ravioli’s were found to be located in the drill holes in the vertebral bodies at 1 month follow-up, despite filling these holes directly after ravioli delivery in the NP space with polyurethane plugs and PMMA cement.

Clinical perspective

From the above, it is concluded that a mayor progress has been made in developing novel treatment concepts in treating mild
and severe degeneration.

From the clinical point of view, the biological regeneration approach appears feasible and safe, and readily applicable in clinical practice once efficacy is accomplished as well. The co-developed 3-needle minimally invasive delivery system (Fig. 5.7) enabling of mixing and injecting the components of the hydrogel and bioactive moiety at the same time while avoiding iatrogenic injury to the AF, is an important additional asset. Intervention radiologists already indicated strong interest in the needle system. Considering the high intrinsic pressure of the NP, a further advancement may be the option to introduce the hydrogel mixture with increased pressure using for example an injection pistol.

Fig. 5.7: Outline of minimal invasive percutaneous delivery system components for biological regeneration. Left panel: dual-syringe mixing unit. Middle panel: 3-needle system consisting of (i) an introduction needle to cross the skin; (ii) a second introduction needle to access the lateral side of the AF without violating it; and (iii) an injection needle to cross the AF laterally to reach the middle of the NP.

Challenges certainly remain: the adequate assessment of the actual degenerative state of the IVD is still a clinical limitation nowadays, although new MRI methodologies are rapidly advancing, as also evaluated by this consortium (i.e. T2* mapping).

Also, creating a proper microenvironment for regeneration should take multiple aspects into account at the same time, such as the avascular nature of the IVD, the local pH, the cellular quantity as well as quality, control of inflammation and cytokine levels, the appropriate level of mechanical loading, etc. Last but not least, one should always consider that animal models may resemble, but will never absolutely mimic the actual human situation, thus extrapolation of animal findings to clinical practice may be extremely difficult. Nevertheless, The research conducted within the NPmimetic project has provided multiple tools and new insights to address these issues, and has generated important new clues to advance therapeutic options for mildly degenerated IVDs, which may ultimately result in an earlier and thereby maybe more effective intervention and thus prolonged maintenance of Quality of Life and working capacity for patients with, or at risk for low back pain.

For more severely degenerated IVDs, simply “re-inflating” and reactivating the disc’s regenerative potential will not be sufficient, and mechanical reconstruction restoring the internal NP pressure should be the method of choice. The NP mimetic project developed an innovative implantable NP replacement (“ravioli”), with a strongly swelling inner core and a mechanically and biologically stable, biocompatible nanopolymeric envelope. An important additional requirement is that prolapse into the neural canal should be prevented at all times, thus maintaining integrity of the AF as much as possible is crucial. Last but not least it must preferably have capabilities to locally deliver biologically active molecules. Because we know from both personal experience and the literature it has been very difficult to repair tears in the AF, the NPmimetic consortium adopted a transpedicular approach via the vertebral endplate to avoid further AF damage. As indicated above, this approach still needs further optimization, but the preliminary results are encouraging. With the ravioli-shaped NP replacement, made clinically visible with CT imaging by the Barium sulphate incorporation, the project has generated a novel NPmimetic implant which will allow restoration by restoring the intradiscal pressure exerted by the NP, pivotal for the primary function of the total IVD.

The in vivo testing was severely hampered by the unavoidable limitations of the animal model. Dimension of the IVD are similar, but still about 50% smaller in an absolute sense. Thus, the endplate approach will likely be more feasible in the human situation. In fact, the consortium has identified a company (Clariance), that already developed this for the human spine (see figure 5.8).

Fig. 5.8: The Clariance platform access® technology, allowing a percutaneous, transpedicular and transcorporeal access to the intervertebral disc, while respecting the integrity of the AF. Thanks to the curved wire guide and a “Flexodrill” two entrance canals are created, allowing introduction of a shaver device and efficient flushing.

For maintenance of the correct position of the ravioli’s, the ‘anchored” versions may be suitable. An “umbrella”-type addition to the anchor may further reduce the risk of the ravioli dislocation to the vertebral access tunnel. Strong attachment of the anchor to the adjacent vertebral bone needs to be achieved to secure the device. An interference screw like in knee ligament reconstruction can be thought of (figure 5.9).

Fig. 5.9: Ravioli’s with anchors (left) and Fig. 5.10: Placement of two ravioli’s per human lumbar IVD. Left: interference screw to secure the anchors (right) front view. Middle: side view, directly post-implantation. Right: side view, after 24h swelling. Arrows indicate ravioli’s
Recently, a partner in the consortium developed a novel AF glue withstanding rigorous mechanical testing. Thus, delivery of the ravioli through the AF and subsequent closure of the AF entry side with annular glue now also may become a feasible option again. The delivery of Barium sulphated ravioli’s via an annular access was already tested in a pilot study in a human cadaveric spine segment (figure 5.10). Looking at the X rays after implantation indicates that (i) the best positioning is the one where two ravioli’s are side by side, and (ii) co-injection of saline solution resulted in swelling and morphological modifications occur in situ after 24h.

Conclusion
The NPmimetic project generated and evaluated important new technologies and treatment modalities to bridge the big gap between conservative treatment and the well-established but highly invasive interbody spinal fusion. Although further studies are certainly warranted, these new concepts will be less or minimally invasive and restore disc functionality, and may enable prolonged maintenance of Quality of Life and working capacity for patients with, or at risk for low back pain.

Potential Impact:
•• Potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results

Project Output
The NPmimetic consortium has developed biomimetic nano-biopolymer based gels for cell therapy of Degenerative Disc Disease. Electro-spinning technology was exploited to design and develop nano-fiber based, biocompatible, biodegradable, synthetic scaffold that mimic the mechanical (intradiscal pressure potentiating) properties of the native Nucleus Pulposus for immediate and short term treatment. Anti-inflammatory drugs have been carried by biodegradable nano-fibers to be gradually released in situ thus healing and preventing inflammation. Furthermore, the synthetic scaffold has been integrated with bioactive-nano-polymer that highly potent in supporting Nucleus Pulposus cells for long-term cure. Last but not least, a virtual model of the IVD was developed, both to describe the mechanical characteristics required for the NP restoration modalities, and to evaluate possible effects of that implant on the IVD mechanical performance.

The consortium has developed structures that mimic the Nucleus Pulposus extra-cellular matrix (ECM) and provide a wide surface area to be loaded with bioactive molecules and cells. These biologically passive constructs were made of nano-fibers and micro-pores, while pores size and distribution can be fully controlled along with mechanical properties and drug carrying activities. Synthetic nano-fibers have been integrated with a novel bioactive agent: the Hyalunoric acid-protein conjugate (HPL).

NPmimetic took the formulation and production of nano-biopolymers from the laboratory to the scale up phase. On the R&D value chain, this is the first step to building up the emerging nano-bio-materials industries and markets. Biomimetic materials, bio-nano-polymers loaded with active agents and cells will enhance Europe competitiveness in the nano-biomaterials markets. These validated compounds for medical application are of a huge asset to the European scientific, medical and pharma communities, as nanotechnology is at the forefront of the new wave of converging industries & technologies.

NPmimetic outputs are at the frontier of the new bio-nano market for medical applications, and reflect the move from an era of development of bio-nano-materials, and their integration into medical applications. The economic and competitive advantages are obvious.

Breakthroughs:
(1) Potent biomimetic products for Degenerative Disc Disease therapy;
(2) Endorse long-term tissue regeneration; and
(3) Proven concepts of biomimetic nano-biopolymers based gels for minimally invasive NP regeneration and NP replacement therapies
Socio-Economic Impact

Employment

NPmimetic is at the intersection of several key developments in biotechnology and biomedical areas which represent a promising field for growth & sustainable employment in Europe, since it is a high-added value industrial area. Cell therapies are expected to become increasingly vital during the next 10-20 years. NPmimetic has contributed to increase job opportunities & competitiveness in biotechnology, regenerative medicine and pharma industry sectors.

Impact on the Quality of life

Regenerative medicine offers unique opportunities for developing new therapeutic approaches to prevent and treat these debilitating and life-threatening diseases, and new ways to explore fundamental questions of biology. The current optimism over potential stem cell therapies is driven by new understanding of genetics and developmental biology. Gradually, the curative and regenerative potential that lies in harnessing stem cells is being realized. Degenerated and herniated disc treatments are a huge part of the back pain industry. Disc degeneration has the reputation as a chronic and treatment-resistant spinal concern, which has led to the development of some incredibly profitable medical and complementary interventions. Disc degeneration and disc degeneration occur in the spines of at least hundreds of millions of patients and are considered one of the most common of all spinal abnormalities. In fact, they are so common that using the term “abnormal” to describe them is deceptive and inaccurate.

List of Websites:
Project website: http://npmimetic.com

List of partners

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