

Biomaterials for intervertebral disc regeneration : past performance and possible future strategies.

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BIOMATERIALS FOR INTERVERTEBRAL DISC REGENERATION: PAST PERFORMANCE AND POSSIBLE FUTURE STRATEGIES

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Abstract

Intervertebral disc (IVD) degeneration is associated with most cases of cervical and lumbar spine pathologies, amongst which chronic low back pain has become the number one cause of loss of quality-adjusted life years. In search of alternatives to the current less than optimal and usually highly invasive treatments, regenerative strategies are being devised, none of which has reached clinical practice as yet. Strategies include the use of stem cells, gene therapy, growth factors and biomaterial carriers. Biomaterial carriers are an important component in musculoskeletal regenerative medicine techniques. Several biomaterials, both from natural and synthetic origin, have been used for regeneration of the IVD *in vitro* and *in vivo*. Aspects such as ease of use, mechanical properties, regenerative capacity, and their applicability as carriers for regenerative and anti-degenerative factors determine their suitability for IVD regeneration. The current review provides an overview of the biomaterials used with respect to these properties, including their drawbacks. In addition, as biomaterial application until now appears to have been based on a mix of mere availability and intuition, a more rational design is proposed for future use of biomaterials for IVD regeneration. Ideally, high-throughput screening is used to identify optimally effective materials, or alternatively medium content comparative studies should be carried out to determine an appropriate reference material for future studies on novel materials.

Keywords: Intervertebral disc degeneration, nucleus pulposus cells, annulus fibrosus cells, regeneration, hydrogels, synthetic polymers, natural hydrogels, delivery, cell-biomaterial interactions.

Introduction

The vertebral column is composed of the rigid bony vertebral bodies, interspersed with intervertebral discs (IVDs) and facet joints. It protects our spinal cord and supports our head, upper extremities and torso while providing flexibility in multiple degrees of freedom. The IVDs, in total making up about a third of the spinal column by height, enable movement of the spinal column and transfer the loads associated with movement. As non-vascular and non-synovial structures with limited repair capacities, the IVDs have been shown to be prone to cumulative damage. Chronic low back pain is strongly linked to lumbar IVD degeneration while radicular pain is associated with bulging of the posterior annulus fibrosus (AF) and nucleus pulposus (NP) herniation (Luoma *et al.*, 2000). Back pain, as a result of IVD degeneration, can start early in life and will affect at least 60 % of people over the age of 70 (Miller *et al.*, 1988).

The healthy IVD: Functional characteristics and relation to tissue structure

The healthy IVD consists of a nucleus pulposus circumferentially surrounded by an AF and axially enclosed by two cartilaginous endplates (CEP) (Fig. 1). The NP mainly consists of proteoglycans and collagen type II, with a few cells interspersed in the matrix. Due to the presence of the highly charged proteoglycans with the concomitant attraction of cations, the tissue osmotic pressure is high. This in turn attracts water and due to the confinement by the AF and endplates generates hydrostatic pressure. When subjected to shear forces, the NP behaves as a viscoelastic material rather than a fluid (Iatridis *et al.*, 1996). The AF is a fibrocartilaginous structure consisting of both collagen I and II and proteoglycans, where the collagenous layers are alternately arranged at angles of about 60° and 150° to the spinal longitudinal axis around the NP. Together with elastin-rich layers in between the collagen lamellae, the AF provides tensile strength and the capacity to withstand disc bulging in response to loading (Isaacs *et al.*, 2014). The cartilaginous endplate that connects the IVD to the vertebral bodies consists of hyaline cartilage and transfers compressive forces due to axial loading to the NP. It is supported by the osseous part of the endplate of the vertebral bodies to which the AF collagen fibres are anchored. As the IVD matures, the large cytoplasmic vesicle-rich notochordal cells inside the NP are replaced by the smaller chondrocyte-like cells,

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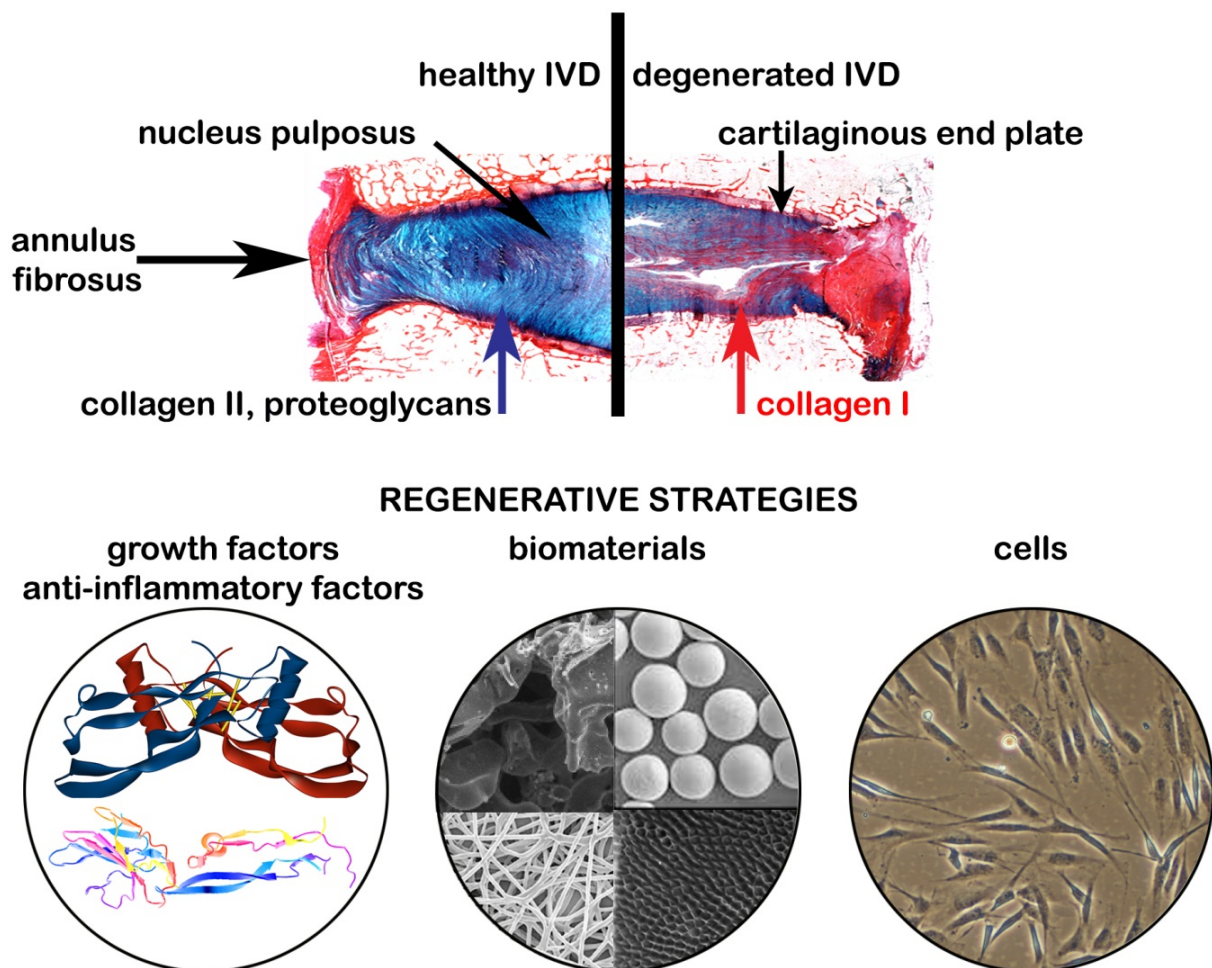


Fig. 1. Tools for regenerative treatments of the IVD. Upper left panel showing a healthy human disc, and on the right a degenerated disc. Below are depicted the main approaches towards regenerative medicine, *i.e.* the application of regenerative factors/factors inhibiting inflammation, biomaterials and exogenously added cells.

possibly by transdifferentiation (Hunter *et al.*, 2004; Kim *et al.*, 2009; McCann *et al.*, 2012; Purmessur *et al.*, 2013). In addition, NP progenitor cells have been identified with mesenchymal stem cell (MSC)-like marker expression and multipotency, also in the degenerate IVD (Risbud *et al.*, 2007; Sakai *et al.*, 2012). The AF contains fibroblast-like cells in its outer rim, whereas cells are more chondrocytic in appearance in the inner annulus.

Pathophysiology and aetiology of IVD degeneration

Intervertebral disc degeneration is characterised by several biochemical and morphological changes. Proteoglycans and collagen type II in the NP are lost and replaced by fibrous tissue rich in collagen type I (Fig. 1). This eventually leads to a reduction in hydration and a loss in the ability to maintain osmotic pressure. The process of proteoglycan loss is due to enzymatic activity by which also collagen and fibronectin become increasingly fragmented (Martin *et al.*, 2002; Urban and Roberts, 2003). Up to 50 % of cells in severely degenerate discs appear necrotic (Trout *et al.*, 1982).

The aetiology of IVD degeneration is still a subject of debate. The most commonly accepted theory is based on the concept that the IVD receives its nutrition from the

cartilaginous endplate by diffusion towards the centre of the NP, although – controversially – diffusion or convection through the AF was also suggested (Cortes *et al.*, 2014; Hutton *et al.*, 2004; Urban *et al.*, 1977; van der Veen *et al.*, 2007). Occlusion of openings containing capillary endings in the bony endplate may reduce nutrient supply and oxygen saturation, affecting the pH, matrix synthesis and eventually cell viability inside the IVD (Urban and Roberts, 2003). Subsequent loss of proteoglycans results in a lower hydration state, leading to a reduced weight bearing capacity. This allows inappropriate weight and stress distribution across the disc, resulting in stress fissures in the nucleus or the AF (Adams *et al.*, 2014). Another mechanism of disc degeneration may be related to mechanical loading. IVD degeneration is associated with physically demanding professions (Luoma *et al.*, 2000), suggesting intensive load bearing may induce degeneration, as supported by *ex vivo* experiments with overloading. Similarly, normal everyday loading may also cause lumbar IVD degeneration. This is supported by the observation that several polymorphisms associated with IVD degeneration involve genes encoding extracellular matrix proteins essential for the biomechanical properties of the IVD; amongst others, collagens (I, IX, XI),

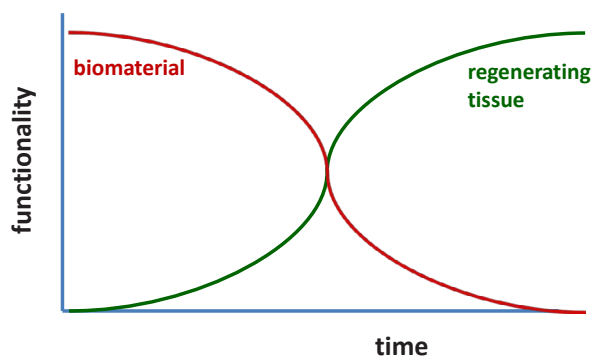


Fig. 2. Paradigm for optimal biomaterial-based regeneration of the IVD. Ideally, functionality of the IVD is maintained by biomaterial degradation occurring at the same speed as tissue regeneration.

aggrecan, hyaluronic acid synthase and also matrix degrading proteases including MMP-1, 2, 3 and 9 (Battie and Videman, 2006; Williams *et al.*, 2012; Mayer *et al.*, 2013; Näkki *et al.*, 2014). These polymorphisms may also act through non-mechanical pathways. However, the current lack of meaningful odds ratios for correlation of disc degeneration and polymorphisms indicates that most likely multiple factors are involved in this condition.

Regenerative medicine (RM) as emerging approach for IVD degeneration

Current treatments of lower back pain mainly aim to treat its symptoms. Options for treatment include physiotherapy, analgesia, muscle relaxants, corticosteroids and surgery (discectomy, disc prosthesis or spinal arthrodesis) (Jacobs *et al.*, 2013). Surgical treatment is highly invasive and although spinal fusion may reduce pain, it does not provide for biological repair and preservation of motion of the treated segment (Etebar and Cahill, 1999). Furthermore, spinal fusion has been associated with degeneration of adjacent IVDs (Higashino *et al.*, 2010; Radcliff *et al.*, 2013), although this partially may reflect the natural course of disc pathobiology (Helgeson *et al.*, 2013). In terms of arthroplasty, no long-term effective and fully safe IVD substitute has been described until now (Thavaneswaran and Vandeppeer, 2014). All in all, the current treatments are mere salvage treatments that are not very effective and/or even entail serious risks.

Therefore, research into novel treatment strategies focuses on regeneration of the IVD rather than addressing the effects of degeneration. For this purpose, three different components are being employed either alone or in combination: growth factors, cells and biomaterials (Fig. 1). Cells, in particular mesenchymal stromal cells (MSCs), and growth factors hold promise to directly regenerate IVD tissue by anabolic effects on the cell population and matrix homeostasis (Chan and Gantenbein-Ritter, 2012; Masuda, 2008; Richardson and Hoyland, 2008; Yim *et al.*, 2014). In contrast, biomaterials can have several roles. They can be used as structural scaffolds (Darwis *et al.*, 2002; Ella *et al.*, 2005; Gloria *et al.*, 2007; Joshi *et al.*, 2006), improving disc height and mechanical stability of the vertebral segments, thereby correcting altered distribution of mechanical loads

affecting cytoskeletal structure, gene transcription, and matrix biosynthesis (Chen *et al.*, 2004; Iatridis *et al.*, 1999; Setton and Chen, 2006). Biomaterials have also been used as cell carriers and release systems for active factors in order to achieve regeneration. Resident cells in the native IVD can migrate into hydrogels, providing a framework for regeneration by native cells (Anderson *et al.*, 2005; Yang and Li, 2009). The focus of this review is to explore the applicability of various biomaterials in IVD regeneration, with a focus on the NP and AF and on biodegradable biomaterials, as biostable materials (materials that do not degrade) are often implants and prostheses rather than elements contributing to regeneration of the IVD.

Biomaterials for IVD regeneration

To stimulate IVD regeneration, a biomaterial ideally possesses many different properties. It should provide some degree of immediate mechanical support and its degradation should occur in parallel with the formation of new and functional tissue, without the production of toxic by-products (Fig. 2). It should provide a permissive environment for seeded cells or for resident cells migrating into the biomaterial, allowing for diffusion of oxygen and nutrients required for tissue production. Cell differentiation and matrix production are achieved, induced either by active factors included or by intrinsic properties of the material or its constituent polymers.

Biomaterials are often divided into natural biomaterials and synthetic biopolymers, and come as hydrogels or solid scaffolds. Natural polymer-based biomaterials used include mainly hydrogels, such as chitosan, alginate, collagen, hyaluronan and agarose. The group of synthetic biomaterials comprises poly-(D,L-lactide) (PLA) and derivatives, polyethylene glycol (PEG), polycarbonate urethane (PU) and poly(epsilon-caprolactone) (PCL). Some of these synthetic biomaterials can serve either as solid scaffolds or as hydrogels. Overviews of the biomaterials used and their effects and properties are given in Tables 1, 2 and 3.

Natural hydrogels

Chitosan

Chitosan is a polysaccharide biopolymer composed of glucosamine and N-acetyl glucosamine units and is derived from partial de-acetylation of chitin (Di Martino *et al.*, 2005; Roughley *et al.*, 2006). Medical applications include wound haemostasis and healing, based on antimicrobial properties as well as drug delivery capabilities (Dai *et al.*, 2011; Patel *et al.*, 2010; Pusateri *et al.*, 2003). Both hydrogels and solid scaffolds can be formed from chitosan (Di Martino *et al.*, 2005). Chitosan is dissolved at low pH, so the final hydrogel needs to be neutralised before application. This presents a challenge as neutralisation of an acidic chitosan solution causes immediate cross-linking of chitosan chains.

Chitosan is degradable *in vivo* through lysozyme activity. Increasing the degree of de-acetylation prolongs its degradation time while enhancing cell adhesion (Mao *et al.*, 2004; Di Martino *et al.*, 2005). The cationic

Table 1. Overview of biomaterials used for IVD regeneration.

Class	Biomaterials	Derived from	Common medical uses	Current stage/use in IVD regeneration	Reference
Natural hydrogels	Chitosan	Arthropod exoskeletons	Wound healing, haemostasis dressings	May mechanically resemble the native NP. <i>In vivo</i> differentiation to NP-like cells.	Bertolo <i>et al.</i> , 2012; Cheng <i>et al.</i> , 2010; Cheng <i>et al.</i> , 2013; Dai <i>et al.</i> , 2011; Iwasaki <i>et al.</i> , 2004; Li <i>et al.</i> , 2005b; Mao <i>et al.</i> , 2004; Di Martino <i>et al.</i> , 2005; Patel <i>et al.</i> , 2010; Pusateri <i>et al.</i> , 2003; Richardson <i>et al.</i> , 2008; Roughley <i>et al.</i> , 2006; Shao and Hunter, 2007; Smith <i>et al.</i> , 2014; Sun <i>et al.</i> , 2014; Zhang <i>et al.</i> , 2014
	Alginate	Brown seaweed	Delayed drug delivery systems	Cells cultured in 3D constructs, tested <i>in vivo</i> in murine model.	Bron <i>et al.</i> , 2011; Chou <i>et al.</i> , 2009; Chou and Nicoll, 2009; Guo <i>et al.</i> , 1989; Larsen and Haug, 1971; Leone <i>et al.</i> , 2008; Li <i>et al.</i> , 2008; Lee and Mooney, 2012; Melrose <i>et al.</i> , 2001; Nunamaker <i>et al.</i> , 2007; Xu <i>et al.</i> , 2012b
	Agarose	Red algae	Culture media	Hybrid scaffold (NP + AF) for <i>in vitro</i> testing.	Cloyd <i>et al.</i> , 2007; Gruber <i>et al.</i> , 2006; Gupta <i>et al.</i> , 2014; Hunt and Grover, 2010; Lazebnik <i>et al.</i> , 2011; Nerurkar <i>et al.</i> , 2010; Tilwani <i>et al.</i> , 2012; Zhang <i>et al.</i> , 2012
	Fibrin	Human plasma	Surgical sealant	Used as a singular or hybrid scaffold for NP and AF <i>in vitro</i> and <i>in vivo</i> testing.	Acosta <i>et al.</i> , 2011; Ahmed <i>et al.</i> , 2008; Colombini <i>et al.</i> , 2014; Diederichs <i>et al.</i> , 2012; Eyrich <i>et al.</i> , 2007; Hegewald <i>et al.</i> , 2010; Ho <i>et al.</i> , 2010; Li <i>et al.</i> , 2014; Likhitpanichkul <i>et al.</i> , 2014; Ma <i>et al.</i> , 2012; Park <i>et al.</i> , 2011b; Pirvu <i>et al.</i> , 2015; Schek <i>et al.</i> , 2011; Stern <i>et al.</i> , 2000; Stern <i>et al.</i> , 2004; Sung <i>et al.</i> , 1999; Wang <i>et al.</i> , 2005
	Hyaluronan	Chicken comb, fermentation	Drug delivery, synovial fluid supplementation	<i>In vivo</i> increase in disc height combined with MSCs.	Chen <i>et al.</i> , 2013; Chung <i>et al.</i> , 2009; Collin <i>et al.</i> , 2011; Erickson <i>et al.</i> , 2009; Kenne <i>et al.</i> , 2013; Kogan <i>et al.</i> , 2007; Su <i>et al.</i> , 2010
Synthetic hydrogels	Collagen & gelatin	Animal tissues	Drug delivery, surgical applications	NP & AF cells differentiation, mechanical properties mimic native NP.	Bron <i>et al.</i> , 2009; Bron <i>et al.</i> , 2012; Cen <i>et al.</i> , 2008; Chen <i>et al.</i> , 2013; Cheng <i>et al.</i> , 2010; Ferreira <i>et al.</i> , 2012; Huang <i>et al.</i> , 2005; Lee <i>et al.</i> , 2001; Malhotra <i>et al.</i> , 2012; Nicodemus and Bryant, 2008; Strange and Oyen, 2012; Wang and Stegmann, 2010; Wilke <i>et al.</i> , 2006
	Polyethylene glycol	Polymerisation of ethylene glycol	Pharmacological: basis for laxatives	Structural component for hydrogel mixtures, <i>in vivo</i> regeneration by IVD cells. The only gel in IVD clinical trial.	Ashley <i>et al.</i> , 2013; Benoit and Anseth, 2005; Collin <i>et al.</i> , 2011; Drira and Yadavalli, 2013; Francisco <i>et al.</i> , 2014; Hern and Hubbell, 1998; Lin and Anseth, 2009; Nguyen <i>et al.</i> , 2012; Raeber <i>et al.</i> , 2005; Zhu, 2010; Scholz <i>et al.</i> , 2010; Benz <i>et al.</i> , 2012a; Benz <i>et al.</i> , 2012b
	Polyurethane	Polymerisation of polyols	Vascular grafts	<i>Ex vivo</i> loading testing as a structural component. Little NP cell research.	Attia <i>et al.</i> , 2011; Dahl <i>et al.</i> , 2010; Hu <i>et al.</i> , 2012; Mauth <i>et al.</i> , 2009; Park <i>et al.</i> , 2013; van Tienen <i>et al.</i> , 2009; Santerre <i>et al.</i> , 2005; Yang <i>et al.</i> , 2009; Yeganegi <i>et al.</i> , 2010
Natural solid biomaterials	Small intestinal submucosa	Acellular small intestine	Research: oesophageal reconstructive surgery	Some animal <i>in vivo</i> tests.	Badyalak <i>et al.</i> , 1995; DeJardin <i>et al.</i> , 2001; Jonsson <i>et al.</i> , 2011; Kim <i>et al.</i> , 2012; Kim <i>et al.</i> , 2014b; Ledet <i>et al.</i> , 2009; Tan <i>et al.</i> , 2012; Visage <i>et al.</i> , 2006
Synthetic solid biomaterials	Poly(lactic/glycolic acid)	Polymerisation of lactide/glycolide	Drug delivery	Some <i>in vivo</i> experiments with NP cells demonstration differentiation.	Agrawal and Athanasiou, 1996; Danhier <i>et al.</i> , 2012; El-Amin <i>et al.</i> , 2003; Endres <i>et al.</i> , 2010; Feng <i>et al.</i> , 2011; Kim <i>et al.</i> , 2014a; Lai <i>et al.</i> , 2014; Liang <i>et al.</i> , 2013; Mizuno <i>et al.</i> , 2004; Ruan <i>et al.</i> , 2010
	Poly-ε-caprolactone	Polymerisation of ε-caprolactone	Sutures. Research; drug delivery	AF cells and MSCs differentiate <i>in vitro</i> . <i>In vivo</i> part of entire IVD constructs	Agrawal and Ray, 2001; Dash and Konkimalla, 2012; Sinha <i>et al.</i> , 2004; Silva <i>et al.</i> , 2007; Li <i>et al.</i> , 2005a; Lopez <i>et al.</i> , 2010; Koepsell <i>et al.</i> , 2011a; Koepsell <i>et al.</i> , 2011b; Martin <i>et al.</i> , 2014

Table 2. Overview of characteristics of natural biomaterials used for IVD regeneration.

Biomaterial	Chitosan	Alginate	Agarose	Fibrin	Hyaluronan	Collagen / gelatin	SIS
Injectable as a liquid	+	+	+	+	+	+	-
Can harden <i>in situ</i> if injected	-	+	+	+	+	+	-
Can provide mechanical support	-	+/-	+/-	+/-	+/-	+	+
Biodegradable	+	+	-	+	+	+	+
Degradation: residue	-	-	+	-	-	-	-
Biocompatible: including cell seedability/migration	+	+	+	+	+	+	+
Stimulation differentiation/ECM production	+	+	+	+	+	+	+
Swelling	-	+	+	+/-	+	+	+/-
Extra properties	Naturally antimicrobial	Degradation not fully understood	Biodegradable only as mixtures	Capability to act as AF defect sealant			

Table 3. Overview of characteristics of synthetic biomaterials used for IVD regeneration.

Biomaterial	PEG	PLA / PGA	PU	PCL
Injectable	+	-	-	-
Can harden <i>in situ</i> if injected	+	-	-	-
Can provide mechanical support	+	+	+	+
Biodegradable	-	+	+	+/-
Degradation: residue	-	-	+	-
Biocompatible: including cell seedability/migration	+	+	+	+
Stimulation differentiation/ECM production	+	+	+	+
Swelling	+/-	+	+	-
Extra properties	Biodegradable as mixture only			

nature of chitosan facilitates interaction with anionic glycosaminoglycans and binding of cytokines and growth factors (Di Martino *et al.*, 2005).

However, chitosan gels tend to be much softer and more flexible than the native NP (Sasson *et al.*, 2012). Biomechanical properties of chitosan scaffolds can be improved by the addition of other biomaterials, in particular alginate (Iwasaki *et al.*, 2004; Li *et al.*, 2005b) and gelatin (Cheng *et al.*, 2010) without affecting cell viability and matrix production. A chitosan/dextran hydrogel implanted into human cadaveric spines and subjected to extensive loading did not show extrusion. Furthermore, Young's modulus and Poisson's ratio were found to be similar to human IVDs under unconfined compression. *In vitro*, NP cells cultured on top of the hydrogel remained viable and MSCs incorporated into the gel were able to chondrogenically differentiate as reflected by collagen type II and aggrecan production (Smith *et al.*, 2014). Bovine NP cells cultured in chitosan scaffolds produced an extracellular matrix (ECM) similar to that of the native

NP, and more so than bovine AF cells (Roughley *et al.*, 2006). Canine AF cells cultured in alginate/chitosan scaffolds formed clusters and produced collagens I, II and aggrecan (Shao and Hunter, 2007). The addition of chitosan to alginate proved to increase degradation time for the scaffold.

Also, undifferentiated cells show a regenerative response to chitosan. Human MSCs differentiated to NP-like cells, produced collagen type II and aggrecan and remained viable up to 70 % after 4 weeks in chitosan-glycerol phosphate hydrogels (Richardson *et al.*, 2008). However, compared to alginate or gelatin, chitosan was less chondrogenic for MSCs (Bertolo *et al.*, 2012). Rabbit adipose tissue-derived MSCs differentiated towards NP-like cells on chitosan/alginate scaffolds under hypoxic conditions (Zhang *et al.*, 2014). Rabbit bone marrow-derived MSCs injected in chitosan-glycerophosphate hydrogels into lumbar NP defects *in vivo*, remained present up to 12 weeks and showed chondrogenic differentiation and ECM production, which was further enhanced by prior

Sox9 transduction (Sun *et al.*, 2014). Unfortunately, MSCs were never directly compared with NP cells in terms of regenerative capacity in chitosan gels.

Although chitosan is a versatile material, it must be used in combination with other materials to improve its biomechanical properties. The added value of using chitosan in this situation is induction of chondrogenic differentiation. The main drawback is that chitosan remains a difficult material to handle, because it forms a hydrogel only when dissolved in an acid.

Highlights:

- Degrades relatively slowly.
- Evidence *in vivo* is limited.
- Hydrogel preparation is complicated as neutralisation causes cross-linking.
- Chitosan is less suitable for MSC-based IVD regeneration than gelatin or alginate.

Alginate

Alginate (alginic acid) is a natural biopolymer derived from brown seaweed, generally composed of beta(1-4)-linked D-mannuronic acid (M) and alpha(1-4) linked L-guluronic acid (G) moieties (Bron *et al.*, 2011; Larsen and Haug, 1971). Alginate-based gels can be cross-linked through ionic, covalent and thermal processes. The latter two require modification of alginate and addition of thermosensitive hydrogels (Lee and Mooney, 2012; Nunamaker *et al.*, 2007). Medical applications of alginate include local drug release and wound dressings (Lee and Mooney, 2012). *In vivo* degradation is dependent on the process of removing links between chains (Lee and Mooney, 2012). *In vivo*, alginate induces negligible immunological reactions (Leone *et al.*, 2008; Li *et al.*, 2008; Nunamaker *et al.*, 2007).

Alginate hydrogel stiffness can be regulated by changing its alginate weight/volume (w/v) percentage (Bron *et al.*, 2011). 2 % (w/v) alginate scaffolds resemble the native NP in terms of stiffness (Bron *et al.*, 2011). Furthermore, 2 % alginate hydrogels cross-linked with 0.025 M CaCl₂ achieved aggregate moduli (1.2 MPa) similar to the native IVD (Foss *et al.*, 2014). Alginate-based materials are often combined with synthetic polymers such as poly (epsilon-caprolactone) (PCL) or polyglycolide (PGA) to further enhance mechanical properties (Xu *et al.*, 2012). Alginate itself is often added to natural hydrogels for its easy to control cross-linking properties (Iwasaki *et al.*, 2004; Shao and Hunter, 2007; Zhang *et al.*, 2014).

Photocrosslinking alginate not only improved mechanical characteristics but also allowed for enhanced ECM synthesis *in vivo* by pre-loaded bovine NP cells, when compared to non-photocrosslinked gels (Chou *et al.*, 2009; Chou and Nicoll, 2009). In addition, AF, NP and CEP-derived stem cells were shown to proliferate and produce ECM in alginate hydrogels, albeit with distinct differences between the cell types (Melrose *et al.*, 2001; Wang *et al.*, 2014). *In vivo*, combination with CEP-derived cells resulted in better NP regeneration in a rabbit model of induced disc degeneration compared to AF- and NP-derived cells (Wang *et al.*, 2014). Further enhancement of NP cell differentiation and matrix production was achieved by adding glucosamine and chondroitin sulphate to alginate

(Foss *et al.*, 2014). The addition of collagen to alginate scaffolds not only promoted proliferation of seeded AF cells and MSCs *in vitro*, but also induced the migration of endogenous AF cells *in vitro*, although in all cases only collagen I and not collagen II was produced (Guillaume *et al.*, 2014). Other cues enhancing regeneration and differentiation inside alginate gels are RGD peptides, which have been shown to facilitate chondrogenic differentiation of human MSCs (Re'em *et al.*, 2010).

Altogether, alginate is a very versatile material that shows definite promise as a scaffold for regeneration of especially the NP. The main drawback of using this material is that the mechanism of degradation *in vivo* is not fully understood yet.

Highlights:

- Alginate is easy to use and its physical and chemical characteristics are well described.
- It is versatile and is very well suited as a structural addition to almost any hydrogel blend.
- Alginate is mostly used for NP regeneration.
- The degradation pathway of alginate *in vivo* is unclear, further research should focus on determining if this interferes with regeneration for the NP.

Hyaluronan

Hyaluronan or hyaluronic acid (HA) is a connective tissue polysaccharide consisting of repetitive units of D-glucuronic acid and N-acetyl-D-glucosamine residues (Kenne *et al.*, 2013), extensively studied for the purpose of tissue regeneration (Collins and Birkinshaw, 2013; Kim *et al.*, 2011). Hyaluronan derivatives are clinically applied for drug delivery and surgical procedures (Kogan *et al.*, 2007).

Biodegradation of HA occurs by the action of hyaluronidases and degradation rate can be adjusted by the incorporation of methacrylate groups also used for photocrosslinking (Kim *et al.*, 2011). Adjusting the w/v percentage of HA hydrogels or using HA with a greater molecular weight increases the stiffness, up to close to that of native NP, although it reduces the ability to withstand shear forces, which negatively influences hydrogel integrity (Chen *et al.*, 2013). In composite hydrogels, ECM production can be influenced by using dynamic hydrogels, *i.e.* hydrogels changing in physicochemical properties, for example at a particular high w/v percentage and hence stiffness, which reduces over time by hydrolytic action on caprolactone units (Chung *et al.*, 2009).

For IVD regeneration with HA, mainly NP cells and MSCs have been studied (Chung *et al.*, 2009; Cloyd *et al.*, 2007; Collin *et al.*, 2011; Su *et al.*, 2010). NP cells retain their rounded morphology while demonstrating a high viability in oxidised-HA gels (Chen *et al.*, 2013). Furthermore, it was demonstrated that the presence of HA facilitates their matrix synthesis as determined by GAG content (Peroglio *et al.*, 2012). HA hydrogels are often mixed with other natural hydrogels such as gelatin or with synthetic polymers, usually polyethylene glycol (PEG) (Collin *et al.*, 2011; Frith *et al.*, 2013). The addition of gelatin provides viscoelastic properties (shear modulus) resembling the native NP, while NP cells cultured on this combination still show regeneration (Chen *et al.*, 2013). In particular, gels containing lower molecular weight HA

combined with PEG were found to facilitate NP and AF cell proliferation (Jeong *et al.*, 2014).

MSCs can bind to HA through the cell surface receptor CD44 (Kota *et al.*, 2014). However, although higher density HA hydrogels enhance ECM production and retention by bone marrow derived MSCs, even distribution of ECM throughout the construct is limited (Erickson *et al.*, 2009). Several animal *in vivo* studies have been performed, each demonstrating that the injection of MSCs with HA into degenerate discs stimulates some regeneration as measured by restoration of disc height (Crevensten *et al.*, 2004; Ganey *et al.*, 2009; Ghosh *et al.*, 2012).

HA is an interesting material for IVD regeneration because it is an integral part of the native IVD. Its main setbacks are the differential effects of molecular weight and the choice of biomaterial to be combined with. Higher molecular weight HA resembles the IVD mechanically but inhibits cell function, which benefits from lower molecular weight formulations.

Highlights:

- Hyaluronic acid is a very versatile biomaterial, is biocompatible and relatively easy to handle.
- Its chemical structure allows for adhesion of many different cell types examined for IVD regeneration.
- HA as scaffold shows great potential for NP regeneration.
- Depending on its molecular weight, HA may also be a good additive to synthetic hydrogels to stimulate ECM formation.

Collagen and gelatin

Collagen is one of the most abundant proteins found in the human body, making up about one third of our total protein weight (Ferreira *et al.*, 2012). Gelatin is a material derived from animal collagen usually through thermal denaturation. Both are biodegradable and their degradation results in non-toxic by-products (Huang *et al.*, 2005; Lee *et al.*, 2001; Nicodemus and Bryant, 2008). Current medical uses include applications in plastic and reconstructive surgery and as a vehicle for drug delivery (Ferreira *et al.*, 2012). Collagen type I as a hydrogel is also investigated for bone and soft tissue regeneration (Badylak *et al.*, 2009; Bron *et al.*, 2012; Cen *et al.*, 2008; Ferreira *et al.*, 2012).

Collagen I hydrogels can attain rheological properties resembling the native NP when compressed (Bron *et al.*, 2009). When implanted inside damaged *ex vivo* bovine IVDs, the collagen matrix was able to restore disc height and mechanical behaviour of the spinal segment. The main issue reported is implant extrusion (Wilke *et al.*, 2006). Hydrogel stiffness can be adjusted by changing the w/v percentage (Bron *et al.*, 2009). Native caprine NP and AF cells successfully migrated into 3.0 % collagen type I scaffolds, which was dependent on collagen digestion by the migrating cells (Bron *et al.*, 2012). Human adipose-derived stromal cells seeded on elastin-glycosaminoglycan-collagen I composite hydrogels attained an NP-like morphology (Mercuri *et al.*, 2014). Gelatin, mostly derived from collagen I, is often used in composite hydrogels to improve biomechanical properties. Addition of gelatin to HA improves viscoelastic properties (Chen *et al.*, 2013; Malhotra *et al.*, 2012). Similarly, addition of gelatin to chitosan hydrogels improved rheological properties and

left cell viability unaffected (Cheng *et al.*, 2010). Addition to agar hydrogels increased equilibrium elastic behaviour to that of the native NP, due to enhanced osmotic swelling (Strange and Oyen, 2012). The addition of gelatin to other hydrogels not only influences the mechanical properties but also has the ability to improve the microenvironment for NP cells, because gelatin, like collagen, contains regions that allow for cell attachment *e.g.* RGD motifs (Chen *et al.*, 2013).

In vivo, gelatin-based hydrogels could suppress the progression of IVD degeneration in a rabbit model of NP aspiration (Nagae *et al.*, 2007). Also, the injection of cell-free gelatin-based microspheres into a rabbit disc degeneration model showed less apoptosis in the NP than IVDs without this treatment (Sawamura *et al.*, 2009).

In conclusion, collagen and gelatin hydrogels, much like HA, are interesting because they form an integral part of the ECM, thereby improving cell adhesion. Collagen hydrogels can prove difficult to work with, because they easily denature at temperatures above 37 °C (Ferreira *et al.*, 2012). They can be blended into other hydrogels in different forms, so as to enhance the drug/growth factor release profiles as well as enhance the compatibility of a composite hydrogel.

Highlights:

- Gelatin/collagen is one of the best-understood natural biomaterials.
- Mechanical properties are well understood.
- Currently widely examined for other RM purposes and controlled release strategies.
- A good addition to any hydrogel blend for its structural and cell binding properties.
- Promising for NP regeneration.
- Further research should focus on studying this material *in vivo*.

Agarose

Agarose is a polysaccharide derived from algae and composed of monomer agarobiose units consisting of one D-galactose and one 3,6-anhydro-L-galactopyranose. Agarose gels when mixed with water provide a 3-dimensional helical structure (Gruber *et al.*, 2006). Agarose by itself is non-degradable *in vivo*. Composites containing agarose degrade by degradation of the added hydrogel components, resulting in fragmentation of agarose over time. Therefore, complete degradation is not guaranteed (Hunt and Grover, 2010). Degradation time is controllable by changing composite component concentrations. None to mild immunological responses were noted *in vivo* in subchondral cartilage defects (Gupta *et al.*, 2014). Human AF cells cultured in agarose produced more GAGs than in collagen sponges but proliferated less. To what extent collagen sponges can be considered truly 3D is unclear; however, as in sponges with large pores, cells may not be surrounded by the biomaterial but rather are attached to the pore surfaces (Gruber *et al.*, 2006). Agarose, in combination with a synthetic electrospun polymer, mimicked the IVD in compression and torsion tests, with agarose as NP and the electrospun polymer as AF (Nerurkar *et al.*, 2010). MSCs seeded on these composite structures produced ECM components (Lazebnik *et al.*,

2011; Nerurkar *et al.*, 2010). Bovine NP cells cultured in agarose hydrogels infused with transforming growth factor-beta (TGF- β) were capable of responding to cyclical compressive loading with increased matrix synthesis and gene expression (Tilwani *et al.*, 2012).

Research groups are combining agarose into natural/synthetic composite scaffolds to allow more comprehensive mechanical testing of NP-AF analogues. Agarose hydrogels singularly may not suffice for regeneration of the IVD, but it holds promise as a structural component to other hydrogel formulations.

Highlights:

- Particularly interesting for NP regeneration as structural component for hydrogel blends.
- It is a relatively difficult material to work with, as it remains liquid only at high temperatures.
- As a single hydrogel, it has limited use, especially since it is non-degradable *in vivo*.

Fibrin

Fibrin is a protein associated with the blood coagulation pathway. It is used in medicine as a sealant. Fibrin is biocompatible and non-immunogenic (Ahmed *et al.*, 2008). Physical scaffold characteristics can be adjusted by adjusting the concentration of Ca²⁺, fibrinogen and thrombin. Fibrinogen concentrations above 25 mg/mL combined with Ca²⁺ 20 mmol/L produced hydrogel constructs that remained stable for up to 3 weeks (Eyrich *et al.*, 2007). Fibrin is often combined with other materials such as silk, HA or PGA (Colombini *et al.*, 2014; Hegewald *et al.*, 2010; Park *et al.*, 2011b; Stern *et al.*, 2004). The addition of silk-fibroin to fibrin/HA hydrogels provides greater mechanical strength than fibrin/HA hydrogels alone while preventing shrinkage *in vivo* (Park *et al.*, 2011b). In fibrin/HA hydrogels, porcine NP cells showed more proliferation and proteoglycan production compared to alginate culture (Stern *et al.*, 2000). Fibrin/HA cultured with bovine NP cells and injected in a nucleotomy defect in organ culture, demonstrated better integration with native NP tissue compared to fibrin hydrogels without the addition of HA, while restoring disc height, with a compressive stiffness similar to native NP levels (Li *et al.*, 2014).

Fibrin/genipin based hydrogels have been tested as adhesives for AF repair. Genipin is an aglycone derived from the Gardenia fruit with excellent cross-linking properties at low toxicity (Sung *et al.*, 1999). A combination of fibrin/genipin hydrogels improved *in vivo* hydrogel longevity compared to fibrin-only hydrogel scaffolds in a subcutaneous model in rats (Likhitanichkul *et al.*, 2014). In a bovine *ex vivo* AF defect model, fibrin/genipin hydrogels prevented disc height loss and remained integrated after more than 10,000 cycles of loading (Likhitanichkul *et al.*, 2014). Furthermore, it was demonstrated that AF cells remained viable and migrated into fibrin/genipin hydrogels (Likhitanichkul *et al.*, 2014; Schek *et al.*, 2011). Fibrin hydrogels functionalised with TGF- β stimulated human MSCs (hMSC) to undergo chondrogenesis, although soluble TGF- β resulted in a more profound induction of regeneration (Diederichs *et al.*, 2012). A fibrin/alginate blend of 40:8 w/w was able to promote MSC proliferation as well as GAG and collagen

type II production while combining physical characteristics of alginate and fibrin hydrogels (Ma *et al.*, 2012). The main problem reported was formation of islands of alginate in the fibrin hydrogel. Moreover, at a w/w% content of 0.6 or above, alginate interfered with chondrogenesis (Ho *et al.*, 2010). Fibrin injected in solid poly(trimethylene carbonate) scaffolds as repair strategy in an *ex vivo* annulus defect model, resulted in GAG deposition after 14 days of culture. However, this appeared independent of the incorporated MSCs (Pirvu *et al.*, 2015).

In vivo fibrin was tested as a cell carrier for IVD regeneration in a mini-pig needle nucleotomy model. Chondrocytes proved to be more effective in restoring disc height and matrix production than MSCs or cell free controls (Acosta *et al.*, 2011).

Fibrin is a promising biomaterial for use as a cellular scaffold. It has excellent biocompatibility, combined with the ability to stimulate chondrogenesis. Fibrin can be considered for any blend of hydrogels for intervertebral disc regeneration. The main associated drawback is that fibrin is readily degraded by enzymatic action *in vivo*; cross-linking, however, can overcome this challenge. Despite the widespread use of fibrin in medical practice, there are as yet no on-going clinical trials using fibrin as a basis for IVD regeneration.

Highlights:

- Fibrin shows excellent promise as a cellular scaffold whereby extensive ECM production can be achieved by a wide variety of cell types.
- It has excellent biocompatibility.
- Fast degradation *in vivo*; requires modification for *in vivo* use for extended periods of time.
- Fibrin can be used for both AF and NP regeneration.
- Further research into understanding the biomechanical properties should be performed.

Synthetic hydrogels

Synthetic hydrogels are mainly based on polymer networks that can absorb large amounts of water. They are characterised by their ease of modification. In addition, self-assembling peptide hydrogels have been a recent focus of attention (Loo *et al.*, 2012); however, as only two publications have described their application for NP incorporation (Tao *et al.*, 2014; Wang *et al.*, 2012), these are not further discussed.

Polyethylene glycol

Polyethylene glycol (PEG) is a synthetic polyether that is manufactured through the polymerisation of ethylene glycol and carries the molecular formula C_{2n}H_{4n+2}O_{n+1}. In medical practice, it forms the basis for laxatives, due to its induction of water absorption, which also makes it an interesting biomaterial for NP regeneration. Like many other hydrogels, PEG is the subject of research in the field of controlled drug delivery (Ashley *et al.*, 2013; Lin and Anseth, 2009). Although PEG-based hydrogels exhibit a similar range of biomechanical properties (compression modulus, tensile strength and hydrostatic swelling) as articular cartilage (Nguyen *et al.*, 2012), it is rarely used for RM research as a stand-alone hydrogel as they are non-cell adhesive (Zhu, 2010). Therefore PEG hydrogels

for RM goals should be functionalised with cell adhesive peptides such as RGD sequences (Benoit and Anseth, 2005; Hern and Hubbell, 1998) or mixed with hydrogels that do allow for adhesion (Collin *et al.*, 2011; Drira and Yadavalli, 2013; Zhu, 2010).

Biodegradation of PEG-based gels can be achieved through the addition of synthetic or natural components such as polylactide or enzyme-sensitive peptides (Raeber *et al.*, 2005; Zhu, 2010). This approach is particularly useful for photopolymerised hydrogels, where cross-linking typically reduces biodegradation. Porcine NP cells encapsulated in laminin-functionalised PEG hydrogels (Francisco *et al.*, 2014) produced collagen and GAGs, which was enhanced in the softer PEG-laminin hydrogels compared to PEG-only hydrogels. Recently, a new PEG-based gel covalently linked to serum albumin and mixed with high molecular weight HA has come close to clinical application. The gel, which has anti-angiogenic properties *in vitro* and *in vivo*, showed an increased expression of collagen II and aggrecan in cells isolated from human IVD tissue harvested in the course of spinal fusion surgery (Benz *et al.*, 2012a; Scholz *et al.*, 2010). In this hydrogel, cartilaginous matrix production was not only found *in vitro*, but also after subcutaneous implantation in immune deficient mice, with most matrix formation around cells of human origin (Benz *et al.*, 2012a). However, application of a gel-IVD cell construct in a sheep model of nucleotomy-induced IVD degeneration could not provide information on the capacity of the gel to mitigate disc degeneration, as all IVDs healed spontaneously, but no adverse effects of gel administration were found either (Benz *et al.*, 2012b). Despite a lack of clear evidence for disc regeneration *in vivo*, a randomised clinical trial was initiated for patients with disc herniation (Web ref. 1).

PEG hydrogels are promising candidates for IVD regeneration because of their proven ease in use and adjustability. Furthermore, PEG hydrogels could be considered as additions to any hydrogel formulation.

Highlights:

- Easy to synthesise and adjust for RM purposes, especially for NP regeneration.
- Biomechanical properties are conducive for IVD regeneration.
- There are no attachment motifs for cells; therefore, functionalisation or blending is required.
- Can be useful as an addition to any hydrogel blend.
- Further research should focus on modifying PEG hydrogels for cell attachment.

Polyurethane

Polyurethanes or polycarbonate urethanes (PU) are materials normally used to produce hard plastics for applications such as electrical circuit housings. They have, however, been widely used in the medical world for years where they form the basis for vascular grafts (Santerre *et al.*, 2005; Tiwari *et al.*, 2002). Current research focuses on PU scaffolds for cartilage repair (Hung *et al.*, 2014; van Tienen *et al.*, 2009). PU and its derivatives are generally biodegradable, *in vivo* degrading to water and carbon dioxide (Santerre *et al.*, 2005; Yang *et al.*, 2009). These by-products do not affect the local pH as much as

poly(lactide-co-glycolide), reducing negative effects on the microenvironment (Yang *et al.*, 2009). Biomechanical functionality appears to be promising, as PU hydrogels injected in human cadaveric spines could withstand loading compression (Dahl *et al.*, 2010). The PU hydrogel translated compression forces to the AF in a similar fashion as the native NP.

Various PU composite scaffolds have been created, with PU/silk fibroin (SF) hydrogels as the main example (Hu *et al.*, 2012; Park *et al.*, 2013). The addition of silk to PU scaffolds provides control over the degradation rate and mechanical properties. PU/SF scaffolds were used to replace the NP in cadaveric porcine spines by injection into the NP cavity (Hu *et al.*, 2012). To scaffolds made of electrospun PU, AF cells attached and produced ECM components (Yeganegi *et al.*, 2010; Attia *et al.*, 2011). The addition of fibrin or fibronectin as cell attachment proteins further improved cell attachment to PU scaffolds (Mauth *et al.*, 2009; Yeganegi *et al.*, 2010). Furthermore, rabbit BMSCs proved to remain viable when seeded in a PU/SF hydrogel and produced extensive ECM.

PU-based scaffolds hold promise for IVD regenerative strategies mainly due to their ease of use and the excellent track record with biocompatibility in medicine and medical research. The disadvantage of PU as a scaffold is mainly that very little research into NP cell and PU scaffold interaction has been conducted.

Highlights:

- PU scaffolds are highly biocompatible and easy to manufacture in different forms (gels and electrospun scaffolds).
- The PU/SF composite has shown promise as a scaffold for NP and electrospun PU for AF regeneration.
- Only few data are available on the response of NP cells to PU.

Natural-based solid biomaterials

Solid biodegradable scaffolds are usually preformed before implantation. They can have many of the same characteristics of hydrogels and function in a similar way. They are, however, not injectable through a needle, but may still be used in minimally invasive surgery. The advantage of using solid scaffolds over hydrogels is that mechanical properties are generally easier to control and better mechanical support can be provided. Among the natural-based solid scaffolds, small intestinal submucosa has been investigated most intensively. In addition, *in vitro* regenerative capacities for AF and NP cells were shown of silk-based scaffolds (Chang *et al.*, 2010; Zeng *et al.*, 2014; Park *et al.*, 2012), which due to the limited number of studies are not further described.

Small intestinal submucosa

Small intestinal submucosa (SIS) is derived from the – usually porcine – intestine. It is currently investigated for oesophageal reconstructive surgery and regenerative restoration of soft tissue and tendon defects (Badylak *et al.*, 1995; Dejardin *et al.*, 2001; Jonsson *et al.*, 2011; Tan *et al.*, 2012).

SIS contains growth factors such as TGF- β (Le Visage *et al.*, 2006). *In vitro*, SIS scaffolds provided suitable

environments for AF and NP cells to produce ECM (Le Visage *et al.*, 2006). Although an organised regenerated structure failed to be produced upon application of a SIS scaffold after box annulotomy in sheep, the scaffold did increase the hydration state of the annulus fibrosus and improved mechanical functionality (Ledet *et al.*, 2009). Biomechanical properties could be further improved by combination with poly(lactide-co-glycolide) (PLGA); this combination also resulted in greater NP cell adhesion and ECM production in the composite scaffold (Kim *et al.*, 2014b).

SIS is a material that needs further research to determine whether it can be used successfully *in vivo* for the regeneration of the IVD. However, the main concern with using decellularised animal matrices is the potential transfer of disease and immunogenicity.

Highlights:

- An ill-defined material that may show promise as a cellular scaffold, in particular for annulus repair.

Synthetic solid biomaterials

There are several synthetic materials that can be used in preformed solid scaffold configuration. Although there only are a few options, they are important to discuss because they hold great promise.

Poly(lactide/glycolide)

Poly(lactide) (PLA) and polyglycolide (PGA) are synthetic polymers with the respective chemical formulae $(C_3H_4O_2)_n$ and $(C_2H_2O_2)_n$. Several different formulations of PLA have been used: poly(L-lactide) (PLLA) and poly(D,L-lactide) (PDLLA) which is a racemic mixture, while PGA is not racemically structured. However, a composite of PLA and PGA termed poly(lactide-co-glycolide) (PLGA) appears most interesting for RM. Osteoblasts adhered better to PLGA scaffolds than single PLA or PGA scaffolds, probably due to better cell adhesion properties of the composite scaffolds (El-Amin *et al.*, 2003). Degradation of PLGA, through hydrolysis of ester linkages, results in the production of lactic and glycolic acids which are naturally present in the human body and hence non-toxic (Danhier *et al.*, 2012), although ensuing local acidification may be harmful (Agrawal and Athanasiou, 1996; Ding and Schwendeman, 2008). PLGA has Food and Drug Administration (FDA) approval for use in the human body for controlled drug release applications, where it is also the subject of much research (Lai *et al.*, 2014). However, very little biomechanical data are available on PLGA scaffolds used for IVD regeneration.

Also, NP cells seeded on PLGA scaffolds and subcutaneously implanted in mice produced extensive ECM in scaffolds with small pores (90–250 μ m) (Kim *et al.*, 2014a). *In vitro*, adipose-derived stem cells have been shown to initiate chondrogenesis when seeded on PLGA scaffolds as measured through collagen type II expression. Upon implantation into pouches in nude mice, chondrogenesis was reflected by mRNA for collagen type II, aggrecan and other chondrogenic genes (Mehlhorn *et al.*, 2009). PLGA constructs seeded with NP cells and inserted into the IVD of beagle dogs maintained disc height better

than no implant or PLGA constructs alone (Ruan *et al.*, 2010), although in a rabbit model of disc degeneration, cell-free scaffolds showed migration of cells and chondrogenic matrix production after 12 months (Endres *et al.*, 2010). PLGA microsphere scaffolds seeded with adipose-derived stem cells were able to partially regenerate the IVD in rats after 24 weeks, where the addition of cells over cell-free scaffolds demonstrated a clear added value (Liang *et al.*, 2013).

PLGA scaffolds are interesting because they have excellent biocompatibility and are widely studied as drug release systems, which has added value for any IVD regenerative implant.

Highlights:

- PLA, PGA and PLGA have good biocompatibility.
- PLGA shows promise for NP regeneration, but AF regeneration was not studied.
- PLGA could be used for combining drug delivery with regeneration.
- Further research should focus on biomechanical properties relevant for IVD regeneration.

Poly(ϵ -caprolactone)

Poly(ϵ -caprolactone) (PCL) is a synthetic polyester produced through the polymerisation of ϵ -caprolactone $(C_6H_{10}O_2)$. PCL is biodegraded through hydrolysis of the ester linkages, but degradation takes months to years, far slower when compared to other materials such as PLGA. The degradation time can be reduced by the addition of poly(vinylalcohol) (PVA) or PGA into the polymer chain (Dash and Konkimalla, 2012; Sinha *et al.*, 2004). PCL is approved for use in the human body by the FDA and as such has been part of various medical applications for years, such as medical sutures. PCL has currently undergone a resurgence in research for the purpose of controlled drug release (Agrawal and Ray, 2001; Dash and Konkimalla, 2012; Silva *et al.*, 2007).

PCL is interesting for regeneration of the AF because electrospun PCL fibres can mimic an AF fibre structural alignment (Koepsell *et al.*, 2011b). It was shown that organised alignment by electrospinning induces ECM production by porcine AF cells on PCL scaffolds (Koepsell *et al.*, 2011a). PCL was previously demonstrated to be a good carrier of human bone marrow derived MSCs (Li *et al.*, 2005a), when cultured in 3D for 21 days extensively produced chondrogenic ECM. PCL as part of a whole IVD replacement scaffold was shown to hold promise for regeneration of the IVD (Martin *et al.*, 2014).

PCL-based scaffolds usually have to be pre-shaped before implantation, but the addition of other synthetic scaffold materials allows the production of injectable hydrogels together with PCL. The addition of PCL into a synthetic hydrogel also allows the hydrogel to function as a scaffold for the longer term (Lopez *et al.*, 2010).

Highlights:

- PCL is an FDA-approved material for use in the human body.
- Holds promise as an AF regeneration component due to its high tensile strength.
- Little use for this material as a NP structural component.

- Can be used as hydrogel in combination only or as a material to reinforce hydrogel structures.
- *In vivo* tests with PCL as part of a composite disc-like structure for whole IVD replacement/regeneration.

Biomaterials as sustained delivery agents in IVD regeneration

In addition to their role in supporting native or exogenously added cells, scaffolds, either solid or hydrogel-based, can be used for the delivery of cues required for regeneration. Several bioactive substances have been incorporated into delivery systems for IVD regeneration, including regenerative factors, such as growth factors and hormones, but also anti-oxidant and anti-inflammatory factors targeting mechanisms of cell senescence and matrix catabolism. Thus far, mostly nano- and microstructured injectable biomaterials have been used to achieve sustained release and are also discussed here, although they do not have a biomechanical nor intrinsic regenerative role in this type of application. For a general overview of materials for potential use as delivery vehicles, readers are referred to (Blanquer *et al.*, 2012).

Natural hydrogels

In the degenerated IVD, apoptosis and senescence related to reactive oxygen species (ROS) production rises with increasing degeneration (Heathfield *et al.*, 2008). Ferulic acid, a strong anti-oxidant, loaded onto an injectable thermosensitive chitosan/gelatin/glycerol phosphate hydrogel was shown *in vitro* to decrease hydrogen peroxide-induced oxidative stress of NP cells and reduce the levels of cell apoptosis. Moreover, sustained release of ferulic acid resulted in matrix anabolism, at the gene and protein level (Cheng *et al.*, 2011; Cheng *et al.*, 2013).

Biodegradable gelatin hydrogel microspheres impregnated with several poly-ionic growth factors released bioactive factors in a sustained fashion (Tabata *et al.*, 1999) and upon impregnation with platelet-rich plasma (PRP), have been shown to inhibit IVD degeneration *in vivo* in a rabbit model of induced IVD degeneration (Nagae *et al.*, 2007). Partial prevention of IVD height loss, increased gene expression levels of proteoglycan core protein and type II collagen, and a lower number of apoptotic cells compared to microsphere-only controls was noted. As this study was performed in a rabbit model with very mild IVD degeneration, additional studies are required to show proof of concept in moderate to severe IVD degeneration.

The release of chemokines to attract endogenous stem cells was shown as a promising alternative approach towards regeneration, as demonstrated by the enhanced attraction of MSCs by injection of stromal cell-derived factor-1 (SDF-1)-loaded HA hydrogels in an *ex vivo* nucleotomy model, with a clear added value over SDF-1 injected directly (Pereira *et al.*, 2014).

Synthetic hydrogels

Intra-discal injection of a poly-N-isopropylacrylamide (pNIPAAm)-LDH-based hydrogel releasing celecoxib to

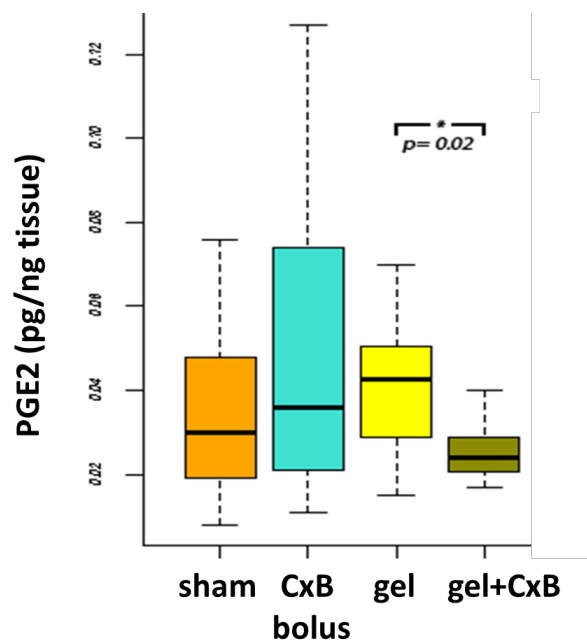


Fig. 3. Inhibition of PGE₂ production by hydrogel-mediated controlled release of celecoxib in a canine model of spontaneous disc degeneration. A PNIPAAm-based thermoreversible hydrogel was loaded with celecoxib (gel+CxB) and injected in a canine model of spontaneous disc degeneration. Controls were unloaded gel (gel), sham injection (sham), and CxB alone (CxB bolus).

specifically inhibit cyclooxygenase-2 (COX-2) activity showed a constant, albeit minor reduction of inflammation in a canine model of spontaneous IVD degeneration (Fig. 3; Willems *et al.*, 2015). The minor effect may have been related to the limited degeneration grade in this model. As this gel was proven to be suitable for delivery and transfection of siRNA, very specific inhibition of various other factors through RNAi-mediated gene silencing is also envisaged (Willems *et al.*, 2015).

Solid synthetic biomaterials

Various studies were performed using PLGA as delivery system. Nanostructured composites of PLGA microspheres loaded with dexamethasone and FGF-embedded heparin/poly(L-lysine) nanoparticles effectively induced chondrogenic differentiation of MSCs seeded on the microspheres (Liang *et al.*, 2012). *In vivo*, in a rat model of puncture-induced IVD degeneration, adipose-derived stem cells were seeded on a similar drug delivery system of PLGA microspheres loaded with dexamethasone and TGF-β₃, with a release for at least 28 d *in vitro*. At 24-weeks follow up, IVDs treated with the delivery system alone and the delivery system seeded with cells, regained height and restored proteoglycan content compared to untreated controls, although not to the levels of healthy controls (Liang *et al.*, 2013). Likewise, PLGA microspheres releasing growth differentiation factor 5 (GDF-5) over the course of 42 days improved IVD height, GAG and DNA content of treated discs compared to punctured untreated

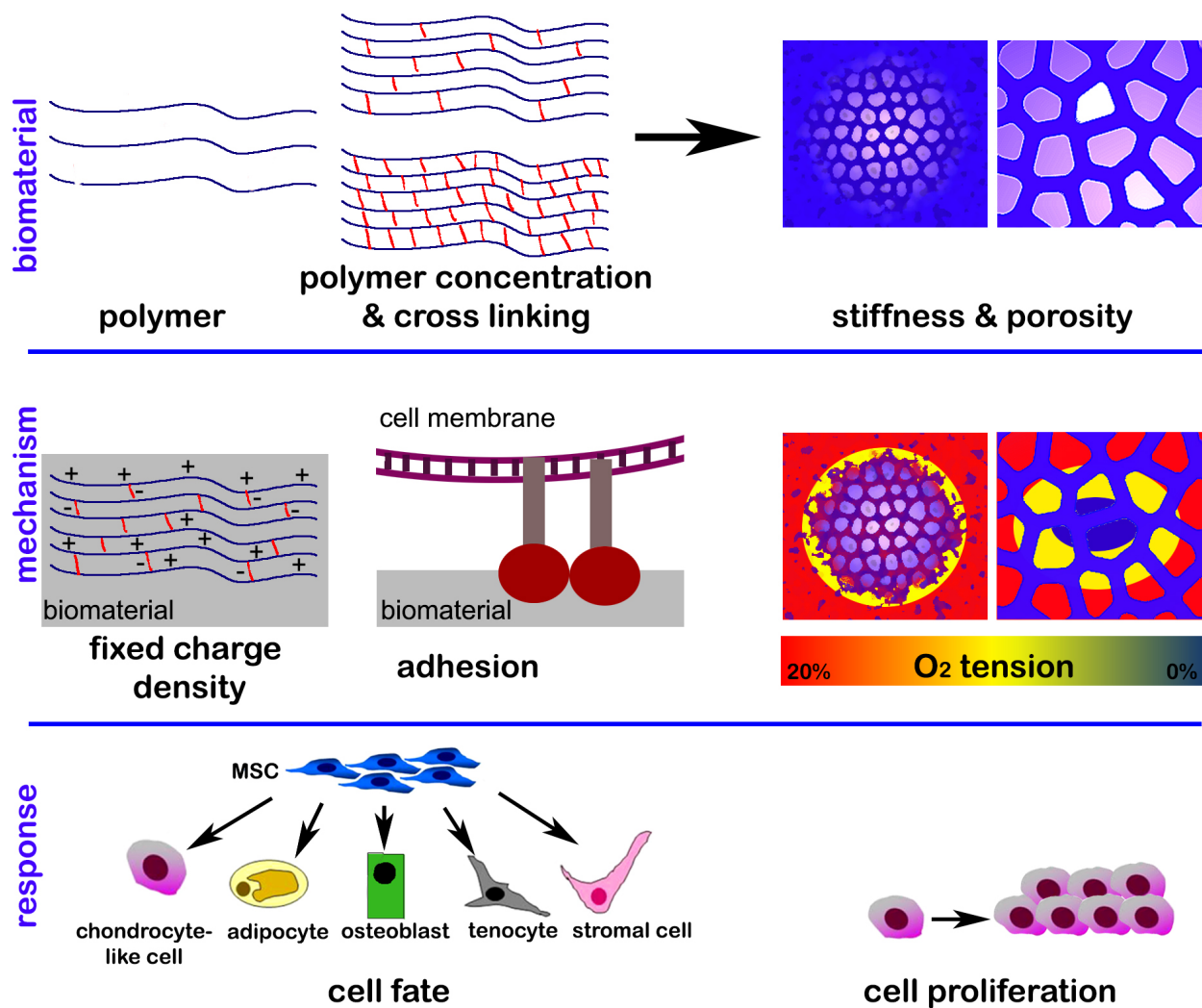


Fig. 4. Scaffold characteristics determining cell behaviour and tissue regeneration. Biomaterial properties affect cell behaviour through various mechanisms, thereby determining the differentiation pathways chosen and the proliferative response.

IVDs in a rat tail degeneration model (Yan *et al.*, 2013). However, no single intra-discal application of growth factors was included as comparator in these studies.

Release of interleukin-1 receptor antagonist (IL-1ra) from PLGA microspheres was shown to attenuate the degradation induced by IL-1 β in agarose-bovine NP cell constructs. The inhibitory effects on inflammation were most obvious during the first week of culture. Strikingly, although beyond 3 days of release proteoglycan content was not restored further, the biomechanical properties of the constructs were restored to control levels (Gorth *et al.*, 2012).

Altogether, it appears that the concept of employing biomaterials for the sustained delivery of agents in IVD regeneration is just emerging. However, local prolonged exposure to factors modulating regeneration and degeneration holds great promise by reduction of systemic side effects and increasing effectivity.

Towards rational design of biomaterials for IVD regeneration

The response of cells to biomaterials depends on a complex set of chemical, physicochemical and physical parameters (Fig. 4). Understanding the intrinsic effects of biomaterials on incorporated cells and addressing these in focussed biomaterial studies is likely to enhance the search for the most effective material.

Extracellular matrix-cell interaction

Mesenchymal cell attachment to its surrounding material strongly affects its behaviour. Most of this attachment will be mediated by the ECM binding integrins present on the cell surface. The ECM motifs are either provided directly by the material used (gelatin, collagen, fibronectin), by serum adhesion proteins (Kobayashi *et al.*, 2005; Yang *et al.*, 2013; Dodo *et al.*, 2013), or early deposition of ECM by the cells themselves (Pearlstein *et al.*, 1980; Desai *et*

al., 2014; Jin *et al.*, 2007; Sharma *et al.*, 2012; Xu *et al.*, 2013). Integrin-mediated cell-matrix binding not only affects chondrogenic cell survival and proliferation *in vitro* and *in vivo* (Gilchrist *et al.*, 2007; Loeser, 2014), but is also involved in cell differentiation by regulating the response to materials with different mechanical properties. High stiffness results in osteogenesis by MSCs (Huebsch *et al.*, 2010), most likely through the availability of ECM binding sites (Trappmann *et al.*, 2012) and a process involving stress fibre formation. Inhibition of this pathway favours adipogenic or chondrogenic differentiation (Dupont *et al.*, 2011; Khetan *et al.*, 2013; McBeath *et al.*, 2004; Xu *et al.*, 2014), probably co-regulated by growth factor signalling (Park *et al.*, 2011a). In addition to integrin receptors, several other cell-material receptors have been described, of which the hyaluronic acid receptor CD44 also appears to mediate chondrogenic differentiation and regeneration by hMSCs (Bian *et al.*, 2013; Wu *et al.*, 2013), although HA binding appears not to be involved in sensing stiffness (Khetan *et al.*, 2013). Toll like receptor (TLR) signalling, originally identified in immune cells, may affect MSC and chondrocyte behaviour by enhancing cytokine production (Shokouhi *et al.*, 2010; Campo *et al.*, 2012; Sillat *et al.*, 2013; Tsuchida *et al.*, 2013), although the ensuing effect on regeneration was not studied yet.

Hypoxia, porosity and fixed charge density

In terms of physical and physicochemical properties affecting cell behaviour, hypoxia is known to enhance chondrocyte redifferentiation through hypoxia-inducible factor 1alpha (HIF1 α) expression and GAG deposition by MSCs (Duval *et al.*, 2009; Buckley *et al.*, 2012). Fixed charge density has been shown to above neutral values inhibit chondrocyte redifferentiation on top of hydrogels (Yang *et al.*, 2010).

Towards efficient biomaterial design

All in all many biomaterial-related properties are known to affect the cell response, of which many cannot be fully dissected from each other. Porosity will for example simultaneously affect hypoxia, nutrient diffusion, and attachment and stress fibre formation. The relative contribution of these properties in determining cell behaviour is unclear and will in addition depend on cell type. It should also be borne in mind that crosslinking of natural biomaterials, such as methacrylation of HA or crosslinking of gelatin, to enhance biomechanical properties may compromise degradability and biocompatibility.

A promising approach towards efficient design of materials for IVD cell regeneration may entail the use of biomaterial arrays. 3D high throughput arrays of over 1000 different biomaterials have been applied to study proliferation and differentiation, until now mainly of MSCs and preosteoblasts (Dolahatshai-Pirouz 2014; Chatterjee 2010). Biomaterial arrays not only can be used to determine the optimal response, in a widely varying array of biomaterials, but also in arrays of very similar polymers to determine essential chemical characteristics. Testing of material blends is also most likely possible, though not undertaken as yet. In addition to measuring

cell differentiation and proliferation, the readout of biomaterial screens can also be biomechanical (Tweedie *et al.*, 2005), which will be crucial as the type and magnitude of biomechanical input to the cell-biomaterial construct, in particular the relation between hydrostatic and shear stress, will affect differentiation (Carter *et al.*, 2004; Neidlinger-Wilke *et al.*, 2009). Final identification of biomaterials or their physicochemical characteristics as regenerative will be achieved by automated multifactorial analysis of the results.

Although the design of these arrays will be a task for skilled engineers, defining the readout parameters and using the right cell type will be the responsibility of the IVD biologist. Here, a key challenge may lie in outlining IVD cell differentiation and regeneration, which until now has mostly been defined in terms of chondrogenic ECM production, which in particular will not distinguish between cartilage and nucleus pulposus tissue. Recently, a consensus paper was published defining the healthy NP cell by several protein markers and functional characteristics (Risbud *et al.*, 2015). As hardly any of these markers are present both exclusively and exhaustively in all NP cells, the ratio of aggrecan/collagen II may possibly be the most reliable parameter to relatively easily and safely define NP-like regeneration. This ratio has been shown to be always above 5:1 in the NP, even around 25:1 in healthy tissue, whereas for cartilage this never exceeds 3:1. Other screen readouts, for some of which other cell types will be used, may be anti-inflammatory, anti-angiogenic and anti-neurogenic properties, given the suggested association between low back pain and nerve ingrowth towards the centre of the NP. Finally, as different stages of IVD degeneration may require different types of biomaterials, the use of cells from different degeneration stages in the biomaterial screens will be required.

Importantly, in the course of validation, *ex vivo* and *in vivo* models in different stages of degeneration will be a prerequisite to match biomaterials to degeneration grade. In addition to the medium-throughput screening of *in vivo* biocompatibility already available (Oliveira *et al.*, 2014) further validation in *ex vivo* IVD organ culture models, preferably of clinically relevant sizes, may allow for standardised and reliable testing before finally using *in vivo* models (Hudson *et al.*, 2013).

Concluding Remarks

In the 21st century, regenerative medicine will become one of the most important strategies of treating disease in general and IVD degeneration in particular. Biomaterials may serve a crucial role in this approach because they provide immediate mechanical support and instruct cells in the IVD to differentiate and produce new extracellular matrix.

The advantages of natural materials include established degradation pathways, biocompatibility and safety. In general, they provide more favourable environments for cell proliferation and regeneration. However, the material properties of natural polymers are difficult to control, and the manufacturing processes are expensive

for recombinant proteins, or are based on animal tissues, with concomitant regulatory issues. Synthetic biomaterials allow for easy and reproducible manufacturing, while their chemical properties are simpler to adjust. However, the by-products of degradation are in some cases harmful and the interaction with cells often limited. In practice, most of the materials mentioned have been tested in mixtures, because this allows blending of properties from each material, thereby improving biodegradability, biocompatibility or biomechanical functionality.

On the whole, the application of biomaterials for regeneration of the IVD appears, until now, to have been mainly directed by intuition and/or mere availability. This has likely not enhanced progress in this area, as illustrated by the fact that, to date, only one hydrogel has made it into a clinical trial. Advancement of the field towards more effective design of biomaterials may be provided by high throughput screening of biomaterials, in which several characteristics of biomaterials are related to their regenerative effects. However, as this will likely involve a substantial lag time in development, an alternative route may possibly lie in extensive comparative studies carried out at several IVD research laboratories, leading to the identification of some kind of reference biomaterials for NP and AF regeneration. Standardisation of characterisation and testing, including relevant biomechanical properties, biocompatibility and regenerative responses, would provide a major advantage here.

Finally, the clinical application of biomaterials may require some further research into the surgical techniques needed. Already, large sized needle injection of large volumes of fluid (Chee *et al.*, 2014) was suggested to accelerate IVD degeneration (Carragee *et al.*, 2009). Therefore, materials for NP regeneration are preferably injectable, using needles with small diameters, thereby excluding solid scaffolds and limiting the injectable volume. Alternatively, an approach through the endplate may be considered. AF regeneration may on the other hand be more feasible with solid scaffolds that at the same time withstand the tensile stresses and are capable of firmly adhering to the native tissue.

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Web Reference

1. <http://clinicaltrials.gov/show/NCT01640457> [18-11-2014]

Discussion with Reviewers

Reviewer I: I would agree with the authors that the ability to perform *in vitro* high throughput screening of biomaterials is required to improve on their selection for use in IVD repair strategy. However, could the authors briefly discuss what are in their opinion the “minimum” and specific *ex vivo* quantitative outcome parameters that should be collected to select the most suitable stiffness or tensile strength close to IVD tissues, EC matrix accumulation / composition, *etc.*?

Authors: High throughput screening is usually based on readout parameters that are easy to measure, often gene expression-based detection systems employing luciferase or GFP. To this end, simple detection of collagen II and aggrecan expression with as negative markers collagen X and collagen I (for the NP) would be useful. The use of medium throughput assays that can measure these genes at the protein level, aiming for the healthy tissues’ ratios of their respective ECM proteins, at least including collagen II, I and aggrecan, could be the next step. Ideally, medium to low throughput systems will be developed that can subsequently measure biomechanical properties of mature biomaterial-cell constructs. Those characterising NP tissue, *i.e.* elastic and shear modulus, and the AF, tensile strength and compressive modulus, would predict functionality and thus be helpful in final selection of materials for more extensive experiments, including *in vivo* models of disease.

Reviewer II: The authors note the need for a ‘standard’ or reference material for comparison studies to be made. The same might also true for a standard media composition and oxygen environment. What would these authors suggest for the field in terms of reference materials and environments by which to compare new materials?

Authors: Although it is tempting to specify a few particular biomaterials, first several studies by independent research groups should be performed comparing the currently most often used biomaterials, be they synthetic or natural. The current review will be helpful at providing some suggestions for candidate materials. Those materials that consistently show most regenerative properties in terms of extensive NP-like matrix formation at the protein level, preferably under relevant biomechanical loads, may subsequently be used as reference materials. Any new materials that, in the future, consistently show to have more regenerative properties than the original materials, may be added to or replace the original set of reference materials. For culture conditions, it will be less easy to define optimal conditions. Ideally, the environment of the (human) degenerated IVD is mimicked, but even to the extent that this environment has been characterised, the importance of and mechanisms behind these characteristics (*e.g.* hypoxia, glucose content) is still not completely elucidated. Moreover, these characteristics may only be relevant if they affect the direction of the response to the biomaterial. This would need to be determined first, for example using the abovementioned reference materials.

Editor’s Note: Scientific Editor in charge of the paper: Brian Johnstone.