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# Delivery systems for the treatment of degenerated intervertebral discs

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## abstract

The intervertebral disc (IVD) is the most avascular and acellular tissue in the body and therefore prone to degeneration. During IVD degeneration, the balance between anabolic and catabolic processes in the disc is deregulated, amongst others leading to alteration of extracellular matrix production, abnormal enzyme activities and production of pro-inflammatory substances like cytokines. The established treatment strategy for IVD degeneration consists of physiotherapy, pain medication by drug therapy and if necessary surgery. This approach, however, has shown limited success. Alternative strategies to increase and prolong the effects of bioactive agents and to reverse the process of IVD degeneration include the use of delivery systems for drugs, proteins, cells and genes. In view of the specific anatomy and physiology of the IVD and depending on the strategy of the therapy, different delivery systems have been developed which are reviewed in this article.

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## 1. Introduction

Around 70–80% of the people are suffering from back pain at a certain stage of their life, of whom 15–20% experience protracted pain and approximately 7–8% develop constant chronic pain [1]. Back pain is generally related to pathologies like intervertebral disc (IVD) herniation, spine stenosis, radiculopathy or sciatica [2,3], leading to pain caused by compression and/or inflammation of nerves in the cervical or lumbosacral area where the spine forms the lordotic curve.

In many cases, back pain can be linked to multifactorial parameters like physical characteristics, lifestyle factors, employment conditions, social factors, heredity and ageing [4–7]. It has been demonstrated that back pain is most-often caused by degenerative conditions of the spine, especially degeneration of the IVD [2,8,9].

The IVD is a heterogeneous, fibrocartilaginous tissue located between each of the 24 vertebrae of the spine, which are subdivided in 7 cervical, 12 thoracic and 5 lumbar vertebrae [10]. An illustration of the human IVD is shown in Fig. 1. The function of the IVD is to absorb and transmit shocks and stresses subjected to the spine, as well as to confer flexibility to the backbone [11,12]. The IVD is structurally and biologically diverse, as it consists of three distinct parts. The centre of the disc, the nucleus pulposus (NP), is a highly hydrated gelatinous tissue mainly composed of aggrecan proteoglycan and collagen type II [13]. Except the upper and lower surface, the NP is completely surrounded by the annulus fibrosus (AF), which is mainly composed of organized and oriented collagen type I fibres [14]. The cartilaginous end-plates of the two adjacent vertebrae are in contact with the upper and lower surface of the IVD and allow the transfer of nutrients, oxygen, metabolic products and water [15]. The cell populations present in the IVD correspond to 1% of the total disc volume, and this limited number of cells impedes the synthesis of extracellular matrix (ECM) [16]. The lack of vascularization of the IVD, with capillaries only supplying the outside of the AF and the upper and lower surface of the disc through the end-plates, makes it the largest avascular structure in the body [17]. In addition, the IVD is largely aneural, with nerve endings only reaching the periphery of the AF [10]. Consequently, the nutritional process is strongly impaired which may explain why the IVD is prone to degeneration [18–20].

Disc degeneration is associated with morphological (decrease of disc height, bulging, ingrowth of blood vessels and nerves) [21–23], physical (decrease of water content inside the disc) [24] and mechanical (loss of

flexibility and elasticity) [25,26] changes leading to disc pathology. The loss of IVD function is essentially the consequence of deregulation of the physiology of the disc, which affects the balance between anabolic and catabolic processes [27–29]. This results in down-regulation of ECM synthesis in the degenerating disc [30], with a drastic drop of proteoglycan production in the NP which induces a fall in osmotic pressure of the disc matrix resulting in loss of hydration [13,31]. In addition, alteration and denaturation of collagen in the AF make the tissue less elastic and less tough [32,33]. Moreover, pro-inflammatory substances such as cytokines are produced which trigger an inflammatory cascade (interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )) [34–37] and an augmentation of enzyme activity which is the main cause of ECM degradation (matrix metalloproteinases (MMPs), aggrecanase, cathepsin) [38–41]. Finally, chemical factors are also involved such as an increasing acidity due to the lack of cell nutrition, which compromises cell function and may cause cell death [42,43].

Established protocols to treat back pain consist of physiotherapy and pain medication by drug therapy, which show limited success in the case of chronic back pain. Surgical treatments are the current alternative [44]. Spinal fusion [45], total discectomy [46] and partial nucleotomy [47] are the most common surgical procedures performed to reduce the pain. However, these interventions do not allow restoration of disc function and are associated with drawbacks such as high invasiveness, high risk of recurrence, loss of mechanical properties and degeneration of adjacent IVDs [48,49]. As both non-surgical and surgical therapies are sub-optimal, it is very important to find more-effective therapeutic strategies for the treatment of back pain.

Taking into account the different mechanisms related to IVD degeneration, current research is focused on different approaches for pain treatment and/or reversal of disc degeneration. A common aspect of all strategies is the use of a therapeutic delivery system, either for drugs, proteins, cells or genes. Although many reviews have been published on delivery systems for the treatment of bone and cartilage defects [50–53], delivery systems to treat IVD degeneration have not been reviewed. In this article, we will discuss the non-sustained delivery of bioactive agents and cells, after which an overview will be given on the development and optimization of specific carrier systems for the (sustained) delivery of bioactive agents, cells and genes, in the context of the treatment of IVD degeneration.

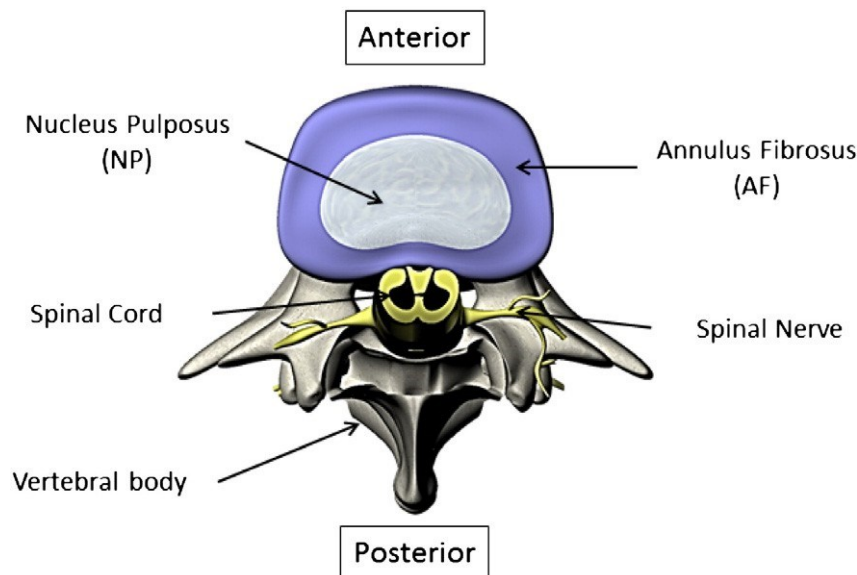


Fig. 1. Illustration of the human IVD anatomy and location.

## 2. Non-sustained delivery of bioactive agents

In advance of surgical intervention, back pain induced by degeneration of the IVD can be relieved or treated by the action of bioactive compounds, which are administered orally, trans-dermally or by injection. A huge variety of therapeutic interventions is available, with different mechanisms of action and various levels of efficiency. The type of therapeutic compound is based on two main approaches: treatment of radicular pain or regeneration of the IVD.

Pain symptoms can be relieved by anti-inflammatory agents such as steroids and non-steroidal drugs, and by opioids and muscle relaxants [54-56]. For regeneration purposes mainly growth factors are being used. This chapter presents an overview of the different approaches and therapeutic agents, which are administered without support of a drug delivery system.

### 2.1. Anti-inflammatory and analgesic agents

Steroids and their specific derivatives corticosteroids are small-molecule hormones in use as clinical treatment for back pain. Steroid solutions are most of the time applied by epidural injection in the inflammatory area via transforaminal, interlaminar and caudal approaches (Fig. 2) [57].

The aim is to bathe the nerve root in the steroid solution which should lead to an immediate and efficient effect. Therefore, steroid injections are mainly being used to treat symptoms of radiculopathy with specific nerve root inflammation [58]. The main corticosteroids clinically used to relieve low back pain are methyl-prednisolone, cortisone, triamcinolone and dexamethasone [59]. Different mechanisms are reported in the literature on the anti-inflammatory action of corticosteroids: inhibition of secretion of inflammatory cytokines and inhibition of other pro-inflammatory substances like catabolic enzymes [60], as well as immunosuppression by inhibition of leukocyte functions [61]. The clinical efficiency of epidural steroid injection for the treatment of back pain is controversial, as both positive and negative results in terms of pain reduction and functional recovery have been reported [62-65].

Besides steroid treatment, another common strategy to treat back pain is based on the use of non-steroidal anti-inflammatory drugs (NSAIDs). These drugs are considered as conservative treatment and belong to the most commonly prescribed oral medications [66]. The basic mechanism of action consists of inhibition of cyclo-oxygenase activity [67].

Several different drugs based on non-steroidal molecules are being used to relieve back pain, such as diclofenac [68], ketoprofen [69], ibuprofen [70], naproxen, celebrex [71], valdecoxib [72] and etoricoxib [73]. NSAID treatment has been shown to be mainly efficient for short-term symptomatic relief in patients with acute back pain [74,75].

Another strategy to treat back pain is based on the use of opioid drugs. Opioids are psychoactive molecules which react with opioid receptors located in the central and peripheral nervous system, resulting in an analgesic effect. Opioid drugs can be administered by oral, parenteral or transdermal ways. Both weak (e.g. tramadol) [76] and strong opioids (e.g. morphine, oxycodone, hydromorphone, hydrocodone, buprenorphine) [77,78] can be discerned. The efficiency of opioid treatment is controversial but seems to be reasonable in patients with chronic non-specific back pain and neuropathic pain [59]. However, the risk of addiction is not negligible and a slow delivery of opioids is preferable compared to immediate release.

Muscle relaxants derived from benzodiazepine have also been advocated for the treatment of back pain. The mechanism of action is not completely known but is probably related to the antispasmodic and antispastic properties of benzodiazepines. Treatment with these muscle relaxants is mainly efficient for the relief of non-specific back pain [79-81].

New lines of medication for the treatment of back pain are currently under investigation, aiming at equal or better efficiencies than the above-mentioned traditional methods without side effects. An overview of potential drugs for the relief of back pain associated with IVD degeneration is shown in Table 1.

Except the compounds mentioned in the last 3 rows of Table 1, all other bioactive agents have been approved by the Food and Drug Administration (FDA) for the treatment of diseases other than back pain. Therefore, the latter agents did not have to enter phase I clinical trials. Most of them are currently being tested in clinical trials to determine their efficacy for the treatment of back pain as compared to established drugs, see Table 2 for registered clinical trials. The compounds in Table 1 without FDA approval, were selected for the treatment of back pain because of their well-documented anti-inflammatory effects in vitro. They are currently being tested in animal models, and have to show significant pain relief versus controls before entering clinical trials. As NF- $\kappa$ B not only mediates inflammatory but also catabolic signalling in the IVD [101], the NF- $\kappa$ B inhibitors are also being tested for inhibition of disc degeneration.

At any stage of IVD degeneration, a pharmacological treatment can be initiated to reduce the inflammatory activity and relieve the pain [55]. A direct epidural injection of bioactive agent seems to be the most efficient and quickest treatment, as it has immediate effect on the inflammatory area. However, although the strategy of direct injection of bioactive agents is currently the most common clinical practice, efficiencies range from moderate to low and there are inherent limitations of the treatment. In addition to determination of the dosage and volume of drug solution, the principal limitations of the treatment are the short duration of activity due to short biological half-lives of the bioactive agents, problems due to repeated injections and insufficient targeting [61]. Therefore, a sustained delivery system should be used

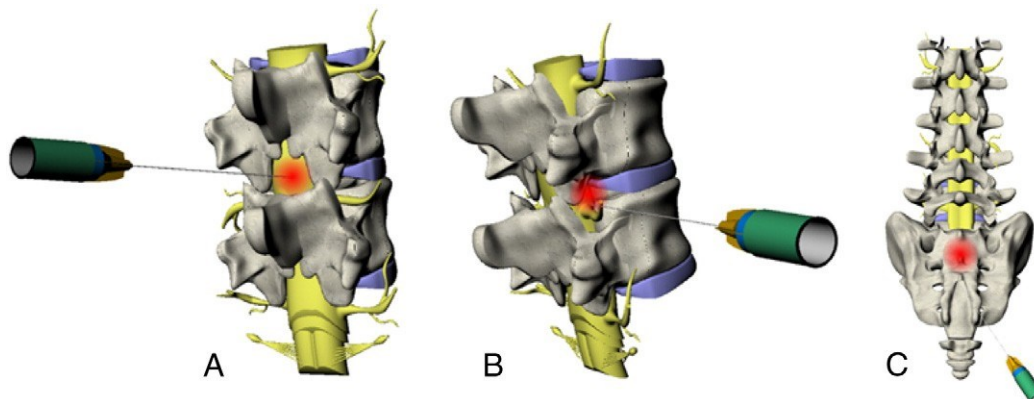


Fig. 2. Epidural injection in clinical practice. A: interlaminar, B: transforaminal, C: caudal approaches. Grey: vertebral body; yellow: spinal nerve; blue: IVD; red: injection area.

Table 1  
Non-conventional drugs for the treatment of discogenic pain symptoms.

Class of drug and way of administration	Drug and FDA approval	Working mechanism	Effective result	Ref.
Tri- and tetracyclic anti-depressant. Oral administration	Imipramine, paroxetine, etc. FDA-approved	Nor-adrenergic and serotonergic activities Inhibition of pain pathways in the brain stem and spinal cord	Relief of neuropathic pain. Neurotrophic effect	[82,83]
Anti-epileptic. Oral administration	Gabapentin, topiramate, pregabalin. FDA-approved	Action on voltage-gated Ca <sup>2+</sup> channels. Enhancement of the function of gamma-aminobutyric acid (GABA)	Relief of neuropathic pain. Neurotrophic effect	[84-86]
Anti-TNF $\alpha$ receptor antagonist. Intravenous or subcutaneous injection	Infliximab, etanercept. FDA-approved	TNF- $\alpha$ inhibition	Attenuation of TNF- $\alpha$ effects. Reduction of inflammation from sciatica	[87] [88]
Anti-TNF $\alpha$ antibody. Subcutaneous injection	Adalimumab. FDA-approved	TNF- $\alpha$ inhibition	Attenuation of TNF- $\alpha$ effects. Reduction of inflammation from sciatica	[89]
Anaesthetic. Wound site injection	Bupivacaine. FDA-approved	Inhibition of ion channels	Long-acting anaesthetic. Reduction of postoperative discomfort	[90]
Adrenergic receptor agonist. Epidural injection	Clonidine. FDA-approved	Activation of type $\alpha$ 2 adrenergic receptors	Relief of neuropathic pain. Anaesthetic effect.	[91,92]
Botulinium toxin. Paravertebral injection	Botox, myobloc. FDA-approved	Deactivation of specific presynaptic protein	Analgesic effect in case of chronic back pain. Improved functional recovery	[93]
Coumarin derivative. Epidural injection	Osthole. Not FDA-approved	Modulation of cyclooxygenase-2 (COX-2) and nitric oxide synthase (NOS) activity	Anti-inflammatory activity in rat model. Reversed hyperalgesia due to herniated disc	[94]
Polyphenol curcuminoid and stibenoid class. Wound site injection	Curcumin, resveratrol. Not FDA-approved	Inhibition of NF- $\kappa$ B, MAP kinases and Toll-like receptors	Anti-inflammatory effect by reduction of IL-1 $\beta$ mediated activation of NF- $\kappa$ B in human IVD cells in vitro and in vivo	[95-97]
NF- $\kappa$ B decoy compound, peptide, oligodeoxyribonucleotide (ODN). Intraperitoneal, intradiscal, epidural injection, resp.	IMD-0560, 8K-NBD peptide, AnGes MG ODN. Not FDA-approved	Inhibition of NF- $\kappa$ B activation	Decreased levels of pro-inflammatory cytokines like IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-8	[98-100]

to prolong the effect and reduce side effects such as toxicity and psychological dependence.

## 2.2. Growth factor therapy

Growth factors (GFs) are proteins secreted by cells to stimulate cell proliferation, differentiation and migration, and to regulate ECM metabolism. GFs bind to specific transmembrane receptors on the surface of cells to induce activation of intracellular signalling cascades [102]. The signalling by GFs can take place in three different ways: paracrine (released by one cell type and acting on another cell type), autocrine (acting on the same cell by which it has been secreted) and endocrine (produced by a specific organ and secreted into the blood circulation).

In addition to development of new therapies for pain relief as mentioned above, recent research has been focused on treatment of the pathogenesis of IVD degeneration. The preponderant role of GFs in IVD physiology has been widely reported in the literature [103,104].

Therefore, the use of GFs as therapeutic agents to treat, reverse or retard the degenerative process is an obvious choice.

Two types of GFs were identified in the IVD, mitogens and morphogens, and subsequently investigated in the context of IVD regeneration. Mitogenic GFs such as insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF) influence the division of cells while morphogenic GFs such as the transforming growth factor beta (TGF- $\beta$ ) superfamily and bone morphogenetic protein (BMP) mainly influence the phenotype and function of cells [105]. Masuda et al. reviewed the effects obtained by the addition or injection of different mitogenic and morphogenic GFs, using different IVD cell types and animal models [103,105,106]. By means of different in vitro culture systems, monolayer and organ culture, it was shown that the above-mentioned GFs stimulate the production and accumulation of proteoglycans, increase the synthesis of collagen, increase AF and NP cell proliferation and reduce apoptotic cell death [106]. The in vivo application of such GFs resulted in a temporary restoration of the height and viscoelasticity of the IVD [107]. In addition, platelet-rich-plasma (PRP) which is a fraction of

Table 2  
Clinical trials to treat degenerative disc disease using non-sustained delivery of bioactive agents or cells.

Trial number	Type of agents or cells	Administration	Phase
NCT00588354	Clonidine vs. triamcinolone	Epidural injection	n.d. <sup>a</sup>
NCT01502644	Oxycodone vs. morphine	Oral ingestion	IV
NCT01694134	Lidocaine vs. hydrocortancyl	Intradiscal injection	III
NCT01052571	Steroid + lidocaine vs. lidocaine	Epidural injection	n.d.
NCT00908375	Pregabalin vs. control	Oral ingestion	IV
EudraCT2006-003564-57	TNF- $\alpha$ antibody vs. control	Subcutaneous injection	II
EudraCT2010-023830-22	Autologous disc chondrocyte transplantation vs. control	Intradiscal injection	I, II
NCT01640457			
EudraCT2010-018335-17	Autologous mesenchymal stem cells	Intradiscal injection	I, II
NCT01860417	Allogeneic mesenchymal stem cells vs. control	Intradiscal injection	I, II
NCT01290367	Allogeneic mesenchymal precursor cells vs. control	Intradiscal injection	II
NCT01771471	Allogeneic juvenile chondrocytes vs. control	Intradiscal injection	II

<sup>a</sup> n.d.: not disclosed.

blood plasma containing high concentrations of mitogenic GFs and cytokines was also used as biologically active substance to stimulate regeneration of the IVD [105].

These studies also demonstrated that the avascular, alymphatic and aneural structure of the IVD might be a suitable site for therapeutic injection. Due to the typical structure of the disc with the confined NP tissue surrounded by the AF collagen fibres, a reasonable amount of biological agent is retained at the site of injection. However, because of AF degeneration and needle puncture during injection, the concentration of the injected compound decreases quickly [108].

Despite the interest in GFs as therapeutic agents for the treatment of IVD degeneration, the therapy may encounter limitations. Due to the different cell populations and low cell density of the IVD, GF therapy is much more challenging for IVD regeneration as compared to regeneration of other tissues such as bone. Moreover, as the degeneration process induces cell apoptosis and down-regulation of ECM synthesis, it was demonstrated that the state of degeneration drastically influences the success of GF treatment [105,106]. In addition, the half-life of GF proteins is relatively short, which may lead to the requirement of multiple intradiscal GF injections. Consequently, the degeneration process may be accelerated [109,110] with a high risk of toxic or undesirable side effects such as ectopic bone formation after direct BMP injection [111]. This is why recent research aiming at GF therapy is mainly focusing on strategies to prolong the residence time of GFs after injection. Thus, sustained delivery systems and gene therapy have been developed to promote the local delivery of GFs and thereby to increase the efficiency of the treatment [104,107].

### 2.3. Cell therapy

Besides the panoply of bioactive agents intended to kill the pain or to induce tissue regeneration, cell transplantation has also been advocated as technique for IVD regeneration [105]. Several cell types have received attention, such as autologous human disc cells derived from therapeutic discectomy [112], autologous articular chondrocytes [105] and autologous mesenchymal stem cells (MSCs) [113]. However, the retention, activity, viability and differentiation of cells directly injected in the IVD are limited and need to be supported by an appropriate cell delivery system [114].

### 2.4. Clinical trials using non-sustained delivery of bioactive agents and cells

Table 2 shows registered clinical trials, retrieved from databases available at [clinicaltrials.gov](http://clinicaltrials.gov) and [clinicaltrialsregister.eu](http://clinicaltrialsregister.eu), on the treatment of back pain and reversal of disc degeneration using non-sustained delivery of bioactive agents or cells.

Except the first trial (NCT00588354), which was terminated because targeted patient enrolment was not reached, all other trials are still ongoing. The outcome of the NCT00588354 trial was that the steroid triamcinolone improved functional recovery of the patient with unclear differences in pain reduction as compared to the adrenergic receptor agonist clonidine [92].

## 3. Sustained delivery systems

Nowadays, it is commonly accepted that the effectiveness of pharmaceutical agents can be greatly improved by controlled delivery with the help of biomaterial carriers. Biomaterials are recognized for their biocompatibility, biofunctionality and bioresorbability in vivo [115-117]. Numerous biomaterials, either from natural sources or synthetic, have been used in clinical applications. Natural materials such as polysaccharides or proteins like collagen [118,119] create a biocompatible environment in vivo due to their analogy to the ECM. Synthetic biodegradable polymers, dominated by the family of aliphatic polyesters like poly(lactide) (PLA), poly(glycolide) (PGA), poly( $\epsilon$ -caprolactone) (PCL) and their copolymers, are used in biomedical applications for their

tunable properties, ease of processing, reproducibility and lack of immunogenicity [120]. The challenge is to find a suitable material which would degrade completely, while sufficiently prolonging drug delivery or facilitating tissue restoration, depending on the application. A smart delivery system for bioactive agents would provide considerable advantages such as a local, sustained delivery of the agent, protection of the loaded compound, improved bioavailability and prevention from systemic side effects. For the treatment of IVD degeneration, sophisticated and innovative biomaterial carriers have been developed to deliver bioactive agents as well as cells. These carrier systems, which include 3D porous or non-porous devices, microspheres and hydrogels are discussed below, with a special interest in minimal invasive approaches.

### 3.1. Devices for the delivery of bioactive agents

Various polymeric devices including pellets, implants or porous scaffolds have been fabricated for the delivery of a variety of drug classes. The design of biomaterials for the formulation into drug carrier or cell support has received a lot of attention for IVD treatment. Drugs can be incorporated in dissolved or dispersed polymer matrices to provide valuable assistance in designing advanced delivery systems.

#### 3.1.1. Polymeric monoliths for the delivery of pharmaceutical drugs

As mentioned above, alternative treatments are needed for steroid and opioid injections. A sustained anti-inflammatory drug release was shown to be more effective than injection to treat the pain caused by radiculopathy. Beall et al. proposed to increase the effect of clonidine treatment by increasing the residence time of the drug in the inflamed area [121]. They investigated the implantation of poly(L-lactide) (PLLA) pellets loaded with clonidine •HCl in the foraminal region in a porcine model, allowing peripheral delivery of the drug resulting in a better treatment outcome.

Zanella et al. investigated the in vivo sustained delivery of the TNF- $\alpha$  inhibitor etanercept incorporated in poly(lactic-co-glycolic acid) (PLGA) mini-cylinders [122]. In a rat chronic sciatic nerve constriction model, the loaded mini-cylinder was implanted near the injured sciatic nerve. It was shown that the activity of the drug increased from weeks to months compared to direct injection. In addition, the sustained delivery approach resulted in a 10-fold higher local concentration of etanercept than reached by repeated subcutaneous injections.

#### 3.1.2. Delivery of bioactive agents from tissue engineering scaffolds

In view of the native structure of AF tissue, restoration of the function and properties of the AF requires the use of biomaterials which are easy to process and able to provide good mechanical properties. Thus, artificial hydrophobic biomaterials such as aliphatic polyesters have been widely used for AF tissue engineering [14]. Very limited studies have been reported, however, on the use of scaffolds in combination with the release of bioactive agents for the regeneration of AF tissue.

The main technique to prepare scaffolds for AF tissue engineering in combination with drug delivery is electrospinning. The use of a biomimetic scaffold made by electrospinning is the most straightforward approach to mimic the oriented collagen fibre structure of the AF [123, 124]. Vadala et al. proposed to use a bioactive electrospun PLLA scaffold containing TGF- $\beta$ 1 (Fig. 3A), that was shown to stimulate IVD cell proliferation as well as collagen and glycosaminoglycan (GAG) production [125]. In vivo, the half-life of TGF- $\beta$ 1 activity after direct injection into tissue is no longer than 2-3 min. Therefore, repeated injections are necessary which may provoke adverse side effects including toxicity. Analysis of the release profile of TGF- $\beta$ 1 incorporated in the PLLA electrospun matrix showed a sustained delivery with a cumulative release reaching a plateau after 7 days. As described in the review by Pham et al. [126], the electrospinning process is affected by the drug concentration. By increasing the GF concentration in the spinning solution, the viscosity of the solution decreases whereas the charge density

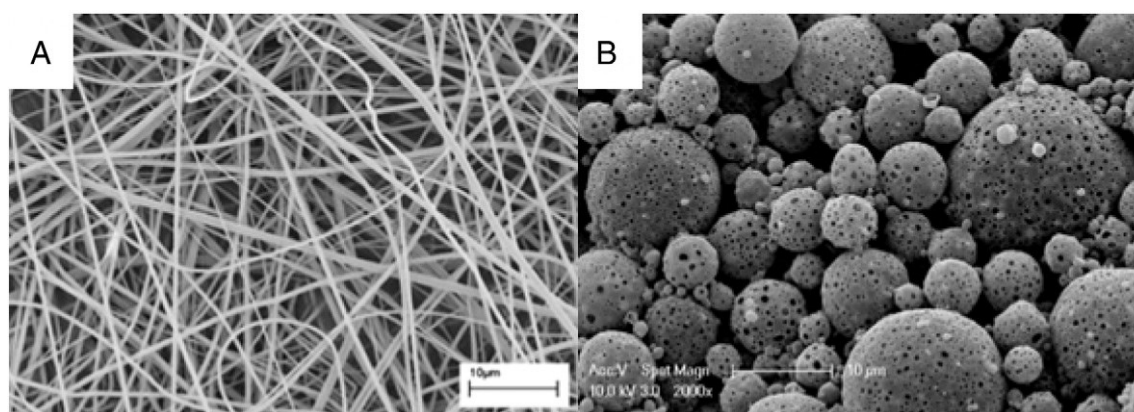


Fig. 3. Scanning electron microscopical images of an electrospun PLLA scaffold loaded with TGF- $\beta$ 1 (A), and PLGA microspheres loaded with IL-1Ra (B). Reproduced with permission from [125] and [134], respectively.

increases, leading to the production of fibre with a smaller diameter and a larger total surface area, and therefore faster release kinetics.

The sustained release of anti-microbial agents was investigated in order to avoid drug resistance due to repeated injections. Surgical procedures like discectomy are often followed by infection of the site of operation [127]. Bao et al. investigated the incorporation of berberine into PCL electrospun scaffolds [128]. Berberine has been reported to have a strong anti-fungal and anti-bacterial activity [129]. Incorporation of berberine in electrospun PCL scaffolds resulted in a broad-spectrum anti-microbial activity sustainable for 33 days. As described above, the release rate was found to decrease with increasing fibre diameter, but also by reducing the solubility of the drug in water by decreasing its ionic strength.

Although a number of approaches to regenerate AF tissue by means of 3D scaffolds other than electrospun scaffolds have been reported, none of them were combined with sustained delivery of active compounds [14]. Currently, our group is developing a biomimetic poly(trimethylene carbonate) (PTMC) scaffold with designed and controlled pore characteristics built by stereolithography [130,131]. A viable strategy to increase the efficiency of AF regeneration would be the combined application of this PTMC scaffold with the sustained release of appropriate GFs such as TGF- $\beta$ 3 or FGF-2 [132,133].

### 3.2. Delivery of bioactive agents from injectable microspheres

Encapsulation of bioactive agents in microspheres facilitates an accurate and controlled delivery of the active compounds [135,136]. In addition to advantages like prolonged and protected biological activity, lower systemic drug concentrations and decreased administration frequencies, these systems offer the possibility to adapt the release profile of the drug. Moreover, administration is carried out by injection, which is less invasive than surgery needed to place a scaffold in the desired location.

Inflammation not only plays a role in the generation of pain, but also in the progression of disc degeneration. Non-sustained corticosteroid injections do not show any effect on cell and matrix catabolism but have clinically demonstrated some pain relief after repeated injections. To prevent the need for multiple injections, sustained release of corticosteroids is widely suggested in the literature. To this end, ceramic microcapsules have been used as corticosteroid carriers. Although ceramics are commonly used as material for drug delivery, engineering in the form of microspheres has not been extensively reported. Ragab et al. investigated the regenerative effect of the sustained release of corticosteroids in a rat traumatized disc model by means of corticosterone-loaded resorbable and non-immunogenic tricalcium phosphate (TCP) microcapsules. The cellular degeneration was significantly reduced by the local injection of the loaded microcapsules [137].

Inhibition of cytokines has also been demonstrated to be a valid approach to reduce both inflammation and disc degeneration. In this respect, interleukin-1 $\beta$  is a key cytokine [138], that can be inhibited by interleukin-1 receptor antagonist (IL-1Ra). Also in this case, the approach is limited by the short biological half-life of the protein inhibitor of around 5 h. Therefore, IL-1Ra was encapsulated in PLGA microspheres (Fig. 3B), after which the effect was investigated in an in vitro model of NP degeneration [134]. To prolong the delivery of the hydrophilic protein, the ratio of lactide to glycolide was chosen in such a way to increase the hydrophobicity of the copolymer. NP degeneration was shown to be inhibited during a period of 20 days.

Another approach which deserves particular interest is the combined delivery of different agents to relieve the pain and to inhibit or reverse the degeneration process. Liang et al. developed PLGA microspheres loaded with dexamethasone and coated with the polycation poly(ethylene imine). The positively charged microspheres were then interacted with negatively charged nanoparticles based on heparin/poly(L-lysine). Subsequently, TGF- $\beta$ 3 [139] or bFGF [140] was bound to the heparin moieties of the nanoparticles. Injection of these dual delivery microspheres in a degenerated disc model in the rat resulted in a significant increase of proteoglycan production and restoration of disc heights to approximately 70% as compared to healthy control animals. The authors also loaded the outside of the dual delivery microspheres with adipose-derived stem cells, but this did not result in an additional effect. It was therefore concluded that the stimulation of proteoglycan production and restoration of disc height were probably mediated by stimulation of host cells or in situ stem cells.

Aliphatic polyesters have been selected for microencapsulation of drugs to treat IVD degeneration or related pain symptoms. Kim et al. investigated the in vitro release of capsaicin from PLGA, PCL and PLLA nano- and microspheres with varying sphere sizes and loading doses [141]. Capsaicin is a hydrophobic drug with potential analgesic properties [142]. The release of capsaicin was shown to be correlated with drug diffusion through amorphous regions of the polymer matrix and not with polymer degradation. Therefore, crystalline PLLA and semi-crystalline PCL microspheres showed a more sustained release profile than amorphous PLGA. Moreover, the rate of capsaicin release increased with decreasing microsphere size, as typically demonstrated in the literature for common drug release from microspheres [135,136].

### 3.3. Injectable hydrogels for the delivery of therapeutic agents and cells

Hydrogels represent an important class of (bio)materials with very wide applicability in biomedical technology and medicine. Hydrogels are networks of hydrophilic polymers capable of absorbing large amounts of water or biological fluids. Hydrogels can be used as matrices to deliver drugs, cytokines, growth factors and even (growth factor-

producing) cells and stem cells in a controlled manner. These biomaterials can be prepared from natural as well as synthetic polymers, and they can display mechanical and structural properties that are similar to those of several soft tissues in the body [143-146]. The crosslinkages in these networks can be covalent in nature (e.g. photo-crosslinked networks) or can be based on physical interactions (e.g. electrostatic and hydrophobic interactions).

In the repair of the IVD, the current focus is on developing injectable formulations that cure in situ to form a hydrogel matrix. A mixture of biologically active molecules or cells in an aqueous solution of the hydrogel precursor can be injected in a minimally invasive manner in the body. After injection of the viscous liquid mixture at the desired location, the hydrogel delivery system is formed upon the occurrence of a sol-gel phase transition at physiological conditions [147,148].

As the nucleus pulposus itself is a gel-like tissue, injectable hydrogel systems have been frequently used to deliver cells and biologically active agents into the disc upon partial discectomy or into the perineural space in a non-invasive way [114,149]. Here we review the different approaches for NP repair, that focus on injectable in situ forming hydrogel systems to deliver bioactive agents or cells in a sustained and minimally invasive manner.

### 3.3.1. Hydrogel microparticles

Hydrogel particles and beads vary in size from approximately 20  $\mu\text{m}$  (microparticles) to 200  $\mu\text{m}$  (microbeads). Initially, the envisaged use of these particles was as a drug carrier that allows targeting, protection and sustained release of the encapsulated drugs upon oral administration or injection [150]. Recent studies have shown that when cells are cultured on hydrogel beads in cell therapy applications, the large surface area of the cell-supporting beads can prevent the problems related to the lack of oxygen and nutrition which the cells can encounter when embedded in traditional hydrogel carriers [151].

Hydrogel beads have been shown to be suitable cell culture substrates to investigate the metabolic activity of IVD cells and to understand the mechanisms involved in IVD cell therapy [152]. Although not intended to be implanted, non-degradable alginate beads have been widely used in vitro to maintain IVD cells in a rounded morphology and to determine the effects of growth factors on the stimulation of ECM synthesis and differentiation of MSCs towards NP cells [153-156].

Based on the studies using alginate beads, biocompatible and degradable gelatin hydrogel microparticles were developed to deliver GFs from PRP after injection into the NP in a rabbit degenerated IVD model. The GFs incorporated in the PRP-containing gelatin microparticles facilitated IVD regeneration by a sustained release as a result of diffusion through the pores of the degrading network [157,158].

### 3.3.2. Hydrogels formed by photo-crosslinking in situ

Photo-crosslinking allows for the in situ formation of hydrogels for the delivery of bioactive compounds and cells [159]. Network formation is based on crosslinking by photo-polymerization upon the formation of free radicals that can initiate radical addition polymerization reactions. A covalently crosslinked hydrogel can be formed when a hydrophilic oligomer or polymer that is functionalized with polymerizable (meth)acrylate groups is subjected to radical polymerization. In situ photo-polymerization of a hydrogel can take place upon injection of a low viscosity aqueous solution of water-soluble macromers with initiator, and crosslinking and gelation can occur upon transdermal illumination via a catheter light guide [160].

One of the most-often used hydrogel biomaterials in NP regeneration is alginate. This polysaccharide is highly hydrophilic and as a rule is physically crosslinked into a hydrogel using divalent cations such as  $\text{Ca}^{2+}$ . These networks are weak and have poor mechanical properties. As an alternative, alginate can be chemically functionalized to allow covalent crosslinking. Chou et al. investigated the potential of photo-crosslinked alginate networks to encapsulate bovine NP cells [161]. Following this in vitro study, the authors assessed the performance of

the network in vivo using a murine subcutaneous pouch model [162]. Comparison between ionically crosslinked and photo-crosslinked alginate not only showed a much higher mechanical stability of the photo-crosslinked network, but also much better encapsulation of cells that resulted in higher amounts of produced ECM.

As already mentioned, alginate is not biodegradable which may lead to problems in NP regeneration at longer times. A possible solution is the use of partially oxidized alginate, yielding  $\text{Ca}^{2+}$ -crosslinkable hydrogels which are degradable by hydrolysis [163]. Alternatively, another biomaterial such as carboxymethylcellulose (CMC) can be used to prepare biodegradable hydrogels [164]. The CMC was functionalized with methacrylate groups, and aqueous CMC macromer solutions of different concentrations were photo-crosslinked to produce a variety of hydrogels in which NP cells [164] and human MSCs [165] could be encapsulated. In these studies, it was shown that the increase in crosslinking density of the network leads to an increase in hydrogel stiffness and a concomitant decrease in swelling and water uptake.

Systems that can be photo-crosslinked in situ possess several advantageous properties, such as fast curing and a minimal invasive approach. However, the crosslinking reactions are exothermic and reducing the amount of heat released is challenging. Also, possible cytotoxicity of the macromers and initiators needs to be taken into account. Furthermore, illumination of the injected macromer solutions containing biologically active agents or cells is hampered by limited space and the difficult access to the disc.

### 3.3.3. Hydrogels formed by reaction in situ

Formation of a hydrogel delivery system upon injecting mixtures of reactive compounds can overcome the limitations of photo-crosslinking in situ, as the cytokines, growth factors and cells present in the mixture are not exposed to potentially cytotoxic photo-initiators and radicals, especially when benign chemistries are used. In addition, the presence of a light source is not required.

As a rule, aqueous solutions of the reactive compounds are mixed directly before injection into the body, and allowed to react to form a hydrogel in situ. This can be done using a double barrelled syringe, equipped with a static mixing head. Network formation can be the result of reversible interactions between the reactants or by covalent bond formation between the molecules. For this, different crosslinking chemistries are used. Examples are enzymatic crosslinking reactions, crosslinking of proteins via genipin, Schiff's base chemistry, Michael-type reactions, or click chemistry. With these chemistries, in situ hydrogel formation can take place under physiological conditions (37  $^{\circ}\text{C}$ , body fluids pH 7.4) and in reasonable time periods (10 to 15 min).

The mild crosslinking conditions make these systems very-well suited for the encapsulation of cells, allowing their delivery to the site of interest. Upon injection in a minimally invasive manner, the in situ formed hydrogels ensure that the cells are maintained within the gel. Subsequently, the cells can be used to produce and deliver biologically active growth factors and differentiation factors or to form new tissue. In regenerative therapy of the NP, this type of hydrogel system is the preferred cellular delivery system. Table 3 gives an overview of the different chemistries used and summarizes the different hydrogels formed for cell delivery to the IVD.

The majority of studies listed in Table 3 concern in vitro experiments using in situ forming hydrogels for regeneration of the NP. Before being tested in animal models, these hydrogel systems should meet the following specifications: have a suitable time to gelation, have a modulus similar to that of native NP or AF tissue, support the viability of IVD cells and production of ECM, be retained at the site of injection (i.e. not leak out of the NP/detach from the AF). Before initiation of clinical trials, animal experiments will have to show evidence of restoration of IVD function. Except histology confirming cell viability and ECM production, these models should show stimulation of disc regeneration (e.g. disc height) and pain reduction.

Table 3

Overview of different chemistries to prepare crosslinked hydrogels and the results of cell incorporation for delivery to the IVD.

Crosslinking chemistry	Biomaterials involved	Encapsulated cell types	Outcome	Ref.
Enzymatic crosslinking (horseradish peroxidase HRP)	Hyaluronic acid (HA), Poly(ethylene glycol) (PEG), Pentosan polysulphate (PPS)	Human mesenchymal precursor cells (MPCs)	Cell viability in vitro. GAG and collagen type II production. Unconfined compressive moduli close to that of human NP Hydrogel tolerated upon subcutaneous implantation in rat model	[166,167]
Enzymatic crosslinking (trans-glutaminase)	HA, Collagen	Bovine calf NP cells	Cell viability in vitro. NP phenotype maintained (high amount of GAG). Resistance to hydrogel volume reduction during cell culturing	[168]
Click-chemistry	HA, Type II atelocollagen, 4N-hydroxy-succinimidyl (NHS)-star PEG	Rabbit adipose stem cells (ASCs), Bovine calf NP cells	Cell viability in vitro. Rounded NP cell morphology. Collagen type I expression maintained. Stable hydrogel in cell culture	[169]
Schiff's base reaction	Oxidized HA, Gelatin, Adipic acid, Dihydrazide	New Zealand white rabbit NP cells	Cell proliferation in vitro. Aggrecan and collagen type II production. Shear moduli close to that of human NP.	[170,171]
Michael-type reaction	Laminin-111, PEG	Porcine NP cells	Cell viability and NP cell retention in IVD organ culture. Dynamic shear moduli close to that of human NP	[172]
Michael-type reaction	Thiol-modified HA, PEG diacrylate, Elastin-like peptide (ELP)	Human NP cells	Cell viability and NP phenotype maintained in vitro. Confined compression improved by ELP addition in vitro. Inhibition of disc volume decrease in rabbit model	[173]
Genipin cross-linking	Chitosan, Genipin	Bovine AF and NP cells	Cell viability in vitro. 80% of produced proteoglycans retained. Biocompatible upon subcutaneous implantation in mouse model. No leakage from NP after injection in human cadaveric IVD	[174]
Genipin cross-linking	Fibrin, Genipin	Human AF cells	Cell viability in vitro. Shear moduli close to that of human AF. Strong adherence to human cadaveric AF tissue	[175]

NP function is typically restored by injecting shock-absorbing hydrogels containing ECM-producing cells or compounds that stimulate endogenous cells to produce ECM. For this, the hydrogels used in NP cell therapy strategies are generally formed using the macromolecules that are predominantly present in the ECM of the NP tissue. Hyaluronic acid (HA) is the natural biomaterial that is most-often investigated for use in NP repair and regeneration, as it is the most abundant water-absorbing molecule in NP tissue. In addition, high molecular weight HA has anti-inflammatory and anti-angiogenic properties and acts as an immunosuppressive [176]. Collagen and collagen derivatives such as gelatin are also frequently used, as it is abundantly present in the IVD. Collagen has several other advantageous properties such as its highly organized structure which leads to good mechanical properties, its weak immunogenicity and its ability to support a variety of cell types [27]. Also proteins such as fibrin are often-used constituents of these hydrogels, combining their capacity to act as a tissue sealant and to stimulate the production of ECM [175,177].

Hydrogels formed in situ by injection of aqueous solutions of natural biomaterials allowed encapsulated cells to be kept within the gel after injection. The benign reaction conditions ensured cell survival and maintained the NP phenotype of the cells in vitro as well as in vivo. It was shown that ECM production, and more specifically proteoglycan production, was enhanced. This led to minimal loss of disc height with the NP cell-containing gels compared to punctured non-treated discs [172-174].

To enhance the mechanical properties of the hydrogels, networks were also prepared in which the natural biomaterials were combined with synthetic polyethylene glycol (PEG) in different manners. Such composite materials would possess the suitable environment for cells to regenerate NP tissue, while at the same time allow tuning of the mechanical properties of the hydrogel by variation of the crosslinking density [166], the composition of the mixture and/or the molecular weight

of the polymers [170]. In this manner, hydrogels with properties similar to those of the native human NP have been prepared (the shear modulus of the human NP is 7-21 kPa [178,179], the compressive modulus of the human NP is 3-7 kPa [180,181]).

It should be realized, however, that even though hydrogels can be prepared with mechanical properties similar to those of native NP tissue, hydrogels in general are relatively weak materials and extrusion of the hydrogel matrix and cells through the injection site often occurs. It is therefore of great importance to also regenerate or repair the AF in the damaged or degenerated disc [14,182,183].

### 3.3.4. *In situ forming hydrogels using thermoresponsive systems*

Physically crosslinked hydrogels are formed via non-covalent interactions in response to an external stimulus. The formation of a hydrogel in which the network is formed through physical interactions in situ can take place via a variety of stimuli [184], and can yield a very gentle method to prepare hydrogel-based delivery systems [185].

A temperature change as a stimulus for network formation is of great importance for application in vivo. A polymer solution that is able to undergo a sol-gel transition upon a change in temperature when injected into the body can be used to prepare a depot for the delivery of drugs, growth factors and cells [186,187]. There is great interest in injectable thermoresponsive hydrogel systems for regenerative therapies of the NP, as the hydrogels are formed in a very mild manner in situ without the need of chemical reactions. A wide variety of thermally responsive biomaterials have been used for NP regeneration. These include recombinant polypeptides, natural biomaterials and synthetic polymers.

For the intended purposes, hydrogel formation with thermoresponsive polymers is based on the aptitude of the polymer to be soluble in water at a temperature that differs from physiological temperatures and to spontaneously undergo a phase transition at body temperature to form a gel-like water-swollen material in situ upon injection.

The classic mechanism is based on the formation of hydrophobic interactions that increase the entropy of the system upon heating from room temperature to body temperature. This leads to a specific organization at the lower critical solution temperature (LCST). The solubility of thermoresponsive polymers is considered to be negatively dependent on the temperature. While most macromolecules tend to increase their solubility in water when temperature increases, the interactions between polymer and solvent molecules in solutions of thermoresponsive polymers are destroyed with increasing temperature in favour of the formation of polymer-polymer interactions. This results in aggregation and shrinkage of the polymer and transition to a gel phase. Therefore, thermoresponsive polymer solutions allow the preparation of hydrogels loaded with biologically active compounds or cells by simple mixing into the polymer solution at room temperature and subsequent heating to body temperature, for example upon injection.

One of the most-often used thermoresponsive polymeric biomaterials for NP regeneration is elastin-like peptide (ELP), a recombinant polypeptide. ELP consists of repeating Val-Pro-Gly-Xaa-Gly (Xaa being any other amino acid) pentapeptide units. ELP is synthesized by genetic engineering, which allows perfect control of the molecular structure, molecular weight and mono-dispersity. It has been recognized that ELP is a biocompatible and biodegradable polymer that is non-immunogenic in nature. It has successfully been used as a drug carrier [188,189].

ELP can readily be conjugated with other bioactive proteins without impairing its thermosensitive properties [190]. By conjugating ELP with anti-inflammatory proteins such as IL1-Ra or soluble TNF Receptor Type II (sTNFR2), Shamji et al. investigated the potential of ELP to be used for protein delivery in NP tissue repair. The polypeptide-protein conjugates were not denatured and maintained their thermoresponsive characteristics [191-193]. It was demonstrated in these studies that injection of soluble ELP-protein conjugates followed by in situ gelation in the perineural area, sustained the release and enhanced the bioactivity of the coupled proteins released upon proteolysis from the ELP part. The combination of functionalities ensured the prolonged presence of these proteins at effective concentrations in the perineural space. Moreover, the risk of systemic exposure and toxicity associated with direct injection of the proteins was overcome by using this system.

To reduce inflammation in the degenerated IVD, the drug curcumin was coupled to ELP in a similar approach. Curcumin is a hydrophobic drug known to prevent neuro-inflammation by inhibition of TNF- $\alpha$  production [95]. Curcumin was chemically modified to be coupled with ELP via a labile carbamate bond. The use of this injectable ELP-curcumin conjugate resolved the problem of the low solubility of curcumin in water. Upon injection and the formation of a hydrogel, a depot was created that released curcumin for a period of 4 days in vivo in a mouse model.

A thermoresponsive hydrogel based on chitosan has also been investigated for application as an injectable in situ forming carrier for use in IVD repair. Chitosan is a cationic polysaccharide that is obtained by alkaline deacetylation of chitin, the main component of the exoskeleton of crustaceans. It has often been used in biomedical applications, as it is biocompatible and biodegradable, bioadhesive and shows antimicrobial characteristics [194,195]. Chitosan is capable of binding the highly anionic aggrecan that is produced by IVD cells and has been used as a cell carrier as its solution behaviour is sensitive to pH and depends on temperature [196]. Chitosan in solution spontaneously undergoes a phase transition above pH = 6.2 (pKa of chitosan is 6.2). Polyols such as  $\beta$ -glycerophosphate maintain chitosan in the liquid state at higher pH values and lower temperatures [197]. Therefore, this thermo- and pH-sensitive hydrogel composed of chitosan-glycerophosphate is liquid at room temperature with physiologic pH and transforms itself to a hydrogel around body temperature. These properties potentially allow chitosan-glycerophosphate thermosensitive hydrogel to be used as NP, AF or MS cell carrier [198,199]. These studies showed that the embedded NP cells produced proteoglycans and that these proteoglycans

were retained in the chitosan matrix. AF cells did not survive the hydrogel formation process [198]. Human MSCs that were cultured in vitro for 4 weeks in a chitosan-glycerophosphate hydrogel without the addition of exogenous factors were found to have differentiated into a cell phenotype that closely resembled NP cells [199].

To improve the mechanical stability of the hydrogel and to accelerate gel formation, Cheng et al. suggested adding gelatin to the thermosensitive chitosan-glycerophosphate mixture [200]. They showed in an in vitro assay that when cultured in a chitosan-gelatin-glycerophosphate hydrogel, the NP cells produced normal levels of GAGs and highly expressed collagen type II and aggrecan mRNA, while collagen type I expression was relatively low. Reactive oxygen species (ROS) may play an essential role in disc degeneration as high levels of ROS were found to be produced during cell apoptosis. The same team of investigators proposed to use chitosan-gelatin-glycerophosphate matrix to release feluric acid, a natural phenolic antioxidant. The controlled long-term release of feluric acid drastically reduced oxidative stress and allowed production of ECM by the cells [201,202].

Also synthetic amphiphilic degradable copolymers with thermoresponsive properties have been used to prepare hydrogels for IVD therapies [203]. These copolymers often comprise hydrophilic polyethylene glycol segments and degradable polyester segments. Due to their biodegradability and biocompatibility, they have been widely investigated. Zhang et al. utilized a thermosensitive poly(ethylene glycol)-poly(lactic-co-glycolic acid)-poly(ethylene glycol) (PEG-PLGA-PEG) blockcopolymer gel to deliver simvastatin within the disc in vitro and in a rat disc stab injury model [204]. In the rat model, the controlled and prolonged release of simvastatin significantly increased BMP-2 mRNA expression. This resulted in aggrecan and collagen type II production without impairing the phenotype of the NP cells.

Poly(N-isopropylacrylamide) (PNIPAM) is the most-often used thermoresponsive polymer in biomedical applications, mainly as a result of its transition temperature of approximately 32 °C [186,205]. Increasing the temperature of solutions in water to above the LCST, results in increased hydrophobic interactions and formation of a hydrogel. In combination with hyaluronic acid, Peroglio et al. used the thermoresponsive properties of PNIPAM to encapsulate and deliver human MSCs into the NP in organ culture [206]. These HA-PNIPAM hydrogels provided a favourable environment for chondrogenic and disc-like differentiation of hMSCs without growth factor supplementation. An injectable colloidal gel-forming dispersion based on PNIPAM and PLGA nanoparticles has been developed, with the aim to improve the mechanical strength of the formed hydrogel [207]. As the viability of bovine NP cells incorporated in this hydrogel was demonstrated, this system might be suitable for cell delivery into the IVD.

From a similar perspective, a drug delivery system composed of microspheres and an injectable thermoresponsive hydrogel has been developed. To control the sustained localized release of bupivacaine, an anaesthetic drug that is under investigation as an alternative to corticosteroids and opioids, Lee et al. embedded a PCL microsphere-based delivery system in a Pluronic F127® hydrogel matrix for the treatment of IVD degeneration [208]. To prevent the polyester microspheres to be dislodged from the site of injection and phagocytised prematurely by macrophages, the microspheres were incorporated in the hydrogel matrix. The authors showed that the in vitro release of bupivacaine encapsulated in the PCL microspheres was unaffected by the hydrogel matrix and could be sustained for up to 42 days.

#### 4. Gene therapy

The efficiency of GF injections to promote IVD regeneration by regulation of cellular metabolic pathways has been clearly demonstrated in vitro as well as in vivo. However, drawbacks of this therapy include the short half-life of GFs in vivo and the lack of controlled and prolonged activity upon GF injection. Therefore, innovative protein delivery systems have been developed as discussed earlier in part 3. However,

the effectiveness of this approach depends on several factors like the amount of loading, the biological stability of the GFs incorporated in the biomaterial structures and the need of suitable degradation of the biomaterials. An alternative strategy addressing this concern would be to genetically reprogram the functions of IVD cells through gene therapy in order to alter or reverse the degenerative processes [209,210].

The principle of gene therapy is based on the delivery of complementary deoxyribonucleic acid sequences (cDNA) in the cells of a specific tissue by means of a vector. The gene coding for a specific protein is integrated into the vector to allow transfection of the host cell. Upon internalization of the genetic material, the gene is translocated to the nucleus of the cell and transcribed into messenger ribonucleic acid (mRNA), which is subsequently translated by ribosomes in the cytoplasm resulting in the synthesis of the desired proteins. These proteins can be secreted by the cell to act in a paracrine way, or stay in the cell to influence pathways in the cell itself. For delivery of the genes, either viral or non-viral vectors can be used. The gene-containing vectors can be introduced directly into the body (in vivo gene transfer), or target cells can be collected, transfected and subsequently re-implanted into the body (ex vivo gene transfer).

#### 4.1. Viral gene delivery systems

In order to deliver genetic material inside cells, the most straightforward and effective approach is considered to be viral transfection [211,212]. For this, the virus must be engineered to prevent pathological infections to take place, and the gene to be delivered has to be inserted in the viral genome.

Gene delivery can take place by means of a retrovirus which incorporates the gene in the genome of the host cell, or by a non-genome-incorporating virus like adenovirus. As random gene insertion in the host genome may lead to the development of cancer, the use of the latter virus type is considered to be more safe.

##### 4.1.1. Delivery of growth factor genes

GF genes are the most-investigated exogenous genes used for gene therapy to reverse the process of IVD degeneration. Nishida et al. studied the effects of in vivo transfection of NP tissue with the human TGF- $\beta$ 1 gene in a rabbit model using an adenoviral vector [213]. After one week, the genetically treated tissue showed an increased TGF- $\beta$ 1 production compared with untreated control tissue, resulting in a significant increase of proteoglycan synthesis.

Transfection of other genes belonging to the TGF- $\beta$  superfamily such as growth differentiation factor-5 (GDF-5), by means of adenoviral vectors, was investigated in vitro using rabbit, mouse and human IVD cells [214,215] and in vivo in a mouse model [216]. The in vitro studies demonstrated a stimulation of AF and NP cell proliferation as well as an increased ECM deposition, in particular of aggrecan and collagen type II, after transfection with GDF-5. These results were confirmed in the mouse model as a recovery of disc height after GDF-5 gene delivery.

BMP gene transfection was investigated by Zhang et al., using cell as well as organ cultures and adenoviral vectors [217-219]. Transfection with BMP-2 and -7 specifically stimulated proteoglycan production, BMP-4 and -14 expression was most effective in stimulating collagen deposition and BMP-2 expression substantially promoted NP cell proliferation.

Despite the efficient gene delivery by adenoviral vectors both in vitro and in vivo, a significant host immune response impairs gene expression upon repeated transfection [220,221]. Therefore, adeno-associated virus (AAV) has been developed as alternative vector for gene therapy. AAV does express less immunogenic properties than adenovirus [222,223], while offering sufficient size for incorporation of TGF- $\beta$  or BMP genes. AAV-mediated delivery of the human BMP-7 gene in canine NP cells significantly increased the deposition of proteoglycans and collagen type II [224].

##### 4.1.2. Delivery of transcription factor genes

In addition to GF gene transfection, the delivery of transcription factor genes was investigated as an approach to treat IVD degeneration. SOX-9 is a transcription factor involved in chondrogenesis and collagen type II expression. Adenoviral transfection of cultured degenerated human disc cells with the SOX-9 gene resulted in a markedly increased collagen type II production by the cells. Moreover, transfection of NP tissue with the same vector/gene construct in a rabbit model of disc degeneration, showed preservation of the chondrogenic appearance of the NP during a period of 5 weeks, whereas non-transfected injured discs severely degenerated [225].

Lim mineralization protein-1 (LMP-1) is an intracellular protein, that has been reported to play a role in the synthesis of BMPs by cells and to potentiate the responsiveness of cells to extracellular BMPs [226]. Adenoviral delivery of the human LMP-1 gene in rabbit IVDs in vivo resulted in increased mRNA levels of BMP-2, BMP-7 and aggrecan [226]. It was hypothesized that increased levels of intracellular LMP-1 resulted in increased synthesis and secretion of BMP-2 and -7, which led to increased aggrecan expression. Transfection of MSCs with the LMP-1 gene promoted differentiation of the cells into the osteogenic lineage, which was successfully used as a strategy to promote spinal fusion in a rat model [227]. This effect of LMP-1 was probably due to expression and subsequent secretion of BMPs by the transfected cells.

##### 4.1.3. Delivery of protein genes inhibiting inflammatory and catabolic pathways

Up-regulation of protein inhibitors of cytokine pathways, such as IL-1 receptor antagonist, has been proposed to inhibit IVD degeneration [228,229]. Adenoviral transfection of human IVDs in organ culture with the IL-1Ra gene was shown to increase the synthesis of IL-1Ra protein, which led to a higher resistance of the IVD cells to IL-1 during two weeks as compared to non-transfected cells [228].

Catabolic processes in the ECM of the disc are most often due to increased matrix metalloproteinase levels. Therefore, transfection with the gene coding for the MMP inhibitor TIMP-1 (tissue inhibitor of matrix metalloproteinase-1), should decrease ECM degradation. Indeed, this effect was shown in vitro by adenoviral transfection of degenerated human IVD cells with the TIMP-1 gene, resulting in an increased proteoglycan content of the ECM [230].

#### 4.2. Non-viral gene delivery systems

Recent research shows a growing interest in non-viral gene carriers, as this avoids the risks of mutagenicity, systemic viral infections and viral immunogenicity [231,232]. In addition, non-viral vectors can be more easily produced in large scale as compared to viral vectors.

Non-viral gene carriers basically consist of polycationic compounds including phospholipids [233], synthetic polymers [234], polypeptides [235], or naked DNA [236]. Despite the advantages of non-viral vectors, their use is still limited due to low transfection efficiencies. Only a few applications have been reported for the treatment of IVD degeneration.

Chung et al. investigated the in vitro transfection of ovine NP cells with the telomerase gene using lipofectamine as non-viral transfection agent, which are liposomes consisting of polycationic lipids [237]. Telomerase is a key enzyme involved in the extension of shortened DNA strands, resulting in an increased cellular lifespan. Indeed, transfection of the NP cells with the telomerase gene resulted in delayed senescence of the cells and prolonged production of ECM, especially collagen types I and II, for more than 9 months. Morey et al. investigated the efficiencies of the in vitro transfection of human IVD chondrocytes with marker genes using various commercially available vectors [238]. The histone polypeptide/lipid-based vector LT1 was found to give the best results, combining a high transfection efficiency with a low cytotoxicity.

A new approach for gene therapy of the IVD has recently been reported by Nishida et al. [239], using phospholipid-stabilized gaseous microbubbles ( $b\ 10\ \mu\text{m}$ ) combined with resonant ultrasound excitation to deliver the genes [240,241]. Ultrasound excitation triggers the release of the genes and simultaneously induces perturbation and permeability of cell membranes by the oscillating microbubbles. However, high excitation pressures of therapeutic ultrasound in association with gaseous carriers may result in cell death. By using this approach, Nishida et al. showed that the expression of the marker protein luciferase was sustained up to 24 weeks in a rat IVD model. Moreover, the same team used this strategy to transfect siRNA (silencing or short interfering RNA) molecules into human NP cells *in vitro* [242] and *in vivo* in the rat IVD model [243]. The results showed that luciferase marker protein was dramatically down-regulated in cells transfected with siRNA interfering with luciferase mRNA for at least 24 weeks [243]. Therefore, transfection with siRNAs targeting proinflammatory cytokines, catabolic enzymes or other proteins involved in degenerative disc disease has great potential to inhibit inflammation and degeneration of the IVD. Using this approach, Seki et al. inhibited the proteolytic enzyme ADAMTS5 (a disintegrin and metalloprotease with thrombospondin-like repeats) [244]. A single injection of ADAMTS5 siRNA in the rabbit annular needle-puncture model showed a suppression of NP tissue degradation. As shown by Han et al., treatment of cultured rat NP cells with siRNA interfering with the expression of FAS, a receptor involved in apoptotic cell death, resulted in the inhibition of NP cell apoptosis [245]. Likewise, down-regulation of caspase 3, a key enzyme involved in apoptosis, inhibited rabbit NP cell apoptosis *in vitro* as well as disc degeneration in the rabbit annular needle-puncture model [246].

Although these are very promising results, the use of non-viral carrier systems for the safe and effective delivery of genes and siRNA into IVD cells is still in its infancy and deserves to be further developed in order to find an optimal strategy for gene therapy and silencing to treat IVD degeneration.

## 5. Conclusions

Intervertebral disc degeneration is recognized as one of the most serious degenerative disorders that dramatically affect the quality of life. The many efforts which have been paid to understand the metabolism, physiology and pathophysiology of IVD degeneration, have formed the basis for the development of therapies based on injections of anti-inflammatory or analgesic drugs, bioactive agents such as growth factors, or cells. However, these therapies are sub-optimal because they lack control of the delivery of the agents. This limitation can be overcome by the use of carrier systems for the delivery of the active compounds. Most of the delivery systems developed for the treatment of IVD degeneration are focused on control of the pharmacokinetics of the agent, targeting of the agent towards the intradiscal or perineural area, prevention of unwanted side effects of the agent and protection of the entrapped agent against clearance by the innate immune system. Due to the complex and delicate location of the IVD nearby the spinal nerves, there is an extensive need to minimize the invasiveness of the administration. Therefore, besides marginal applications of implantable delivery devices, the majority of the approaches are based on the use of injectable systems such as microparticles and hydrogels, that have also been used for the delivery of cells. Gene therapy, which is another strategy to treat IVD degeneration, also requires a carrier system in the form of a viral or non-viral vector. These different approaches are summarized in Fig. 4, from conventional therapy to sophisticated carrier systems to control the delivery of bioactive agents, cells and genes to the IVD.

A large number of anti-inflammatory and analgesic drugs have been approved by the FDA, and these compounds are being clinically used for the relief of back pain by means of injection. Interesting agents without FDA approval in the preclinical stages of development, are NF- $\kappa$ B antagonists and inhibitors of NF- $\kappa$ B activation, see Table 1. These compounds are very promising, as NF- $\kappa$ B not only mediates inflammatory but also catabolic signalling in the degenerating IVD.

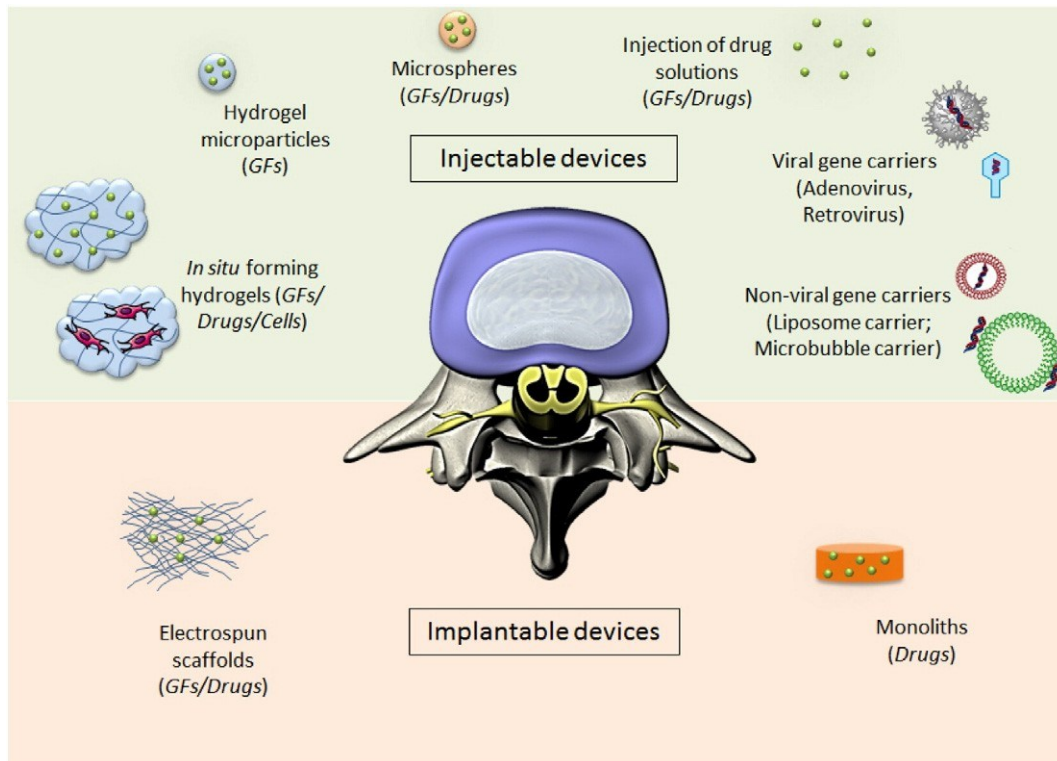


Fig. 4. Overview of strategies to deliver bioactive agents, cells and genes to the IVD.

With respect to the delivery of bioactive agents and cells to the IVD, microparticles and in situ forming hydrogels are most-often used. These systems are also in the preclinical stages of development. In view of the mild crosslinking conditions and the support of cell viability and ECM production, hydrogels formed by reaction in situ are the preferred cellular delivery systems. Moreover, these hydrogels have very suitable mechanical properties, see Table 3.

Although gene therapy for the treatment of degenerative disc disease using viral vectors shows promising preclinical results, major concerns of this approach include the development of cancer and immune responses. Therefore, non-viral gene carriers are the preferred delivery systems, although transfection efficiencies are lower than those of viral vectors. An interesting approach is transfection of IVD cells with the LMP-1 gene, which may already be successful at moderate transfection efficiencies. Transfection of cells with the LMP-1 gene, results in expression and subsequent secretion of BMPs by the transfected cells, which may influence the phenotype and function of surrounding cells promoting IVD regeneration.

Finally, as loss of IVD function generally starts with degeneration of the NP, major advances have been reported on regeneration of the NP while regeneration of the AF has received less attention. However, to restore the function of the whole IVD, it is essential to combine the regeneration of NP and AF tissue. Therefore, a new challenge for future research would be the development of a minimally invasive method able to facilitate regeneration of the NP and at the same time to restore the structure and function of the AF.

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