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A REVIEW OF THE APPLICATION OF REINFORCED HYDROGELS AND SILK AS BIOMATERIALS FOR INTERVERTEBRAL DISC REPAIR

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Abstract

The degeneration of the intervertebral disc (IVD) within the spinal column represents a major pain source for many patients. Biological restoration or repair of the IVD using "compressive-force-resistant" and at the same time "cytocompatible" materials would be desirable over current purely mechanical solutions, such as spinal fusion or IVD implants. This review provides an overview of recent research on the repair of the inner (nucleus pulposus = NP) and the outer (annulus fibrous = AF) parts of the IVD tissue. Many studies have addressed NP repair using hydrogel-like materials. However, only a few studies have so far focused on AF repair. As the AF possesses an extremely low self-healing capacity and special attention to shear-force resistance is essential, special repair designs are required. In our review, we stated the challenges in IVD repair and highlighted the use of composite materials such as silk biomaterials and fibrin cross-linked reinforced hydrogels. We elaborated on the origin of silk and its many in tissue engineering. Furthermore, techniques such as electrospinning and 3D printing technologies allow the fabrication of versatile and functionalised 3D scaffolds. We summarised the research that has been conducted in the field of regenerative medicine over the recent years, with a special focus on the potential application and the potential of combining silk and reinforced – and thus mechanically tailored – hydrogels for IVD repair.

Keywords: Intervertebral disc, fibrin, silk, hydrogel, nucleus pulposus, annulus fibrosus, repair.

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Introduction

The clinical problem

Low back pain (LBP) is one of the most common diseases in today's society and affects up to 70 % of the Western population at least once in their lifetime (Dagenais *et al.*, 2008; Hoy *et al.*, 2010). The high incidence of this condition not only has negative effects on the quality of life of the people affected but also on the whole economy. Hence, the socioeconomic burden associated with LBP is immense (Duthey, 2013; Hong *et al.*, 2013; Wieser *et al.*, 2011). The vast majority of back pain instances are functional and temporary. For chronic LBP, advanced intervertebral disc (IVD) degeneration caused by aging, impaired nutrient supply, or IVD trauma either caused by direct impact or indirectly by fractures of the cartilaginous endplate (EP) is believed to be the most important factor initiating the degenerative cascade (Alkhatib *et al.*, 2014; Dudli *et al.*, 2014; Luoma *et al.*, 2000; Weber *et al.*, 2015). In order to treat degenerated IVD tissues, numerous surgical approaches and materials have been investigated so far. These range from injectable biomaterials to IVD implants in order to restore the disc height (Chan and Gantenbein-Ritter, 2012; Long *et al.*, 2016a; Sakai and Grad, 2014; Sharifi *et al.*, 2015). Nevertheless, no fully satisfactory solution has been identified yet that results in reconstitution of the IVD to its natural properties, thus leaving the patient with a disc that

is not able to restore its initial height or has a risk of re-herniation. Hence, the current gold standard treatment in case of persistent IVD-associated pain is to perform a spinal fusion of two or more adjacent vertebrae to stabilise segmental instability and to remove the damaged disc material (Benneker *et al.*, 2014). This is done by removal of the disc material and filling the disc space with a cage containing bone grafts or substitutes. Although this temporarily relieves the back pain, it seems that, at a later stage, the neighbouring discs are more prone to accelerated degeneration (adjacent segment degeneration), which again can become symptomatic (adjacent segment disease) (Burkhardt *et al.*, 2017; Donk *et al.*, 2017). This observation can partially be explained by normal ageing (Maldonado *et al.*, 2011) but can also be directly linked to the surgical intervention and the mechanical stresses that are subsequently generated (Matsumoto *et al.*, 2010). Moreover, spinal fusion does not always succeed and an incomplete fusion might result in the reappearance of back pain (Korovessis *et al.*, 2005; Lee *et al.*, 2011; Li *et al.*, 2012; Watkins *et al.*, 2014). The reason for this is not yet fully understood, but residual IVD tissue seems to alter and/or block the ossification of the spine (Chan *et al.*, 2015; Tekari *et al.*, 2017), a fact that could explain why BMP-2-coated implants have a rather neutral effect in meta-analyses (Carragee *et al.*, 2011; Simmonds *et al.*, 2013). Therefore, disc replacement became more popular over recent decades and seems to perform in the same way as spinal fusion (Blumenthal *et al.*, 2005; Zigler *et al.*, 2007).

There are many reviews available on IVD repair. However, none of these summarises the recent advances using reinforced hydrogels in combination with resorbable biomaterials such as silk. A combination of biomaterials might be necessary due to the complex nature and composition of the IVD, with silk offering versatility in scaffold design and decades of experience in the medical field while hydrogels can be injected to fill up the core of the IVD and restore the disc height. The aim of this review is to provide an overview on reinforced natural hydrogels for the repair of the centre of the IVD. Repair can be achieved in two ways 1) a substitute material can restore the disc height and sustain the mechanical loading encountered *in vivo* and/or 2) a substrate for growing and differentiation of cells. Thereby, native or added cells such as human mesenchymal stem cells (hMSC), IVD cells or progenitor cells in general are possible candidates. Further, the review updated recent research of silk in combination with reinforced hydrogels with a focus on IVD repair. We examined the challenges and requirements of IVD repair, the hydrogels already used for tissue engineering, and specifications of silk and its possible uses for nucleus pulposus (NP) and annulus fibrosus (AF) repair when combined with hydrogels.

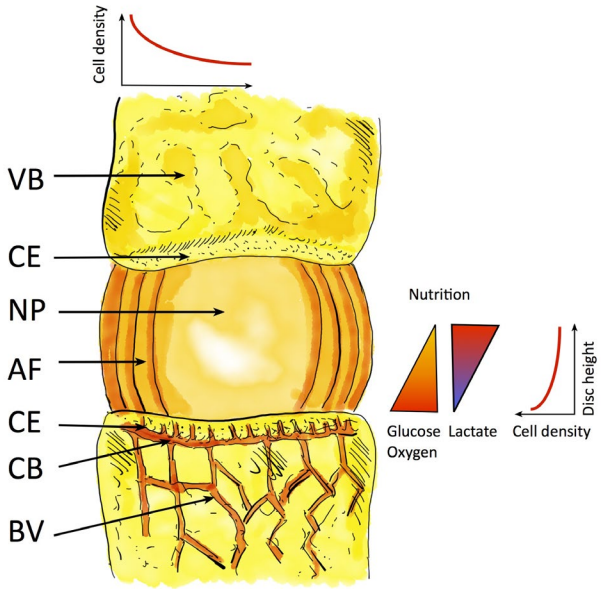

Fig. 1. Schematic drawing of the intervertebral disc (IVD), illustrating the most important structures. The blood vessel supply is only indicated on the lower vertebral body (VB). The centre of the IVD, *i.e.* the gelatinous nucleus pulposus (NP), consists mainly of a proteoglycan and collagen type 2-rich matrix that retains water. The milieu is acidic, low in glucose, and hypoxic. The outer centre of the IVD, *i.e.* the annulus fibrosus (AF) is more fibrotic in nature and is linearly arranged and consists of a higher content of collagen type I than collagen type II and also contains fewer proteoglycans. Finally, the two cartilaginous endplates (EP) contain chondrocytes and form a thin layer of hyaline cartilage with approximately twice as much proteoglycans than collagen type II and a water content of approximately 50-60 %. The EP fills the border between the IVD-tissue niche and the bone-tissue niche of the adjacent vertebrae. Abbreviations are NP = nucleus pulposus, AF = annulus fibrosus, CE = cartilaginous endplate, CB = capillary buds, BV = blood vessels, VB = vertebral body. Cell density, glucose, oxygen, and lactate concentration propagation follows the research work of (Huang *et al.*, 2014; Maroudas *et al.*, 1975; Urban *et al.*, 2004).

The anatomical structure of the intervertebral disc

The IVD is located between the vertebral bodies and allows bending and twisting motions of the spine. Additionally, it acts as a shock absorber during movement, which is possible due to the unique composition of the IVD. In the core of the IVD a gelatinous tissue called NP is present. It is a tissue of high glycosaminoglycan (GAG) content; these polysaccharides are negatively charged with a high capacity of binding to water molecules. This allows the IVD to act as a shock absorber, because the NP can withstand and recover from compressive impacts. The AF is a fibrous tissue surrounding the NP. In contrast to the NP it is highly concentrated in collagen type I, which makes the tissue resistant to tensile strength and holds the softer NP in place. The connection of the NP and AF to the vertebrae is ensured by cartilaginous EPs (Eyre, 1979; Oegema, 1993). Nearly all nutrient uptake occurs through the EP, as there is no blood vessel supply to the IVD (see Fig. 1 for an overview).

The most common causes of LBP are disc herniation and IVD degeneration, but modic changes, other abnormal spinal structures or musculature may also result in LBP (Alkhatib *et al.*, 2014; Deyo and Weinstein, 2001; Dudli *et al.*, 2017; Luoma *et al.*, 2000; Määttä *et al.*, 2015). In the case of disc herniation, the NP is extruded through the AF. The extruded tissue can press against a nerve or cause inflammation when in proximity of a nerve, which may result in pain and may necessitate a surgical intervention. Additionally, IVD degeneration may occur spontaneously due to normal ageing, loading history, inadequate nutrition or impaired metabolite transport. Genetics can also trigger the onset of IVD degeneration (Adams and Roughley, 2006; Buckwalter, 1995; Huang *et al.*, 2014). All of these factors lead to a loss in water content and degradation of the extracellular matrix (ECM) content. Further, cell density decreases and a shift from collagen type II to collagen type I fibres occurs in the core of the disc, thus leading to a stiffer IVD. In disc degeneration and/or trauma, pain can be caused by neovascularisation of the IVD or arthrosis of the faced joints due to the IVD losing height (Adams and Roughley, 2006).

Current challenges for IVD repair

Identifying new strategies for IVD repair is a demanding task, due to their biomechanical and biological properties (Kandel *et al.*, 2008). The IVD possesses a specialised ECM-rich environment with limited nutrients. Therefore, major solutes, such as glucose and oxygen, reach the centre of the disc only by diffusion (Huang *et al.*, 2014). Additionally, due to the avascular property of the IVD tissue, the oxygen content in the centre of the IVD as compared to outer AF is reduced by 2-5 % (Bartels *et al.*, 1998; Holm *et al.*, 1981; Stairmand *et al.*, 1991), a concentration which is recommended to improve culture conditions for IVD cells as well as cells from other tissues, *e.g.* articular cartilage (Mwale *et al.*, 2011; Risbud *et al.*, 2015). Proposed tissue engineering approaches, for instance a combination of biomaterials with IVD cells or cells that can differentiate into IVD cells (MSC and IVD progenitor cells), face the difficulty that once the IVD cells are isolated from their native environment they need to be maintained in hypoxic conditions (Feng *et al.*, 2013; Mwale *et al.*, 2011; Risbud *et al.*, 2015). Furthermore, the cells of the IVD do not possess remarkable self-healing potential (Li *et al.*, 2015a). Moreover, the IVDs are subjected to complex loading forces, *i.e.* a combination of compression, torsion, flexion and tension resulting from body motion. For example, normal movement causes intradiscal pressures in the range of 0.1 MPa when reclining, 0.46 MPa for unsupported sitting, and 0.5 MPa for relaxed standing (Nachemson and Elfstrom, 1970; White and Panjabi., 1990; Wilke *et al.*, 1999). Hence, a successful approach has to incorporate a biomaterial that can fulfil the requirements of withstanding a motion in six degrees of freedom (Costi *et al.*, 2007; Guterl *et al.*, 2013; Long *et al.*, 2016b; Nerurkar *et al.*, 2010), while providing nutrient delivery to the centre of the IVD and simultaneously allowing the transportation of metabolites out of the disc (Huang *et al.*, 2014; Kandel *et al.*, 2008). Due to this challenging task, approaches that rely solely on biomaterials to restore disc height are more closely investigated. Additionally, the different types of tissue that are combined in the IVD require different treatment approaches. For example, an AF repair requires a higher order of structure and must be able to withstand tensional and torsional forces or shear forces. The NP, on the other hand, has to be able to maintain disc height throughout mainly compressional loading.

Reinforced hydrogels for intervertebral disc tissue engineering

Natural hydrogels for tissue engineering applications

Hydrogels are divided into two groups, based on their origin and are considered either natural or synthetic. Here, we focused on natural hydrogels that can be derived from several natural sources, such as agarose from seaweed, alginate from brown algae, hyaluronate and collagen from connective tissues, and chitosan derived mainly from chitin of shrimp shells. Natural hydrogels often possess good cytocompatible properties. Hence, these are frequently included in possible tissue engineering treatment approaches (Priyadarshani *et al.*, 2015), *e.g.* agarose (Awad *et al.*, 2004; Bougault *et al.*, 2012; Iwata *et al.*, 2013; Luo *et al.*, 2015; Mauck *et al.*, 2006), collagen (Borde *et al.*, 2015; Calderon *et al.*, 2010; Mercuri *et al.*, 2014; Omlor *et al.*, 2012; Tsaryk *et al.*, 2015), alginate (Bron *et al.*, 2011; Duggal *et al.*, 2009; Xu *et al.*, 2008; Zeng *et al.*, 2015a), chitosan (Naqvi and Buckley, 2015; Ngoenkam *et al.*, 2010), and combinations of alginate and chitosan have been investigated (Shao

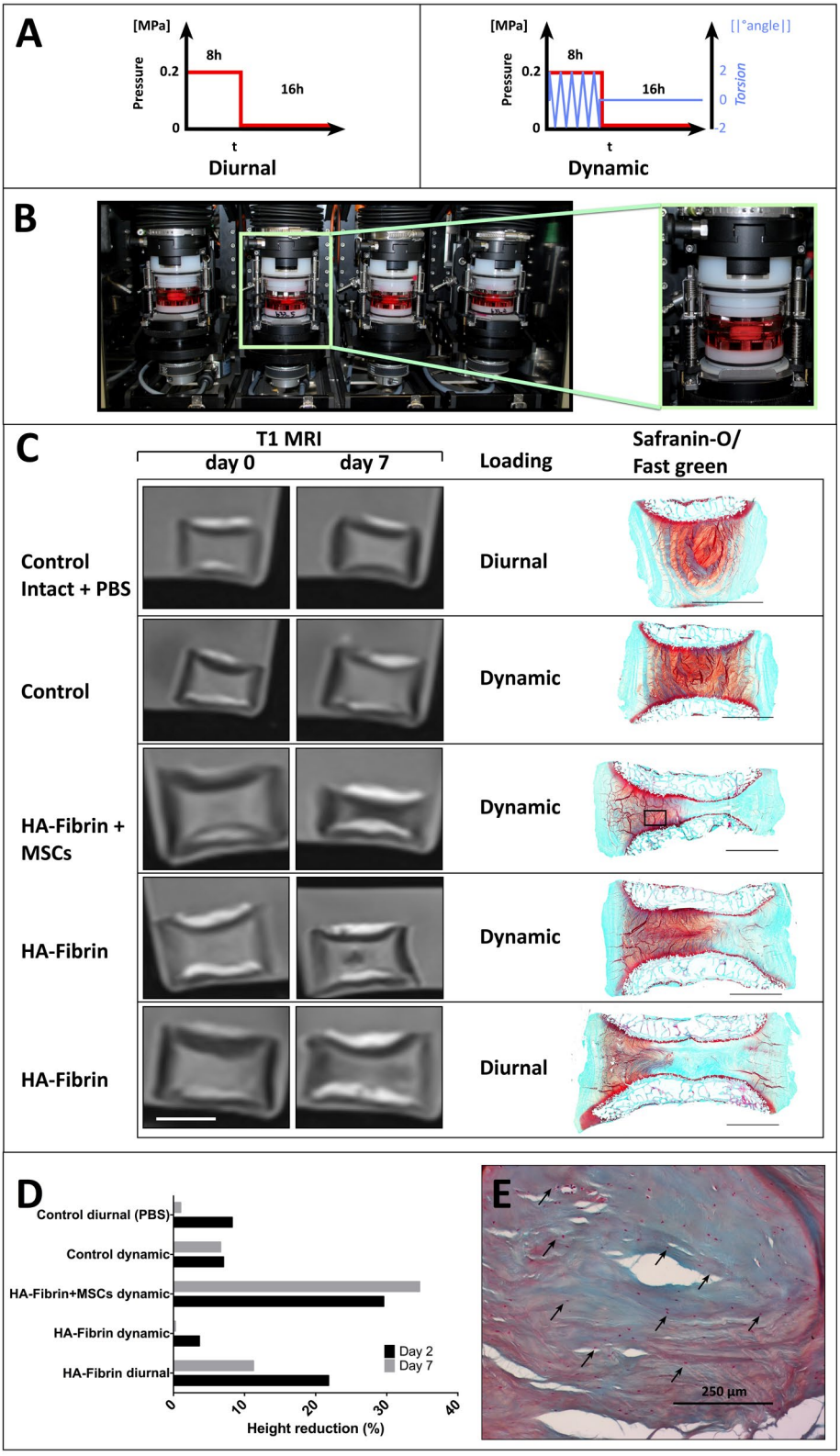

Fig. 2. Proof-of-concept ($n = 1$) of an *ex vivo* organ culture feasibility study of injected hyaluronic-acid fibrin-based hydrogel (HA-Fibrin) materials for nucleus pulposus repair in a papain-induced cavity model for IVD repair. The IVDs were subjected to physiological mechanical loading involving compression and torsion. **A**) Performance of a "biomimetic" hyaluronan-based fibrin reinforced hydrogel after 7 d of two different loading regimes: diurnal loading with 0.2 MPa for 8 h and dynamic loading consisting of 8 h of compression (0.2 MPa) and torsion (0 ± 2° at 0.2 Hz) and no loading for the 16 h phase according to Chan *et al*. (2013). **B**) Bioreactor used to apply dynamic loading on IVDs. **C**) Left column: T1-weighted Magnetic Resonance Images (MRI) before and after loading, right column, Safranin-O/Fast green staining of sagittal paraffin-embedded IVD sections. Scale bars indicate 5 mm. **D**) Reduction in disc height before and after loading. **E**) A close-up of the cavity core region that was filled with HA-Fibrin biomimetic hydrogel. Scale bar = 250 µm. The hydrogel has been studied in an *in vivo* animal model in Peeters *et al*. (2015).

and Hunter, 2007). Although natural hydrogels generally offer good cytocompatibility, they often possess inferior mechanical properties (Schutgens *et al.*, 2015). Nevertheless, by combining them with other synthetic hydrogels or functionalising them, they can gain superior properties. For example, Hong *et al.* (2015) combined alginate with poly(ethylene glycol) and nanoclay to fabricate a hydrogel that was stiffer than natural cartilage. Also, functionalisation of alginate with extracellular matrix components such as collagen type 1 or 2 is possible (Almeida *et al.*, 2017).

Another important natural hydrogel is the fibrin-clot, which has been proposed for use either untouched, in combination with hyaluronic acid (HA) (Li *et al.*, 2014; Peeters *et al.*, 2015) (Fig. 2), or reinforced by the addition of a cross-linker such as genipin (Colombini *et al.*, 2014; Frauchiger *et al.*, 2015; Guterl *et al.*, 2014; Likhitpanichkul *et al.*, 2014; Likhitpanichkul *et al.*, 2015) (Table 1). Fibrin is very important to mention, especially in the context of 3D cell culture, as it is derived from the wound-healing process. The technique is commercialised, and fibrin sealant has been in common use for decades to close bleeding wounds during open surgery. Its use to culture cells in 3D for tissue engineering approaches is proposed (Bensaïd *et al.*, 2003; Hunter *et al.*, 2004; Perka *et al.*, 2001), by mixing cells with the fibrinogen solution prior to the addition of thrombin or injection of cells within the carrier. The clotting is initiated by mixing fibrinogen with thrombin to obtain the insoluble fibrin. Depending on the thrombin concentration and the concentration of the factor XIII, the speed of the reaction can be manipulated (ranging from a few seconds to several minutes). Fibrin hydrogels are also successfully used for *in vitro* 3D culture of different cell types (Gantenbein-Ritter *et al.*, 2008; Li *et al.*, 2009; Li *et al.*, 2014; Zeiter *et al.*, 2009). However, although the gel is modifiable by adjusting the concentrations of fibrinogen and factor XIII, it is not a realistic choice for IVD repair due to its softness (*i.e.* dynamic stiffness at 1 Hz dynamic loading ~10 kPa, (Hunter *et al.*, 2004)) and poor mechanical properties as compared to the IVD tissue (normalised stiffness of IVD is estimated to be around ~10 MPa (Beckstein *et al.*, 2008; Showalter *et al.*, 2012)). Thus, compressive stiffness of most hydrogels is usually too low by a magnitude of ~1,000×, many hydrogels often having a stiffness in the range of ~10 kPa. On the other hand, depending on the concentration of the fibrinogen and factor XIII, the resulting hydrogel could become too dense and make it difficult for chondrocytes/chondrocyte-like cells and MSC to thrive (Zeiter *et al.*, 2009).

Recently, novel research has emerged around the natural, plant-derived cross-linker genipin, which allows tailoring the stiffness of the too-soft fibrin gels to obtain properties comparable to AF or NP tissue (Colombini *et al.*, 2014; Guterl *et al.*, 2014; Likhitpanichkul *et al.*, 2014; Schek *et al.*, 2011). Recently, how the genipin diffuses inside the fibrin network was modelled (Ninh *et al.*, 2015). So far, genipin does not show toxic effects upon human vascular wall MSC either cultured *in vitro* or implanted *in vivo* in nude mice experiments (Likhitpanichkul *et al.*, 2014; Panzavolta *et al.*, 2011). However, genipin causes problems for using fluorescence microscopy as it is auto-fluorescent. This may cause difficulty during assessment of cell viability using the classical calcein AM and ethidium-homodimer LIVE/DEAD stain (Matcham and Novakovic, 2016). Difficulties are also noted in diffusion models because the genipin cross-linker may considerably reduce diffusion, possibly reducing cell viability.

Another very promising material for NP repair is Gellan gum hydrogel. Gellan gum is bacterial-derived and shows extremely high cytocompatibility *in vitro* and *in vivo* (Ahearne and Kelly, 2013; Anderson *et al.*, 1988; Khang *et al.*, 2015; Reitmaier *et al.*, 2014). Recently, this material was reinforced to withstand higher compressive forces by addition of methacrylated modifications, resulting in a much stiffer hydrogel tuneable in the range of 0.15-148 kPa (Coutinho *et al.*, 2010). This is similar to the cross-linking genipin approach that leads to shear stiffness in the range of 47-61.1 kPa, depending on the genipin concentration or up to 73 kPa, when fibrin is combined with genipin and fibronectin (Guterl *et al.*, 2014). For instance, for NP repair it was shown by Silva-Correia, *et al.* (2012) that rabbit NP cells thrive perfectly for an extended period, without much loss of cell viability, in methacrylated Gellan gum (Coutinho *et al.*, 2010; Khang *et al.*, 2015; Silva-Correia *et al.*, 2011; Silva-

Table 1. List of previous research articles and reviews using fibrin hydrogels for either AF and NP repair or 3D culture of these cells.

Location	Material	Publication
Annulus Fibrosus	Enhanced with genipin	Likhitpanichkul *et al.*, 2014; Likhitpanichkul *et al.*, 2015; Long *et al.*, 2016a; Schek *et al.*, 2011
Nucleus Pulposus	Combined with hyaluronic acid	Li *et al.*, 2014; Park *et al.*, 2012
3D Culture	Combined with poly(lactic-co-glycolic acid)	Sha'ban *et al.*, 2008; Stern *et al.*, 2000; Stich *et al.*, 2015
	Enriched with collagen	Colombini *et al.*, 2015
	Alone	Yang *et al.*, 2008
	Combined with alginate	Perka *et al.*, 2001

Correia *et al.*, 2013). It has yet to be shown whether Gellan gum could withstand compressive forces in long-term IVD repair experiments.

Other "smart" synthetic hydrogels to regenerate the nucleus pulposus

Where the adaptability of natural hydrogels is somewhat limited, synthetic hydrogels allow for the tailoring of specific needs. This represents a hot topic for IVD repair, to further "fine-tune" the hydrogels and use artificial and so-called "smart" gels. One of these designed gels is poly(*N*-isopropylacrylamide) (pNIPAM), which is a thermo-responsive hydrogel that solidifies upon reaching 32 °C (Mortisen *et al.*, 2010). An aspect that allows for 3D encapsulation and culture of cells, followed by liquefying and injection into the host tissue, such as IVDs, when the hydrogel is cooled to around 5-10 °C (Malonzo *et al.*, 2015; Peroglio *et al.*, 2012; Peroglio *et al.*, 2013; Thorpe *et al.*, 2016). In addition, a combination of pNIPAM with poly($_{D,L}$-lactide-co-glycolide) particles is possible and leads to a thermosensitive particle gel, whose elasticity can be adjusted by changing the concentration of the two components (Fraylich *et al.*, 2010). Such hydrogels can serve as pre-conditioning material to direct the cells towards a desired phenotype in a 3D environment, prior to transplantation into the IVD. For example, MSCs can be differentiated towards IVD-like cells in a 3D culture by the addition of selected growth factors, *e.g.* growth and differentiation factor 6, to better condition the cells and to stimulate the production of ECM for subsequent injection into the IVD (Clarke *et al.*, 2014). This can be especially attractive if the gels can be re-dissolved and the cells can be easily separated from their hydrogel, a property that is found in thermo-responsive hydrogels (Peroglio *et al.*, 2013). This is also possible with natural algae-derived alginate, which can be dissolved by the removal of the Ca^{2+} cations, by the addition of ethylene diamine tetra acetic acid (EDTA), unless the cells produce a large amount of ECM over a prolonged culture period (Maldonado and Oegema, 1992).

Recently, another synthesised polyethylene glycol composite material reinforced with nano-cellulose was developed. It can be polymerised by light using optical fibres directly within the IVD, and is proposed for NP replacement (Schmocker *et al.*, 2016). For the application of the liquid hydrogel, both the injection and illumination are combined within a single needle. The stiffness of the hydrogel can also be tuned to match the compressive stiffness of a native NP.

Silk for Tissue Engineering

Background

Silk is promoted as a promising biomaterial for a wide range of tissue engineering applications. This is in part due to its unique biomechanical properties, namely its strength, elasticity and high cytocompatibility (Wöltje and Böbel, 2017). Silk is of great interest because its production is well-established, primarily in the textile industry. Due to millennia of silk production, the sericulture of silk worms, *i.e. Bombyx mori* (Linnaeus, 1758, Lepidoptera: Bombycidae) and hence silk harvesting is widespread, and a homogenous quality obtained. Silk is relatively easy to obtain from cocoons, is cytocompatible, and is slowly degraded by enzymatic processes *in vivo*, thus allowing ECM formation when it is used as a cell culture substrate. Although silk is considered to be a nondegradable biomaterial, according to the US Pharmacopeia's definition, its degradation can be induced *in vitro* by the incorporation of enzymes *e.g.* proteases, collagenases or α-chymotrypsin (Horan *et al.*, 2005; Li *et al.*, 2003). The *in vivo* degradation rate seems to be influenced by the design and concentration of the silk and is mainly driven by foreign body reaction (Altman *et al.*, 2003). An electrospun silk scaffold degrades over eight weeks when implanted subcutaneously in rats, without adverse side effects for the animal (Zhou *et al.*, 2010). Wang *et al.* uses a different silk scaffold where residual pieces can still be found after six and twelve months following subcutaneous implantation in rats (Wang *et al.*, 2008). Closer examination of the immune response to silk, shows it to be mainly inert as long as it is not combined with sericin (Meinel *et al.*, 2005; Panilaitis *et al.*, 2003). *In vitro*, silk can be degraded and liquefied by the addition of 9.3 M LiBr and incubation at 60 °C for 4 h (Rockwood *et al.*, 2011). Moreover, silk can be moulded into different shapes, which allows for its application to a wide range of clinical problems. The fine structure of *B. mori* silk cocoons is composed of threads with diameters in the range of 10-20 μm, and each thread is, in fact a duplet of two individual fibres. Each of these fibres has a sericin sheath and an inner core composed of fibroin. Fibroin consists of parallel fibrils with diameters of 100-400 nm, providing excellent mechanical properties regarding tensile strength and elasticity (Altman *et al.*, 2003).

Silk biomaterials are nowadays investigated for many applications in tissue engineering, *e.g.* for bone repair (Hardy *et al.*, 2015; Li *et al.*, 2006; Park *et al.*, 2010; Sofia *et al.*, 2001; Zhang *et al.*, 2014), for artificial skin (Sheikh *et al.*, 2015), vascular grafts (Catto *et al.*, 2015; Wang *et al.*, 2015; Zhang *et al.*, 2008), substrate for growing neuronal cells (Sun *et al.*, 2015), IVD repair (Buser *et al.*, 2011; Chang *et al.*, 2010; Chen *et al.*, 2015; Du *et al.*, 2014; Hu *et al.*, 2012; Park *et al.*, 2011; Zeng *et al.*, 2014; Web Ref.1) (Table 2) and cartilage repair (Bhardwaj and Kundu, 2012; Hofmann *et al.*, 2006a; Wang *et al.*, 2005). Additionally, silk can also be used as a drug delivery platform (Mwangi *et al.*, 2015; Qu *et al.*, 2014) for antibiotics (Pritchard *et al.*, 2013) and proteins or small molecule drugs (Meinel and Kaplan, 2012). Furthermore, silk fibroin scaffolds can be functionalised by covalent binding, *e.g.* with growth factors such as bone morphogenetic protein 2 (BMP-2) (Karageorgiou *et al.*, 2004) for bone repair, or by immobilisation of the fibroblast growth factor 2

Table 2. List of publications that use silk or silk composites for either AF or NP repair.

Location	Material	Publication
Annulus Fibrosus	Biphasic silk fibroin scaffold	Du *et al*., 2014
	Porous silk scaffold	Chang *et al*., 2010
	Porous silk scaffold	Chang *et al*., 2007
	Silk	Park *et al*., 2012
Nucleus Pulposus	Porous silk scaffold	Zeng *et al*., 2014
	Silk fibrin/hyaluronic acid composite	Park *et al*., 2011; Park *et al*., 2012
	Silk fibroin/polyurethane composite	Hu *et al*., 2012

(FGF-2) by "click-chemistry" (Zhao *et al*., 2014). Also, functionalisation of silk materials with antibodies is possible, using the avidin/biotin non-covalent linkage system (Wang and Kaplan, 2011).

Silk origin

Silk is mainly produced from *B. mori* and, in smaller quantities, from other animals (Chung *et al*., 2015; Kuwana *et al*., 2014; Li *et al*., 2015b). The species *B. mori* was domesticated in China, thousands of years ago, and belongs to the mulberry silkworms. Therefore, *B. mori* became completely dependent on human care and could not survive in the wild. There is a high probability that *B. mori* originates from the wild silkworm *B. mandarina* (Moore, 1872), which undergoes complete metamorphosis, meaning it passes through the egg, larva, pupa and moth phases (Aruga, 1994). Apart from these domesticated silkworms, wild-type silkworms also exist. The group of non-mulberry silkworms, silkworms that do not depend on mulberry leaves, covers mostly wild silkworms such as the Chinese oak silk moth, *Antherae pernyi* (Guérin-Méneville, 1855, Lepidoptera, Saturniidae) that produces so-called muga silk and the Ailanthus silk moth *Samia cynthia* (Drury, 1773, Lepidoptera, Saturniidae) that produces so-called eri silk (reviewed in Kundu *et al*., 2012). The use of these silkworms for commercial biomedical applications is not common because of their limited availability (Panda *et al*., 2015; Patra *et al*., 2012). Apart from the *B. mori* silkworms, other insects including caddisflies, midges, glow worms, sawflies, bees, wasps (reviewed in Sutherland *et al*., 2010) and spiders can produce silk. Spider silks are of special interest, as they offer a broader range of different silks with different properties (reviewed by Fu *et al*., 2009 and Vollrath, 2000). Nevertheless, their use in biomedical applications is limited due to the lack of appropriate sericulture conditions for spiders, because of their cannibalistic behaviour. Hence, the fabrication of recombinant silk of mainly special spider and other silks, from insects that are difficult to culture, is still a growing field. By using an expression vector in *Escherichia coli*, the production of hornet silk is possible (Kambe *et al*., 2014) or the fabrication of spider silk in mammalian cells (Lazaris *et al*., 2002). Transgenic silk worms are also used to produce spider silk (Kuwana *et al*., 2014). By using such methods, silk can be produced, as a natural product, in a highly-controlled process that makes quality control easier. Nevertheless, producing fibres from recombinant silk poses new challenges, as the spinning process is crucial to maintaining its unique properties (Lazaris *et al*., 2002).

Silk harvest and production

The most abundant source of silk is the *B. mori* cocoon. In order to produce silk of the highest quality with respect to purity, *i.e.* long, regular, single white fibres, silk worms need to be sericultured, and the silkworm cocoons collected before the larvae hatch. This is done either by boiling the cocoons in hot water or pricking the larva with a needle to kill the worm inside. By doing so, the silk moth is prevented from eating its way through the cocoon during hatching and destroying it. The cocoons are then washed in boiling water. This dissolves the sericin, which forms a layer surrounding the silk threads acting as a glue to keep the two silk threads together. By this process, it is possible to uncoil the cocoon and obtain two strings of silk. In contrast, wild silk is obtained by collecting cocoons from already hatched silk moths. Hence, the final product is rougher and not as fine as silk from sericulture. After sericin removal, silk can be dried and directly used for weaving fabrics.

Another option is to produce liquid silk for further applications, *e.g.* fabrication of silk films for drug delivery (Hofmann *et al*., 2006b; Uebersax *et al*., 2007), porous blended silk scaffolds (Rao *et al*., 2009), or bead fabrication (Yildirim *et al*., 2010). Solubilised silk can be obtained by treatment with aqueous 9.3 M LiBr solution followed by sequential membrane dialysis to remove all salt residues. With this method, an aqueous silk solution is obtained, which can be stored at 4 °C for a shorter time period (Rockwood *et al*., 2011). Although, after prolonged storage time, spontaneous β-sheet forming takes place and results in a solidification of the silk, which could be a major issue for off-the-shelf usage for clinical application.

Molecular structure of silk fibroin

The *B. mori* silk consists of two main components: silk fibroin (72-81 %) and sericin (19-28 %) (Rockwood *et al*., 2011). Sericin acts as an interface to adhere the silk fibroin threads, produced by the two silk glands, to each other therefore maintaining the structure of

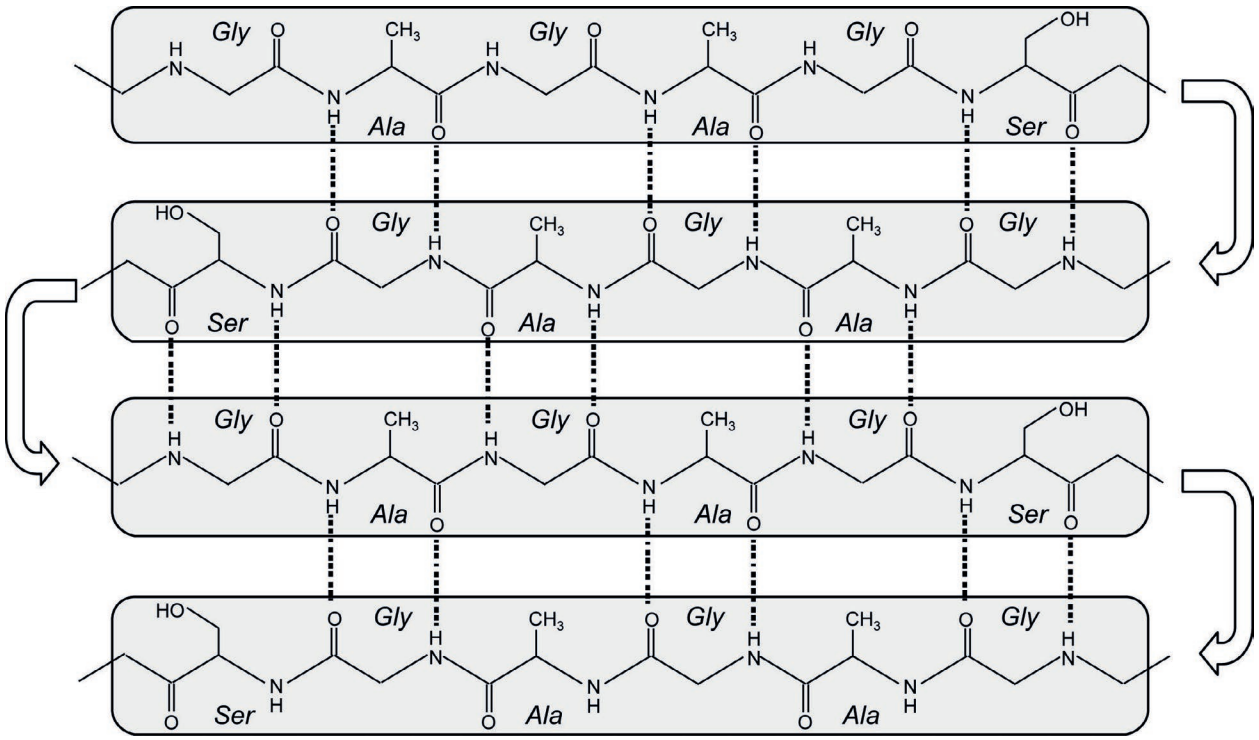

Fig. 3. Molecular structure of silk and those hydrogen bonds between the crystalline structures of fibroin.

the cocoon. The silk fibroin from the silk gland is composed of six heavy chains (350 kDa) and six light chains (26 kDa), which are connected by disulphide bonds forming six heterodimers (Fig. 3). Those dimers are then combined by a central protein called fibrohexamerin/p25 (30 kDa), forming the fibroin elementary unit in a molar ratio of 6:6:1 (Inoue *et al.*, 2000; Inoue *et al.*, 2004). The amino acid composition and the properties of silk fibroin from non-mulberry silkworms, or other silk sources (*e.g.* spiders), can differ widely from that sourced from *B. mori* (Reddy and Yang, 2010).

Silk scaffold development for intervertebral disc tissue engineering applications

Silk can be processed in many different ways, geared to Good Manufacturing Practice (GMP) as required for clinical and translational research applications. One possibility is to solubilise the silk material and print it controllably with a 3D ink-jet printer. This also allows the combination of the silk base with, for example, small particles and antibodies (Tao *et al.*, 2015). Another way to process silk is to use electrospinning to allow the fibre networks to produce an extracellular collagen-like matrix, composed of fibres with diameters in the nano- to micron-scale range, with high specific surface area and high porosity, thus being predestined candidates for enhanced cell attachment (Catto *et al.*, 2015; Meinel *et al.*, 2009; Min *et al.*, 2004; Sheikh *et al.*, 2015; Zhang *et al.*, 2009). Moreover, silk composite fibres can be realised with collagen proteins (Zhu *et al.*, 2015) or nanoparticles such as hydroxyapatite for bone tissue engineering (Kim *et al.*, 2014; Yang *et al.*, 2014) silver (Sheikh *et al.*, 2014), or fibres incorporating specific growth factors for sustained drug delivery (Li *et al.*, 2006). Additionally, liquid silk can be directly injected into the NP (Boyd and Carter, 2006; Hu *et al.*, 2012; Murab *et al.*, 2015). Apart from these techniques silk, can also be produced as a porous scaffold by wet chemical procedures (Bhardwaj and Kundu, 2012; Chang *et al.*, 2007; Chang *et al.*, 2010; Chen *et al.*, 2014; Chen *et al.*, 2015; Hofmann *et al.*, 2007; Yang *et al.*, 2012; Zeng *et al.*, 2014; Zeng *et al.*, 2015b; Zhang *et al.*, 2014), sponges (Rnjak-Kovacina *et al.*, 2015; Zeng *et al.*, 2014), or aerogels (Mallepally *et al.*, 2015).

Electrospinning of silk for intervertebral disc applications

Silk can be liquefied by dissolving it in 5.5-9.3 M LiBr for 2-4 h, to break the Van der Waals forces within the β-sheet structure. Subsequently, the silk can be purified by high-speed centrifugation (~13 ×*g*) (Meinel *et al.*, 2009). For electrospinning of liquid silk, the material is usually mixed with high molecular weight polymers to increase its viscosity. The LiBr salt is exchanged by dialysis against pure water (Meinel *et al.*, 2009). After lyophilising, selected solvents, such as formic acid or 1,1,1,3,3,3-hexafluoro-2-propanol, can be used for homogeneous fibre formation (Jeong *et al.*, 2006; Kim *et al.*, 2003). Improved spinning of aqueous silk solutions is obtained by the application of mild shear stresses under dehumidified air in a post-processing procedure (Singh *et al.*, 2016). Shearing increases the β-sheet content within the solutions remarkably, leading to high strength silk

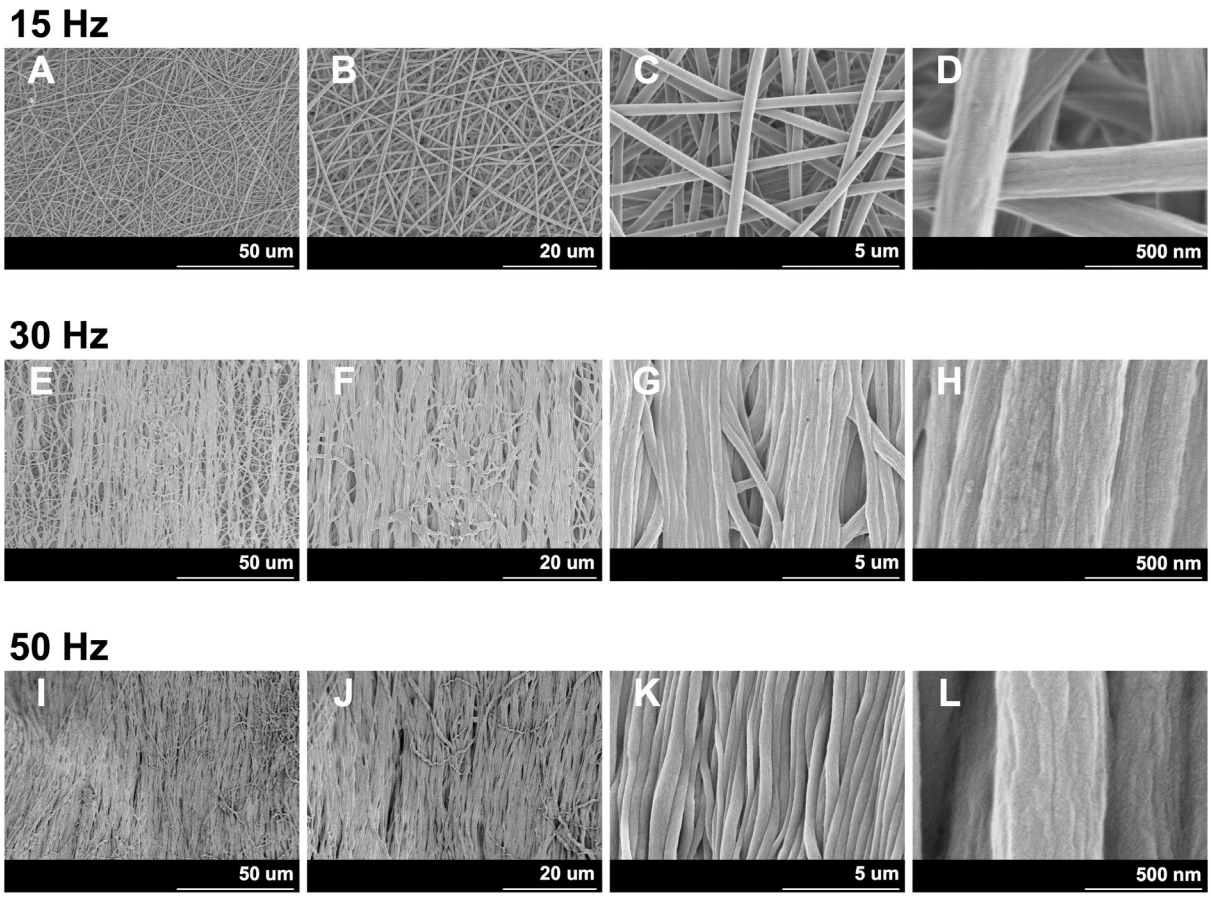

Fig. 4. Production of electrospun silk scaffolds on a rotating mandrel to generate microenvironments for IVD cells. Demonstrating the effect of rotation frequency: Electrospun nanofibers on a rotating mandrel at a speed of 15 Hz (**A**-**D**), 30 Hz (**E**-**H**) and 50 Hz (**I**-**L**). Images taken by SEM from Studer *et al.* (Web Ref. 1).

fibroin fibres. In addition, Zhu *et al.* (2008) reports the effects upon spinnability and respective fibre morphologies of selected pH values in aqueous silk fibroin solutions. For instance, a decrease in the pH value allows for a decrease in the silk concentration within the spinning solution for homogeneous fibre formation. Furthermore, a decrease of the average fibre diameter is observed.

As a further approach, an improved spinning in terms of stability and throughput is obtained by the use of polymeric spinning aids. The silk solutions are mixed with secondary polymers known to incorporate enhanced spinning properties to establish a stable jet from the syringe towards the counter electrode (Jin *et al*., 2002). Typical polymers used are high molecular weight poly-(ethylene-oxide) (PEO) or poly-(vinyl-pyrrolidones) (PVP). Fig. 4 illustrates the effect of the use of selected frequencies (15, 30 and 50 Hz) of a rotating mandrel (diameter 25 cm) by the use of a 7 % aqueous silk fibroin solution with 5 % PEO (200,000 g/mol) as the spinning helps to obtain selectively aligned silk fibre scaffolds, mimicking the AF (Web Ref.1). Thereby, parameters such as environmental humidity, applied potential, flow rate, distance of needle to collector, frequency of rotating mandrel, and the age of the fibroin influence fibre diameter and uniformity. On a flat-collector, randomly aligned fibre scaffolds can be generated, providing an environment that would better suit an NP cell phenotype, rather than the highly aligned fibres which are better for the AF types.

Dong *et al.* (2016) shows a novel route to obtain stable silk spinning solutions, by careful tuning of the dissolution solvent system. Interestingly, the spinning reveals the formation of silk nanofibers incorporating amorphous structures, as compared to the aforementioned β-sheet rich fibres.

Further functionalities are introduced for silk-based scaffolds by application of coaxial electrospinning. Tian *et al.* (2015) spun poly-(lactic acid) as the sheath material and silk fibroin as the core structure, introducing *in situ* active molecules such as nerve growth factors into the core. A sustained release of the growth factor is realised, leading to enhanced attachment and the differentiation of cells (Zhu *et al*., 2008).

Silk and reinforced hydrogels for NP repair

For repair of the NP tissue, specific demands such as a gel-like structure that is able to retain water are required. For this, several studies attempt to produce an injectable liquid silk scaffold (Boyd and Carter, 2006). Hu *et al.* (2012) reports a silk fibroin/polyurethane gel, tested *in vitro* on rabbit MSC,

where it shows excellent cytocompatibility. Also, the feasibility of injection into isolated porcine IVDs by a small AF incision is tested where the hydrogel is able to refill the defect prior to disc degeneration. Nevertheless, neither the impact of mechanical load nor the disc behaviour is investigated after prolonged organ culture. Some studies go further, with the focus on a biphasic scaffold construct that simultaneously mimics both the AF and NP structure (Fig. 5, left column) (Park *et al*., 2012). An ECM for the inner and outer AF, realised by a lamellar silk fibroin scaffold and a fibrin/hyaluronic acid gel for the NP structure, is generated and tested *in vitro* using porcine cells. Over four weeks of culture, the integration of the two components is recorded as well as enhanced *Collagen type 1* and *Aggrecan* expression in the AF, while there is significantly higher expression of *Collagen type 2* in the NP region. Also, the GAG content is two times higher in the NP region of the biphasic scaffold than in the AF region. Another attempt to mimic the environment of the NP even more closely is the formation of a triple composite, incorporating hyaluronic acid into a fibrin hydrogel enforced with silk fibroin (Park *et al*., 2011) (Fig. 5). Here, human chondrocytes are mixed with different gel compositions and analysed over four weeks. A formulation with 1 % silk shows greater DNA and GAG content, as well as *Collagen type I* and *Aggrecan* gene expression when compared to a composition with 2 % silk.

One interesting investigation of NP repair is the clinical trial of the material NuCore® (Spine Wave Inc., Shelton, CT, USA) that is injected into human patients (Fig. 5). This material consists of a recombinant protein copolymer consisting of an elastin hydrogel that is combined and reinforced with silk (Berlemann and Schwarzenbach, 2009; Boyd and Carter, 2006). This material is the only material that has been injected into human patients to date. In a two-year follow-up study, NuCore® is

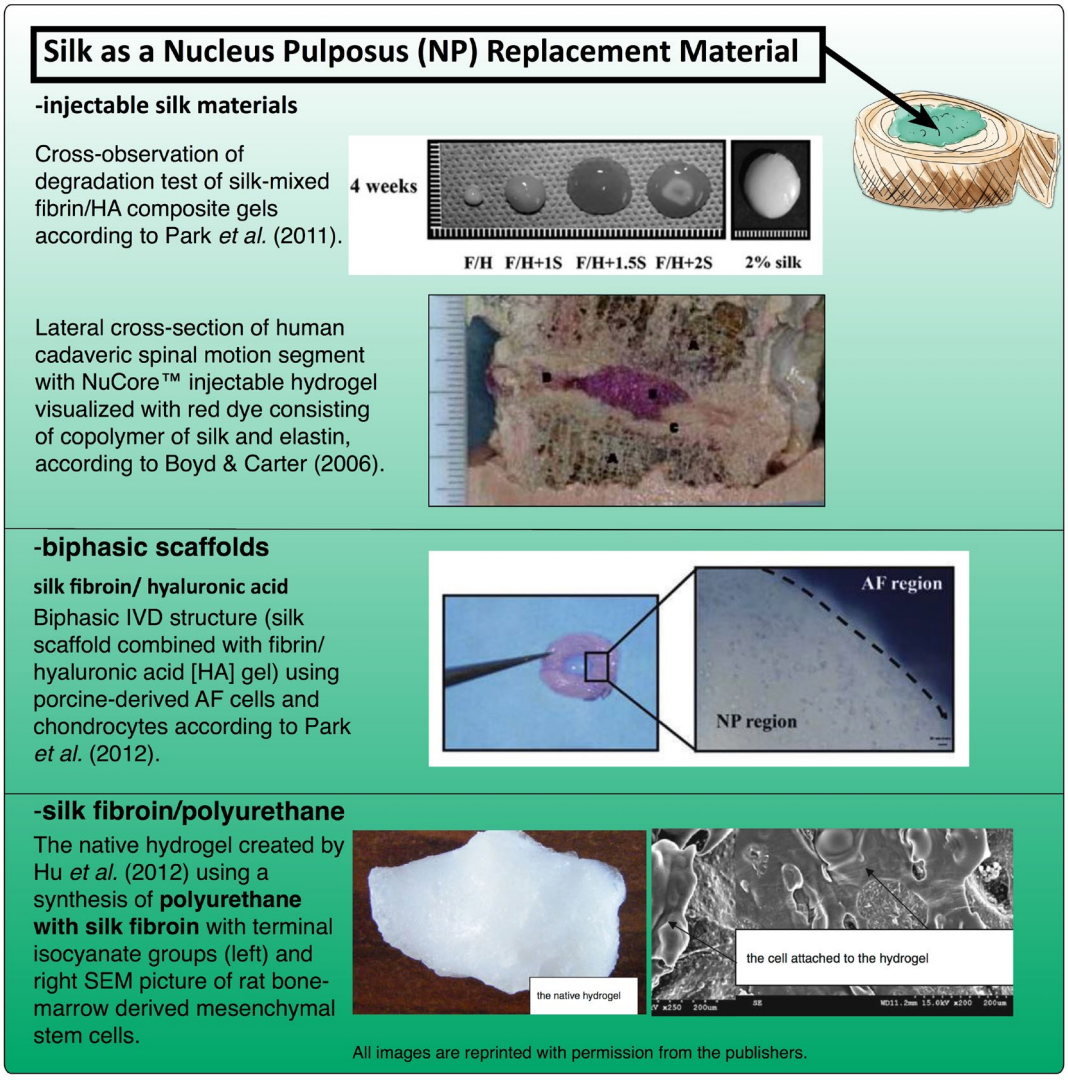

Fig. 5. An overview of silk scaffolds with different material properties produced for nucleus pulposus (NP) repair. Abbreviations: (F/H): fibrin/HA gel, (F/H+1S): fibrin/HA gel with 1 % silk, (F/H+1.5S): fibrin/HA gel with 1.5 % silk, (F/H+2S): fibrin/HA gel with 2 % silk, SEM: Scanning electron microscope. Images were reproduced with permission from the publishers and were taken from Boyd and Carter (2006), Hu *et al*. (2012), Park *et al*. (2011) and Park *et al*. (2012).

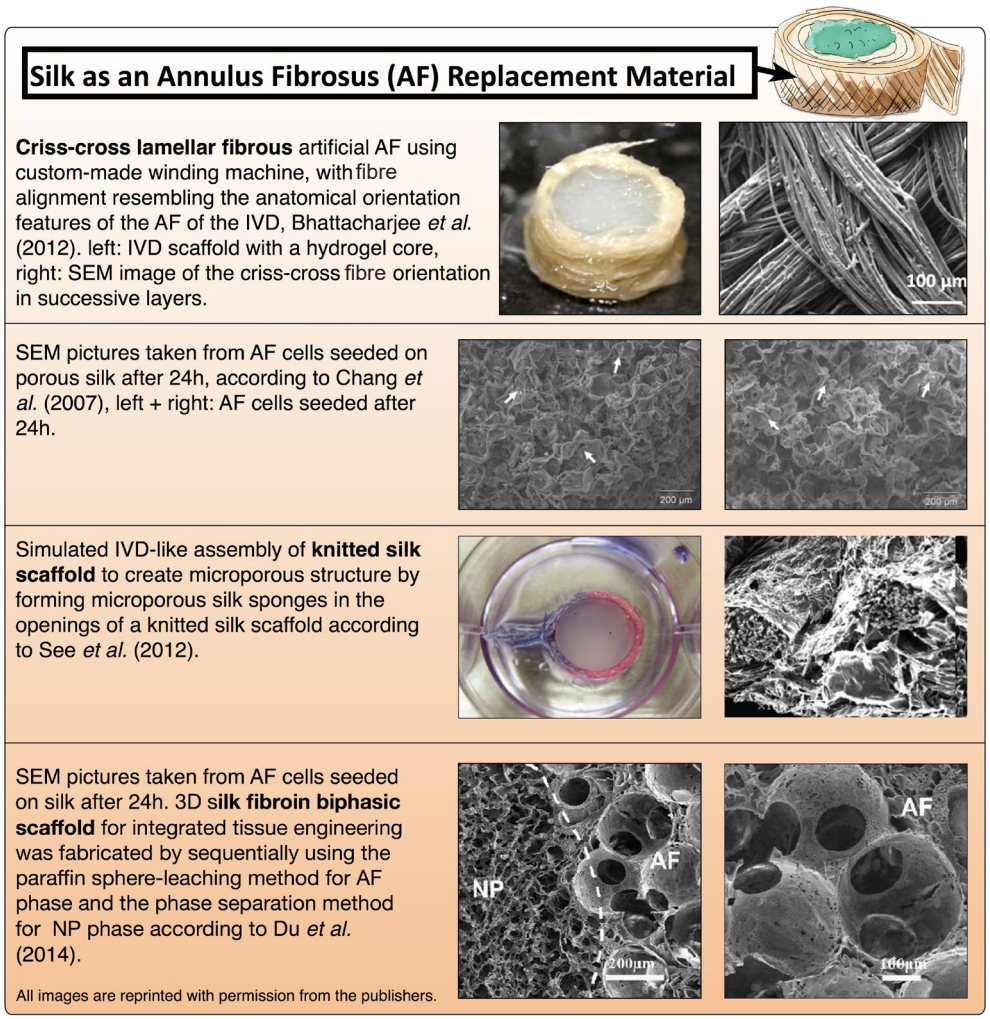

Fig. 6. An overview of silk scaffolds with different material properties produced for annulus fibrosus (AF) repair. Images were reproduced with permission from the publishers and were taken from Bhattacharjee *et al*. (2012), Chang *et al*. (2007), Du *et al*. (2014) and See *et al*. (2012). Abbreviations: SEM = Scanning electron microscope.

indicated to be a safe treatment option that is better able to maintain disc height than discectomy alone (Berlemann and Schwarzenbach, 2009). This material has been extensively tested for cytotoxicity following the ISO 10993 guidelines. A two-year follow-up of the clinical trial is published, and no major failures are reported from the patients (Berlemann, 2015). To the best of our knowledge, no other biomaterial could be directly tested in human patients for NP repair. The necessary hurdles to directly test novel biomaterials in patients have become higher, so most investigations are limited to small and, in the best cases, large animal models. Therefore, the costs have increased considerably.

Silk and reinforced hydrogels for AF repair

It is well appreciated that NP repair is certainly a major clinical challenge and AF repair even more puzzling. Several reviews of the challenges for this organ are published (Bron *et al*., 2010; Li *et al*., 2016; Long *et al*., 2016a). There are several commercial solutions for AF repair, *e.g.* Inclose® and Xclose®, which can be seen as modified sutures with anchors (Gantenbein-Ritter and Sakai, 2011). None of these AF repairs seem to withstand the relatively high physiological tensile forces, and none of the currently available commercial solutions seem valid for a clinical use (Bron *et al*., 2010; Gantenbein-Ritter and Sakai, 2011). Using silk as an AF scaffold is proposed in several studies, as the material properties seem well-suited for this purpose (Bhattacharjee *et al*., 2012; Chang *et al*., 2007; Chang *et al*., 2010; Du *et al*., 2014; Park *et al*., 2012; See *et al*., 2011; See *et al*., 2012; Web Ref.2) (Fig. 6). Among these is a porous biphasic scaffold whose compressive modulus reached ~151 kPa. Although it is still weaker than native IVD tissue, it is a step forward towards IVD repair (Du *et al*., 2014). In addition, a silk fleece-membrane composite is able to withstand the loads in an organ culture model under physiological compression and torsion for two weeks (Web Ref.2).

The reason for this lies in the very good characteristics of these fibres, such as their very low cytotoxicity and high tensile elasticity. Among

the tissue-engineered approaches for IVD repair, biphasic materials are of interest where the natural lamellar structure of the AF is mimicked.

Another, not yet very common, approach is to combine the two biomaterials fibrin and silk (Elliott *et al*., 2015; Frauchiger *et al*., 2015). On its own, fibrin is too weak, but when enhanced with genipin, it reaches a stiffness comparable to that of native IVD tissue. This approach is very promising for IVD repair as the fibrin acts as a glue from the AF side and interacts with the inner AF.

A scientific consortium has been formed to target AF repair as a major priority, and these efforts have resulted in several scientific articles targeting AF repair using biomaterials: (Cruz *et al*., 2017; Guterl *et al*., 2014; Likhitpanichkul *et al*., 2014; Likhitpanichkul *et al*., 2015; Schek *et al*., 2011). These studies conclude that genipin-reinforced fibrin hydrogel seems to be an ideal solution to link the extruded NP material of damaged discs. Similarly, the enhanced fibrin hydrogel shows superior mechanical strength and is able to bear physiological loading. Also, it can be conveniently injected into bovine IVDs, either by a double barrel syringe with a mixing tip or by mixing in thrombin directly prior to application (Frauchiger *et al*., 2015; Long *et al*., 2016a). Future research and clinical trials will need to demonstrate its clinical application in translational medicine.

Conclusion

Silk is an ancient biomaterial whose usage goes as far back as 4000 BC. More recently, silk was tested for biomedical applications for the repair of many musculoskeletal joints. This can be attributed to the high cytocompatibility, the relatively slow rate of resorption, and the high elasticity and tensile strength of the silk fibres, which make it an interesting material for AF repair.

Most hydrogels tested for 3D culture models are cytocompatible and would be suitable for NP repair. However, they have a relatively moderate stiffness for IVD repair purposes. Hence, reinforced hydrogels in combination with selected resorbable biomaterials, such as silk, could be an interesting therapeutic approach. Here, the combination of fibrin hydrogel cross-linked with genipin and silk offers excellent stiffness.

Despite much research, only one product consisting of silk and elastin (NuCore®) is successfully and currently used as NP replacement in clinical settings.

Acknowledgements

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References

Adams MA, Roughley PJ (2006) What is intervertebral disc degeneration, and what causes it? Spine **31**: 2151-2161.

Ahearne M, Kelly DJ (2013) A comparison of fibrin, agarose and Gellan gum hydrogels as carriers of stem cells and growth factor delivery microspheres for cartilage regeneration. Biomed Mater **8**: 035004.

Alkhatib B, Rosenzweig DH, Krock E, Roughley PJ, Beckman L, Steffen T, Weber MH, Ouellet JA, Haglund L (2014) Acute mechanical injury of the human intervertebral disc: link to degeneration and pain. Eur Cell Mater **28**: 98-110.

Almeida HV, Sathy BN, Dudurych I, Buckley CT, O'Brien FJ, Kelly DJ (2017) Anisotropic shape-memory alginate scaffolds functionalized with either type I or type II collagen for cartilage tissue engineering. Tissue Eng Part A **23**: 55-68.

Altman GH, Diaz F, Jakuba C, Calabro T, Horan RL, Chen J, Lu H, Richmond J, Kaplan DL (2003) Silk-based biomaterials. Biomaterials **24**: 401-416.

Anderson DM, Brydon WG, Eastwood MA (1988) The dietary effects of gellan gum in humans. Food Addit Contam **5**: 237-249.

Aruga H (1994) Silkworm and its strains, In Principles of sericulture, CRC Press/Balkema, Rotterdam. Chapter 3: 97-111.

Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F (2004) Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials **25**: 3211-3222.

Bartels EM, Fairbank JC, Winlove CP, Urban JP (1998) Oxygen and lactate concentrations measured *in vivo* in the intervertebral discs of patients with scoliosis and back pain. Spine **23**: 1-7.

Beckstein JC, Sen S, Schaer TP, Vresilovic EJ, Elliott DM (2008) Comparison of animal discs used in disc research to human lumbar disc: axial compression mechanics and glycosaminoglycan content. Spine **33**: E166-E173.

Benneker LM, Andersson G, Iatridis JC, Sakai D, Härtl R, Ito K, Grad S (2014) Cell therapy for intervertebral disc repair: advancing cell therapy from bench to clinics. Eur Cell Mater **27**: 5-11.

Bensaïd W, Triffitt JT, Blanchat C, Oudina K, Sedel L, Petite H (2003) A biodegradable fibrin scaffold for mesenchymal stem cell transplantation. Biomaterials **24**: 2497-2502.

Berlemann U (2015) Nucleus augmentation with NuCore® Injectable Nucleus – previous experience and long-term follow-up. Eur Spine J **24**: 624-660.

Berlemann U, Schwarzenbach O (2009) An injectable nucleus replacement as an adjunct to

microdiscectomy: 2 year follow-up in a pilot clinical study. Eur Spine J **18**: 1706-1712.

Bhardwaj N, Kundu SC (2012) Chondrogenic differentiation of rat MSCs on porous scaffolds of silk fibroin/chitosan blends. Biomaterials **33**: 2848-2857.

Bhattacharjee M, Miot S, Gorecka A, Singha K, Loparic M, Dickinson S, Das A, Bhavesh NS, Ray AR, Martin I, Ghosh S (2012) Oriented lamellar silk fibrous scaffolds to drive cartilage matrix orientation: towards annulus fibrosus tissue engineering. Acta Biomater **8**: 3313-3325.

Blumenthal S, McAfee PC, Guyer RD, Hochschuler SH, Geisler FH, Holt RT, Garcia R, Regan JJ, Ohnmeiss DD (2005) A prospective, randomized, multicenter Food and Drug Administration investigational device exemptions study of lumbar total disc replacement with the CHARITE artificial disc *versus* lumbar fusion: part I: evaluation of clinical outcomes. Spine **30**: 1565-1575.

Borde B, Grunert P, Härtl R, Bonassar LJ (2015) Injectable, high-density collagen gels for annulus fibrosus repair: an *in vitro* rat tail model. J Biomed Mater Res A **103**: 2571-2581.

Bougault C, Aubert-Foucher E, Paumier A, Perrier-Groult E, Huot L, Hot D, Duterque-Coquillaud M, Mallein-Gerin F (2012) Dynamic compression of chondrocyte-agarose constructs reveals new candidate mechanosensitive genes. PLoS ONE **7**: e36964.

Boyd LM, Carter AJ (2006) Injectable biomaterials and vertebral endplate treatment for repair and regeneration of the intervertebral disc. Eur Spine J **15 Suppl 3**: S414-S421.

Bron JL, van der Veen AJ, Helder MN, van Royen BJ, Smit TH, (2010) Biomechanical and *in vivo* evaluation of experimental closure devices of the annulus fibrosus designed for a goat nucleus replacement model. Eur Spine J **19**: 1347-1355.

Bron JL, Vonk LA, Smit TH, Koenderink GH (2011) Engineering alginate for intervertebral disc repair. J Mech Behav Biomed Mater **4**: 1196-1205.

Buckwalter JA (1995) Aging and degeneration of the human intervertebral disc. Spine **20**: 1307-1314.

Burkhardt BW, Simgen A, Wagenpfeil G, Reith W, Oertel JM (2017) Adjacent segment degeneration after anterior cervical discectomy and fusion with an autologous iliac crest graft: a Magnetic Resonance Imaging study of 59 patients with a mean follow-up of 27 yr. Neurosurgery, doi: 10.1093/neuros/nyx304.

Buser Z, Kuelling F, Liu J, Liebenberg E, Thorne KJ, Coughlin D, Lotz JC (2011) Biological and biomechanical effects of fibrin injection into porcine intervertebral discs. Spine **36**: E1201-E1209.

Calderon L, Collin E, Velasco-Bayon D, Murphy M, O'Halloran D, Pandit A (2010) Type II collagen-hyaluronan hydrogel – a step towards a scaffold for intervertebral disc tissue engineering. Eur Cell Mater **20**: 134-148.

Carragee EJ, Hurwitz EL, Weiner BK (2011) A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: emerging safety concerns and lessons learned. Spine J **11**: 471-491.

Catto V, Farè S, Cattaneo I, Figliuzzi M, Alessandrino A, Freddi G, Remuzzi A, Tanzi MC (2015) Small diameter electrospun silk fibroin vascular grafts: mechanical properties, *in vitro* biodegradability, and *in vivo* biocompatibility. Mater Sci Eng C Mater Biol Appl **54**: 101-111.

Chan SC, Gantenbein-Ritter B (2012) Intervertebral disc regeneration or repair with biomaterials and stem cell therapy – feasible or fiction? Swiss Med Wkly **142**: w13598.

Chan SC, Tekari A, Benneker LM, Heini PF, Gantenbein B (2015) Osteogenic differentiation of bone marrow stromal cells is hindered by the presence of intervertebral disc cells. Arthritis Res Ther **18**: 29.

Chan SCW, Walser J, Käppeli P, Shamsollahi MJ, Ferguson SJ, Gantenbein-Ritter B (2013) Region specific response of intervertebral disc cells to complex dynamic loading: an organ culture study using a dynamic torsion-compression bioreactor. PLoS ONE **8**: e72489.

Chang G, Kim HJ, Kaplan D, Vunjak-Novakovic G, Kandel RA (2007) Porous silk scaffolds can be used for tissue engineering annulus fibrosus. Eur Spine J **16**: 1848-1857.

Chang G, Kim HJ, Vunjak-Novakovic G, Kaplan DL, Kandel R (2010) Enhancing annulus fibrosus tissue formation in porous silk scaffolds. J Biomed Mater Res A **92**: 43-51.

Chen L, Gu Y, Feng Y, Zhu XS, Wang CZ, Liu HL, Niu HY, Zhang C, Yang HL (2014) Bioactivity of porous biphasic calcium phosphate enhanced by recombinant human bone morphogenetic protein 2/silk fibroin microsphere. J Mater Sci Mater Med **25**: 1709-1719.

Chen L, Liu HL, Gu Y, Feng Y, Yang HL (2015) Lumbar interbody fusion with porous biphasic calcium phosphate enhanced by recombinant bone morphogenetic protein-2/silk fibroin sustained-released microsphere: an experimental study on sheep model. J Mater Sci Mater Med **26**: 126.

Chung DE, Kim HH, Kim MK, Lee KH, Park YH, Um IC (2015) Effects of different *Bombyx mori* silkworm varieties on the structural characteristics and properties of silk. Int J Biol Macromol **79**: 943-951.

Clarke LE, McConnell JC, Sherratt MJ, Derby B, Richardson SM, Hoyland JA (2014) Growth differentiation factor 6 and transforming growth factor-beta differentially mediate mesenchymal stem cell differentiation, composition and micromechanical properties of nucleus pulposus constructs. Arthritis Res Ther **16**: R67.

Colombini A, Ceriani C, Banfi G, Brayda-Bruno M, Moretti M (2014) Fibrin in intervertebral disc tissue engineering. Tissue Eng Part B Rev **20**: 713-721.

Colombini A, Lopa S, Ceriani C, Lovati AB, Croiset SJ, Di Giancamillo A, Lombardi G, Banfi G, Moretti M (2015) *In vitro* characterization and *in vivo* behavior of human nucleus pulposus and annulus fibrosus cells

in clinical-grade fibrin and collagen-enriched fibrin gels. Tissue Eng Part A **21**: 793-802.

Costi JJ, Stokes IA, Gardner-Morse M, Laible JP, Scoffone HM, Iatridis JC (2007) Direct measurement of intervertebral disc maximum shear strain in six degrees of freedom: motions that place disc tissue at risk of injury. J Biomech **40**: 2457-2466.

Coutinho DF, Sant SV, Shin H, Oliveira JT, Gomes ME, Neves NM, Khademhosseini A, Reis RL (2010) Modified Gellan gum hydrogels with tunable physical and mechanical properties. Biomaterials **31**: 7494-7502.

Cruz MA, McAnany S, Gupta N, Long R, Nasser PR, Eglin D, Hecht A, Illien-Junger S, Iatridis JC (2017) Structural and chemical modification to improve adhesive and material properties of fibrin-genipin for repair of annulus fibrosus defects in intervertebral discs. J Biomech Eng **139**. doi: 10.1115/1.4036623.

Dagenais S, Caro J, Haldeman S (2008) A systematic review of low back pain cost of illness studies in the United States and internationally. Spine J **8**: 8-20.

Deyo RA, Weinstein JN (2001) Low back pain. N Engl J Med 344: 363-370.

Dong X, Zhao Q, Xiao L, Lu Q, Kaplan DL (2016) Amorphous silk nanofiber solutions for fabricating silk-based functional materials. Biomacromolecules **17**: 3000-3006.

Donk RD, Verhagen WIM, Hosman AJF, Verbeek A, Bartels RHMA (2017) Symptomatic adjacent segment disease after anterior cervical discectomy for single-level degenerative disk disease. Clin Spine Surg. doi: 10.1097/BSD.0000000000000551.

Du L, Zhu M, Yang Q, Zhang J, Ma X, Kong D, Li X, Xu B, Wang L (2014) A novel integrated biphasic silk fibroin scaffold for intervertebral disc tissue engineering. Materials Letters **117**: 237-240.

Dudli S, Haschtmann D, Ferguson SJ (2014) Persistent degenerative changes in the intervertebral disc after burst fracture in an *in vitro* model mimicking physiological post-traumatic conditions. Eur Spine J **24**: 1901-1908.

Dudli S, Sing DC, Hu SS, Berven SH, Burch S, Deviren V, Cheng I, Tay BKB, Alamin TF, Ith MAM, Pietras EM, Lotz JC (2017) ISSLS prize in basic science 2017: Intervertebral disc/bone marrow cross-talk with modic changes. Eur Spine J **26**: 1362-1373.

Duggal S, Frønsdal KB, Szöke K, Shahdadfar A, Melvik JE, Brinchmann JE (2009) Phenotype and gene expression of human mesenchymal stem cells in alginate scaffolds. Tissue Eng Part A **15**: 1763-1773.

Duthey B (2013) Low back pain. In: Priority Medicines for Europe and the World 2013 Update. World Health Organization, Geneva. ISBN 978 92 4 150575 8 Chapter 6.24: 165-168.

Elliott WH, Bonani W, Maniglio D, Motta A, Tan W, Migliaresi C (2015) Silk hydrogels of tunable structure and viscoelastic properties using different chronological orders of genipin and physical cross-linking. ACS Appl Mater Interfaces **7**: 12099-12108.

Eyre DR (1979) Biochemistry of the intervertebral disc. Int Rev Connect Tissue Res **8**: 227-291.

Feng G, Li L, Liu H, Song Y, Huang F, Tu C, Shen B, Gong Q, Li T, Liu L, Zeng J, Kong Q, Yi M, Gupte M, Ma PX, Pei F (2013) Hypoxia differentially regulates human nucleus pulposus and annulus fibrosus cell extracellular matrix production in 3D scaffolds. Osteoarthritis Cartilage **21**: 582-588.

Frauchiger DA, Chan SCW, Benneker LM, Gantenbein B (2015) Repair of annulus fibrosus with genipin enhanced fibrin hydrogel and silk membrane fleece. Eur Spine J **24**: 624-660.

Fraylich MR, Liu R, Richardson SM, Baird P, Hoyland J, Freemont AJ, Alexander C, Shakesheff K, Cellesi F, Saunders BR (2010) Thermally-triggered gelation of PLGA dispersions: towards an injectable colloidal cell delivery system. J Colloid Interface Sci **344**: 61-69.

Fu C, Shao Z, Fritz V (2009) Animal silks: their structures, properties and artificial production. Chem Commun (Camb) **43**: 6515-6529.

Gantenbein-Ritter B, Potier E, Zeiter S, van der Werf M, Sprecher CM, Ito K (2008) Accuracy of three techniques to determine cell viability in 3D tissues or scaffolds. Tissue Eng Part C Methods **14**: 353-358.

Gantenbein-Ritter B, Sakai D (2011). Biomaterials for intervertebral disc regeneration. In: Comprehensive Biomaterials (eds) Ducheyne P, Healy KE, Hutmacher D, Grainger DW, Kirkpatrick CJ. Elsevier, Amsterdam. pp161-169.

Guterl CC, See EY, Blanquer SB, Pandit A, Ferguson SJ, Benneker LM, Grijpma DW, Sakai D, Eglin D, Alini M, Iatridis JC, Grad S (2013) Challenges and strategies in the repair of ruptured annulus fibrosus. Eur Cell Mater **25**: 1-21.

Guterl CC, Torre OM, Purmessur D, Dave K, Likhitpanichkul M, Hecht A, Nicoll S, Iatridis JC (2014) Characterization of mechanics and cytocompatibility of fibrin-genipin annulus fibrosus sealant with the addition of cell adhesion molecules. Tissue Eng Part A **20**: 2536-2545.

Hardy JG, Geissler SA, Aguilar D, Villancio-Wolter MK, Mouser DJ, Sukhavasi RC, Cornelison RC, Tien LW, Preda RC, Hayden RS, Chow JK, Nguy L, Kaplan DL, Schmidt CE (2015) Instructive conductive 3D silk foam-based bone tissue scaffolds enable electrical stimulation of stem cells for enhanced osteogenic differentiation. Macromol Biosci **15**: 1490-1496.

Hofmann S, Foo CT, Rossetti F, Textor M, Vunjak-Novakovic G, Kaplan DL, Merkle HP, Meinel L (2006b) Silk fibroin as an organic polymer for controlled drug delivery. J Control Release **111**: 219-227.

Hofmann S, Hagenmüller H, Koch AM, Müller R, Vunjak-Novakovic G, Kaplan DL, Merkle HP, Meinel L (2007) Control of *in vitro* tissue-engineered bone-like structures using human mesenchymal stem cells and porous silk scaffolds. Biomaterials **28**: 1152-1162.

Hofmann S, Knecht S, Langer R, Kaplan DL, Vunjak-Novakovic G, Merkle HP, Meinel L (2006a)

Cartilage-like tissue engineering using silk scaffolds and mesenchymal stem cells. Tissue Eng **12**: 2729-2738.

Holm S, Maroudas A, Urban JP, Selstam G, Nachemson A (1981) Nutrition of the intervertebral disc: solute transport and metabolism. Connect Tissue Res **8**:101-119.

Hong J, Reed C, Novick D, Happich M (2013) Costs associated with treatment of chronic low back pain: an analysis of the UK general practice research database. Spine **38**: 75-82.

Hong S, Sycks D, Chan HF, Lin S, Lopez GP, Guilak F, Leong KW, Zhao X (2015) 3D printing of highly stretchable and tough hydrogels into complex, cellularized structures. Adv Mater **27**: 4035-4040.

Horan RL, Antle K, Collette AL, Wang Y, Huang J, Moreau JE, Volloch V, Kaplan DL, Altman GH (2005) *In vitro* degradation of silk fibroin. Biomaterials **26**: 3385-3393.

Hoy D, Brooks P, Blyth F, Buchbinder R (2010) The epidemiology of low back pain. Best Pract Res Clin Rheumatol **24**: 769-781.

Hu J, Chen B, Guo F, Du J, Gu P, Lin X, Yang W, Zhang H, Lu M, Huang Y, Xu G (2012) Injectable silk fibroin/polyurethane composite hydrogel for nucleus pulposus replacement. J Mater Sci Mater Med **23**: 711-722.

Huang YC, Urban JP, Luk KD (2014) Intervertebral disc regeneration: do nutrients lead the way? Nat Rev Rheumatol **10**: 561-566.

Hunter CJ, Mouw JK, Levenston ME (2004) Dynamic compression of chondrocyte-seeded fibrin gels: effects on matrix accumulation and mechanical stiffness. Osteoarthritis Cartilage **12**: 117-130.

Inoue S, Tanaka K, Arisaka F, Kimura S, Ohtomo K, Mizuno S (2000) Silk fibroin of *Bombyx mori* is secreted, assembling a high molecular mass elementary unit consisting of H-chain, L-chain, and P25, with a 6:6:1 molar ratio. J Biol Chem **275**: 40517-40528.

Inoue S, Tanaka K, Tanaka H, Ohtomo K, Kanda T, Imamura M, Quan GX, Kojima K, Yamashita T, Nakajima T, Taira H, Tamura T, Mizuno S (2004) Assembly of the silk fibroin elementary unit in endoplasmic reticulum and a role of L-chain for protection of alpha1,2-mannose residues in N-linked oligosaccharide chains of fibrohexamerin/P25. Eur J Biochem **271**: 356-366.

Iwata M, Ochi H, Asou Y, Haro H, Aikawa T, Harada Y, Nezu Y, Yogo T, Tagawa M, Hara Y (2013) Variations in gene and protein expression in canine chondrodystrophic nucleus pulposus cells following long-term three-dimensional culture. PLoS ONE **8**: e63120.

Jeong L, Lee KY, Liu JW, Park WH (2006) Time-resolved structural investigation of regenerated silk fibroin nanofibers treated with solvent vapor. Int J Biol Macromol **38**: 140-144.

Jin HJ, Fridrikh SV, Rutledge GC, Kaplan DL (2002) Electrospinning *Bombyx mori* silk with poly(ethylene oxide). Biomacromolecules **3**: 1233-1239.

Kambe Y, Sutherland TD, Kameda T (2014) Recombinant production and film properties of full-length hornet silk proteins. Acta Biomater **10**: 3590-3598.

Kandel R, Roberts S, Urban JP (2008) Tissue engineering and the intervertebral disc: the challenges. Eur Spine J **17 Suppl 4**: 480-491.

Karageorgiou V, Meinel L, Hofmann S, Malhotra A, Volloch V, Kaplan D (2004) Bone morphogenetic protein-2 decorated silk fibroin films induce osteogenic differentiation of human bone marrow stromal cells. J Biomed Mater Res A **71**: 528-537.

Khang G, Lee SK, Kim HN, Silva-Correia J, Gomes ME, Viegas CA, Dias IR, Oliveira JM, Reis RL (2015) Biological evaluation of intervertebral disc cells in different formulations of Gellan gum-based hydrogels. J Tissue Eng Regen Med **9**: 265-275.

Kim KW, Lim TH, Kim JG, Jeong ST, Masuda K, An HS (2003) The origin of chondrocytes in the nucleus pulposus and histologic findings associated with the transition of a notochordal nucleus pulposus to a fibrocartilaginous nucleus pulposus in intact rabbit intervertebral discs. Spine **28**: 982-990.

Kim HH, Park JB, Kang MJ, Park YH (2014) Surface-modified silk hydrogel containing hydroxyapatite nanoparticle with hyaluronic acid-dopamine conjugate. Int J Biol Macromol **70**: 516-522.

Korovessis P, Koureas G, Zacharatos S, Papazisis Z, Lambiris E (2005) Correlative radiological, self-assessment and clinical analysis of evolution in instrumented dorsal and lateral fusion for degenerative lumbar spine disease. Autograft *versus* coralline hydroxyapatite. Eur Spine J **14**: 630-638.

Kundu SC, Kundu B, Talukdar S, Bano S, Nayak S, Kundu J, Mandal BB, Bhardwaj N, Botlagunta M, Dash BC, Acharya C, Ghosh AK (2012) Invited review. Nonmulberry silk biopolymers. Biopolymers **97**: 455-467.

Kuwana Y, Sezutsu H, Nakajima K, Tamada Y, Kojima K (2014) High-toughness silk produced by a transgenic silkworm expressing spider (*Araneus ventricosus*) dragline silk protein. PLoS ONE **9**: e105325.

Lazaris A, Arcidiacono S, Huang Y, Zhou JF, Duguay F, Chretien N, Welsh EA, Soares JW, Karatzas CN (2002) Spider silk fibers spun from soluble recombinant silk produced in mammalian cells. Science **295**: 472-476.

Lee CS, Hwang CJ, Lee DH, Kim YT, Lee HS (2011) Fusion rates of instrumented lumbar spinal arthrodesis according to surgical approach: a systematic review of randomized trials. Clin Orthop Surg **3**: 39-47.

Li X, Dou Q, Kong Q (2016) Repair and regenerative therapies of the annulus fibrosus of the intervertebral disc. J Coll Physicians Surg Pak **26**: 138-144.

Li Z, Jiang Y, Cao G, Li J, Xue R, Gong C (2015 b) Construction of transgenic silkworm spinning antibacterial silk with fluorescence. Mol Biol Rep **42**: 19-25.

Li Z, Kaplan KM, Wertzel A, Peroglio M, Amit B, Alini M, Grad S, Yayon A (2014) Biomimetic fibrin-hyaluronan hydrogels for nucleus pulposus regeneration. Regen Med **9**: 309-326.

Li Z, Kupcsik L, Yao SJ, Alini M, Stoddart MJ (2009) Chondrogenesis of human bone marrow mesenchymal stem cells in fibrin-polyurethane composites. Tissue Eng Part A **15**: 1729-1737.

Li M, Ogiso M, Minoura N (2003) Enzymatic degradation behavior of porous silk fibroin sheets. Biomaterials **24**: 357-365.

Li Z, Peroglio M, Alini M, Grad S (2015a) Potential and limitations of intervertebral disc endogenous repair. Curr Stem Cell Res Ther **10**: 329-338.

Li C, Vepari C, Jin HJ, Kim HJ, Kaplan DL (2006) Electrospun silk-BMP-2 scaffolds for bone tissue engineering. Biomaterials **27**: 3115-3124.

Li C, Wang HR, Li XL, Zhou XG, Dong J (2012) The relation between zoledronic acid infusion and interbody fusion in patients undergoing transforaminal lumbar interbody fusion surgery. Acta Neurochir (Wien) **154**: 731-738.

Likhitpanichkul M, Dreischarf M, Illien-Junger S, Walter BA, Nukaga T, Long RG, Sakai D, Hecht AC, Iatridis JC (2014) Fibrin-genipin adhesive hydrogel for annulus fibrosus repair: performance evaluation with large animal organ culture, *in situ* biomechanics, and *in vivo* degradation tests. Eur Cell Mater **28**: 25-38.

Likhitpanichkul M, Kim Y, Torre OM, See E, Kazezian Z, Pandit A, Hecht AC, Iatridis JC (2015) Fibrin-genipin annulus fibrosus sealant as a delivery system for anti-TNFα drug. Spine J **15**: 2045-2054.

Long RG, Bürki A, Zysset P, Eglin D, Grijpma DW, Blanquer SB, Hecht AC, Iatridis JC (2016a) Mechanical restoration and failure analyses of a hydrogel and scaffold composite strategy for annulus fibrosus repair. Acta Biomater **30**: 116-125.

Long RG, Torre OM, Hom WW, Assael DJ, Iatridis JC (2016b) Design requirements for annulus fibrosus repair: review of forces, displacements, and material properties of the intervertebral disk and a summary of candidate hydrogels for repair. J Biomech Eng **138**: 021007.

Luo L, Thorpe SD, Buckley CT, Kelly DJ (2015) The effects of dynamic compression on the development of cartilage grafts engineered using bone marrow and infrapatellar fat pad derived stem cells. Biomed Mater **10**: 055011.

Luoma K, Riihimäki H, Luukkonen R, Raininko R, Viikari-Juntura E, Lamminen A (2000) Low back pain in relation to lumbar disc degeneration. Spine **25**: 487-492.

Maldonado BA, Oegema TR, (1992) Initial characterization of the metabolism of intervertebral disc cells encapsulated in microspheres. J Orthop Res **10**: 677-690.

Maldonado CV, Paz RD, Martin CB (2011) Adjacent-level degeneration after cervical disc arthroplasty versus fusion. Eur Spine J **20 Suppl 3**: 403-407.

Mallepally RR, Marin MA, Surampudi V, Subia B, Rao RR, Kundu SC, McHugh MA (2015) Silk fibroin aerogels: potential scaffolds for tissue engineering applications. Biomed Mater **10**: 035002.

Malonzo C, Chan SC, Kabiri A, Eglin D, Grad S, Bonél HM, Benneker LM, Gantenbein-Ritter B (2015) A papain-induced disc degeneration model for the assessment of thermo-reversible hydrogel-cells therapeutic approach. J Tissue Eng Regen Med **9**: E167-E176.

Maroudas A, Stockwell RA, Nachemson A, Urban J (1975) Factors involved in the nutrition of the human lumbar intervertebral disc: cellularity and diffusion of glucose *in vitro*. J Anat **120**: 113-130.

Matcham S, Novakovic K (2016) Fluorescence imaging in genipin crosslinked chitosan-poly (vinyl pyrrolidone) hydrogels. Polymers **8**: 385.

Matsumoto M, Okada E, Ichihara D, Watanabe K, Chiba K, Toyama Y, Fujiwara H, Momoshima S, Nishiwaki Y, Iwanami A, Ikegami T, Takahata T, Hashimoto T (2010) Anterior cervical decompression and fusion accelerates adjacent segment degeneration: comparison with asymptomatic volunteers in a ten-year magnetic resonance imaging follow-up study. Spine **35**: 36-43.

Mauck RL, Yuan X, Tuan RS (2006) Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. Osteoarthritis Cartilage **14**: 179-189.

Määttä JH, Wadge S, MacGregor A, Karppinen J, Williams FM (2015) Vertebral endplate (Modic) change is an independent risk factor for episodes of severe and disabling low back pain. Spine **40**: 1187-1193.

Meinel L, Hofmann S, Karageorgiou V, Kirker-Head C, McCool J, Gronowicz G, Zichner L, Langer R, Vunjak-Novakovic G, Kaplan DL (2005) The inflammatory responses to silk films *in vitro* and *in vivo*. Biomaterials **26**: 147-155.

Meinel L, Kaplan DL (2012) Silk constructs for delivery of musculoskeletal therapeutics. Adv Drug Deliv Rev **64**: 1111-1122.

Meinel AJ, Kubow KE, Klotzsch E, Garcia-Fuentes M, Smith ML, Vogel V, Merkle HP, Meinel L (2009) Optimization strategies for electrospun silk fibroin tissue engineering scaffolds. Biomaterials **30**: 3058-3067.

Mercuri J, Addington C, Iii RP, Gill S, Simionescu D (2014) Development and Initial characterization of a chemically stabilized elastin-glycosaminoglycan-collagen composite shape-memory hydrogel for nucleus pulposus regeneration. J Biomed Mater Res A **102**: 4380-4393.

Min B-M, Lee G, Kim SH, Nam YS, Lee TS, Park WH (2004) Electrospinning of silk fibroin nanofibers and its effect on the adhesion and spreading of normal human keratinocytes and fibroblasts *in vitro*. Biomaterials **25**: 1289-1297.

Mortisen D, Peroglio M, Alini M, Eglin D (2010) Tailoring thermoreversible hyaluronan hydrogels by

"click" chemistry and RAFT polymerization for cell and drug therapy. Biomacromolecules **11**: 1261-1272.

Murab S, Samal J, Shrivastava A, Ray AR, Pandit A, Ghosh S (2015) Glucosamine loaded injectable silk-in-silk integrated system modulate mechanical properties in bovine *ex-vivo* degenerated intervertebral disc model. Biomaterials **55**: 64-83.

Mwale F, Ciobanu I, Giannitsios D, Roughley P, Steffen T, Antoniou J (2011) Effect of oxygen levels on proteoglycan synthesis by intervertebral disc cells. Spine **36**: E131-E138.

Mwangi TK, Bowles RD, Tainter DM, Bell RD, Kaplan DL, Setton LA (2015) Synthesis and characterization of silk fibroin microparticles for intra-articular drug delivery. Int J Pharm **485**: 7-14.

Nachemson A, Elfstrom G (1970) Intravital dynamic pressure measurements in lumbar discs. A study of common movements, maneuvers and exercises. Scand J Rehabil Med Suppl **1**: 1-40.

Naqvi SM, Buckley CT (2015) Differential response of encapsulated nucleus pulposus and bone marrow stem cells in isolation and coculture in alginate and chitosan hydrogels. Tissue Eng Part A **21**: 288-299.

Nerurkar NL, Elliott DM, Mauck RL (2010) Mechanical design criteria for intervertebral disc tissue engineering. J Biomech **43**: 1017-1030.

Ngoenkam J, Faikrua A, Yasothornsrikul S, Viyoch J (2010) Potential of an injectable chitosan/starch/beta-glycerol phosphate hydrogel for sustaining normal chondrocyte function. Int J Pharm **391**: 115-124.

Ninh C, Iftikhar A, Cramer M, Bettinger CJ (2015) Diffusion--reaction models of genipin incorporation into fibrin networks. Journal of Materials Chemistry B **3**: 4607-4615.

Oegema TR (1993) Biochemistry of the intervertebral disc. Clin Sports Med **12**: 419-439.

Omlor GW, Nerlich AG, Lorenz H, Bruckner T, Richter W, Pfeiffer M, Gühring T (2012) Injection of a polymerized hyaluronic acid/collagen hydrogel matrix in an *in vivo* porcine disc degeneration model. Eur Spine J **21**: 1700-1708.

Panda NN, Biswas A, Pramanik K, Jonnalagadda S (2015) Enhanced osteogenic potential of human mesenchymal stem cells on electrospun nanofibrous scaffolds prepared from eri-tasar silk fibroin. J Biomed Mater Res B Appl Biomater **103**: 971-982.

Panilaitis B, Altman GH, Chen J, Jin HJ, Karageorgiou V, Kaplan DL (2003) Macrophage responses to silk. Biomaterials **24**: 3079-3085.

Panzavolta S, Gioffrè M, Focarete ML, Gualandi C, Foroni L, Bigi A (2011) Electrospun gelatin nanofibers: optimization of genipin cross-linking to preserve fiber morphology after exposure to water. Acta Biomater **7**: 1702-1709.

Park SH, Cho H, Gil ES, Mandal BB, Min BH, Kaplan DL (2011) Silk-fibrin/hyaluronic acid composite gels for nucleus pulposus tissue regeneration. Tissue Eng Part A **17**: 2999-3009.

Park SH, Gil ES, Cho H, Mandal BB, Tien LW, Min BH, Kaplan DL (2012) Intervertebral disk tissue engineering using biphasic silk composite scaffolds. Tissue Eng Part A **18**: 447-458.

Park SY, Ki CS, Park YH, Jung HM, Woo KM, Kim HJ (2010) Electrospun silk fibroin scaffolds with macropores for bone regeneration: an *in vitro* and *in vivo* study. Tissue Eng Part A **16**: 1271-1279.

Patra C, Talukdar S, Novoyatleva T, Velagala SR, Mühlfeld C, Kundu B, Kundu SC, Engel FB (2012) Silk protein fibroin from *Antheraea mylitta* for cardiac tissue engineering. Biomaterials **33**: 2673-2680.

Peeters M, Detiger SE, Karfeld-Sulzer LS, Smit TH, Yayon A, Weber FE, Helder MN (2015) BMP-2 and BMP-2/7 heterodimers conjugated to a fibrin/hyaluronic acid hydrogel in a large animal model of mild intervertebral disc degeneration. Biores Open Access **4**: 398-406.

Perka C, Arnold U, Spitzer RS, Lindenhayn K (2001) The use of fibrin beads for tissue engineering and subsequential transplantation. Tissue Eng **7**: 359-361.

Peroglio M, Eglin D, Benneker LM, Alini M, Grad S (2013) Thermoreversible hyaluronan-based hydrogel supports *in vitro* and *ex vivo* disc-like differentiation of human mesenchymal stem cells. Spine J **13**: 1627-1639.

Peroglio M, Grad S, Mortisen D, Sprecher CM, Illien-Jünger S, Alini M, Eglin D (2012) Injectable thermoreversible hyaluronan-based hydrogels for nucleus pulposus cell encapsulation. Eur Spine J **21 Suppl 6**: S839-S849.

Pritchard EM, Valentin T, Panilaitis B, Omenetto F, Kaplan DL (2013) Antibiotic-releasing silk biomaterials for infection prevention and treatment. Adv Funct Mater **23**: 854-861.

Priyadarshani P, Li Y, Yao L (2015) Advances in biological therapy for nucleus pulposus regeneration. Osteoarthritis Cartilage **24**: 206-212.

Qu J, Liu Y, Yu Y, Li J, Luo J, Li M (2014) Silk fibroin nanoparticles prepared by electrospray as controlled release carriers of cisplatin. Mater Sci Eng C Mater Biol Appl **44**: 166-174.

Rao J, Shen J, Quan D, Xu Y (2009) [Property studies on three-dimensional porous blended silk scaffolds]. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi **23**: 1264-1270.

Reddy N, Yang Y (2010) Morphology and tensile properties of silk fibers produced by uncommon *Saturniidae*. Int J Biol Macromol **46**: 419-424.

Reitmaier S, Kreja L, Gruchenberg K, Kanter B, Silva-Correia J, Oliveira JM, Reis RL, Perugini V, Santin M, Ignatius A, Wilke HJ (2014) *In vivo* biofunctional evaluation of hydrogels for disc regeneration. Eur Spine J **23**: 19-26.

Risbud MV, Schoepflin ZR, Mwale F, Kandel RA, Grad S, Iatridis JC, Sakai D, Hoyland JA (2015) Defining the phenotype of young healthy nucleus pulposus cells: recommendations of the Spine Research Interest Group at the 2014 annual ORS meeting. J Orthop Res **33**: 283-293.

Rnjak-Kovacina J, Wray LS, Burke KA, Torregrosa T, Golinski JM, Huang W, Kaplan DL (2015)

Lyophilized silk sponges: a versatile biomaterial platform for soft tissue engineering. ACS Biomater Sci Eng **1**: 260-270.

Rockwood DN, Preda RC, Yücel T, Wang X, Lovett ML, Kaplan DL (2011) Materials fabrication from *Bombyx mori* silk fibroin. Nat Protoc **6**: 1612-1631.

Sakai D, Grad S (2014) Advancing the cellular and molecular therapy for intervertebral disc disease. Adv Drug Deliv Rev **84**: 159-171.

Schek RM, Michalek AJ, Iatridis JC (2011) Genipin-crosslinked fibrin hydrogels as a potential adhesive to augment intervertebral disc annulus repair. Eur Cell Mater **21**: 373-383.

Schmocker AM, Khoushabi A, Frauchiger DA, Gantenbein B, Schizas C, Moser C, Bourban P-E, Pioletti D (2016) A photopolymerized Poly-Ethlyene-Glycol composite hydrogel and surgical implanting tool for a nucleus pulposus replacement. Biomaterials **88**: 110-119.

Schutgens EM, Tryfonidou MA, Smit TH, Öner FC, Krouwels A, Ito K, Creemers LB (2015) Biomaterials for intervertebral disc regeneration: past performance and possible future strategies. Eur Cell Mater **30**: 210-231.

See EY, Toh SL, Goh JC (2011) Effects of radial compression on a novel simulated intervertebral disc-like assembly using bone marrow-derived mesenchymal stem cell cell-sheets for annulus fibrosus regeneration. Spine **36**: 1744-1751.

See EY, Toh SL, Goh JC (2012) Simulated intervertebral disc-like assembly using bone marrow-derived mesenchymal stem cell sheets and silk scaffolds for annulus fibrosus regeneration. J Tissue Eng Regen Med **6**: 528-535.

Sha'ban M, Yoon SJ, Ko YK, Ha HJ, Kim SH, So JW, Idrus RB, Khang G (2008) Fibrin promotes proliferation and matrix production of intervertebral disc cells cultured in three-dimensional poly(lactic-co-glycolic acid) scaffold. J Biomater Sci Polym Ed **19**: 1219-1237.

Shao X, Hunter CJ (2007) Developing an alginate/chitosan hybrid fiber scaffold for annulus fibrosus cells. J Biomed Mater Res A **82**: 701-710.

Sharifi S, Bulstra SK, Grijpma DW, Kuijer R (2015) Treatment of the degenerated intervertebral disc; closure, repair and regeneration of the annulus fibrosus. J Tissue Eng Regen Med **9**: 1120-1132.

Sheikh FA, Ju HW, Lee JM, Moon BM, Park HJ, Lee OJ, Kim JH, Kim DK, Park CH (2015) 3D electrospun silk fibroin nanofibers for fabrication of artificial skin. Nanomedicine **11**: 681-691.

Sheikh FA, Woo Ju H, Mi Moon B, Jung Park H, Kim J-H, Joo Lee O, Hum Park C (2014) Facile and highly efficient approach for the fabrication of multifunctional silk nanofibers containing hydroxyapatite and silver nanoparticles. J Biomed Mater Res **102**: 3459-3469.

Showalter BL, Beckstein JC, Martin JT, Beattie EE, Espinoza Orías AA, Schaer TP, Vresilovic EJ, Elliott DM (2012) Comparison of animal discs used in disc research to human lumbar disc: Torsion mechanics and collagen content. Spine **37**: E900-E907.

Silva-Correia J, Gloria A, Oliveira MB, Mano JF, Oliveira JM, Ambrosio L, Reis RL (2013) Rheological and mechanical properties of acellular and cell-laden methacrylated Gellan gum hydrogels. J Biomed Mater Res A **101**: 3438-3446.

Silva-Correia J, Miranda-Gonçalves V, Salgado AJ, Sousa N, Oliveira JM, Reis RM, Reis RL (2012) Angiogenic potential of Gellan-gum-based hydrogels for application in nucleus pulposus regeneration: *in vivo* study. Tissue Eng Part A **18**: 1203-1212.

Silva-Correia J, Oliveira JM, Caridade SG, Oliveira JT, Sousa RA, Mano JF, Reis RL (2011) Gellan gum-based hydrogels for intervertebral disc tissue-engineering applications. J Tissue Eng Regen Med **5**: e97-107.

Simmonds MC, Brown JV, Heirs MK, Higgins JP, Mannion RJ, Rodgers MA, Stewart LA (2013) Safety and effectiveness of recombinant human bone morphogenetic protein-2 for spinal fusion: A meta-analysis of individual-participant data. Ann Intern Med **158**: 877-889.

Singh BN, Panda NN, Pramanik K (2016) A novel electrospinning approach to fabricate high strength aqueous silk fibroin nanofibers. Int J Biol Macromol **87**: 201-207.

Sofia S, McCarthy MB, Gronowicz G, Kaplan DL (2001) Functionalized silk-based biomaterials for bone formation. J Biomed Mater Res **54**: 139-148.

Stairmand JW, Holm S, Urban JP (1991) Factors influencing oxygen concentration gradients in the intervertebral disc. A theoretical analysis. Spine (Phila Pa 1976) **16**: 444-449.

Stern S, Lindenhayn K, Schultz O, Perka C (2000) Cultivation of porcine cells from the nucleus pulposus in a fibrin/hyaluronic acid matrix. Acta Orthop Scand **71**: 496-502.

Stich S, Stolk M, Girod PP, Thomé C, Sittinger M, Ringe J, Seifert M, Hegewald AA (2015) Regenerative and immunogenic characteristics of cultured nucleus pulposus cells from human cervical intervertebral discs. PLoS ONE **10**: e0126954.

Sun W, Incitti T, Migliaresi C, Quattrone A, Casarosa S, Motta A (2015) Viability and neuronal differentiation of neural stem cells encapsulated in silk fibroin hydrogel functionalized with an IKVAV peptide. J Tissue Eng Regen Med **11**: 1532-1541.

Sutherland TD, Young JH, Weisman S, Hayashi CY, Merritt DJ (2010) Insect silk: one name, many materials. Annu Rev Entomol **55**: 171-188.

Tao H, Marelli B, Yang M, An B, Onses S, Rogers JA, Kaplan DL, Omenetto FG (2015) Inkjet printing of regenerated silk fibroin: From printable forms to printable functions. Adv Mater **27**: 4273-4279.

Tekari A, May RD, Frauchiger DA, Chan SCW, Benneker LM, Gantenbein B (2017) The BMP2 variant L51P restores the osteogenic differentiation of human mesenchymal stromal cells in the presence of intervertebral disc cells. Eur Cell Mater **33**: 197-210.

Thorpe AA, Boyes VL, Sammon C, Le Maitre CL (2016) Thermally triggered injectable hydrogel, which induces mesenchymal stem cell differentiation to nucleus pulposus cells: Potential for regeneration of the intervertebral disc. Acta Biomater **36**: 99-111.

Tian L, Prabhakaran MP, Hu J, Chen M, Besenbacher F, Ramakrishna S (2015) Coaxial electrospun poly(lactic acid)/silk fibroin nanofibers incorporated with nerve growth factor support the differentiation of neuronal stem cells. RSC Advances **5**: 49838-49848.

Tsaryk R, Gloria A, Russo T, Anspach L, De Santis R, Ghanaati S, Unger RE, Ambrosio L, James Kirkpatrick C (2015) Collagen-low molecular weight hyaluronic acid semi-interpenetrating network loaded with gelatin microspheres for cell and growth factor delivery for nucleus pulposus regeneration. Acta Biomater **20**: 10-21.

Uebersax L, Mattotti M, Papaloïzos M, Merkle HP, Gander B, Meinel L (2007) Silk fibroin matrices for the controlled release of nerve growth factor (NGF). Biomaterials **28**: 4449-4460.

Urban JP, Smith S, Fairbank JC (2004) Nutrition of the intervertebral disc. Spine **29**: 2700-2709.

Vollrath F (2000) Strength and structure of spiders' silks. J Biotechnol **74**: 67-83.

Wang X, Kaplan DL (2011) Functionalization of silk fibroin with NeutrAvidin and biotin. Macromol Biosci **11**: 100-110.

Wang Y, Kim UJ, Blasioli DJ, Kim HJ, Kaplan DL (2005) *In vitro* cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. Biomaterials **26**: 7082-7094.

Wang D, Liu H, Fan Y (2015) Silk fibroin for vascular regeneration. Microsc Res Tech **11**: 100-110.

Wang Y, Rudym DD, Walsh A, Abrahamsen L, Kim HJ, Kim HS, Kirker-Head C, Kaplan DL (2008) *In vivo* degradation of three-dimensional silk fibroin scaffolds. Biomaterials **29**: 3415-3428.

Watkins R, Watkins R, Hanna R (2014) Non-union rate with stand-alone lateral lumbar interbody fusion. Medicine (Baltimore) **93**: e275.

Weber KT, Jacobsen TD, Maidhof R, Virojanapa J, Overby C, Bloom O, Quraishi S, Levine M, Chahine NO (2015) Developments in intervertebral disc disease research: pathophysiology, mechanobiology, and therapeutics. Curr Rev Musculoskelet Med **8**: 18-31.

White AA, Panjabi MM (1990) Clinical Biomechanics of the Spine. B Lippincott Company, Philadelphia, Pennsylvania. ISBN-13: 978-0397507207

Wieser S, Horisberger B, Schmidhauser S, Eisenring C, Brügger U, Ruckstuhl A, Dietrich J, Mannion AF, Elfering A, Tamcan O, Müller U (2011) Cost of low back pain in Switzerland in 2005. Eur J Health Econ **12**: 455-467.

Wilke HJ, Neef P, Caimi M, Hoogland T, Claes LE (1999) New *in vivo* measurements of pressures in the intervertebral disc in daily life. Spine **24**: 755-762.

Wöltje M, Böbel M. (2017) Natural biodegradable medical polymers: silk, In: Science and principles of biodegradable and bioresorbable medical polymers (ed. X C Zhang). Woodhead publishing series in biomaterials. Chapter 12: 351-376.

Xu J, Wang W, Ludeman M, Cheng K, Hayami T, Lotz JC, Kapila S (2008) Chondrogenic differentiation of human mesenchymal stem cells in three-dimensional alginate gels. Tissue Eng Part A **14**: 667-680.

Yang M, Shuai Y, He W, Min S, Zhu L (2012) Preparation of porous scaffolds from silk fibroin extracted from the silk gland of *Bombyx mori* (*B. mori*). Int J Mol Sci **13**: 7762-7775.

Yang M, Shuai Y, Zhang C, Chen Y, Zhu L, Mao C, Ouyang H (2014) Biomimetic nucleation of hydroxyapatite crystals mediated by *Antheraea pernyi* silk sericin promotes osteogenic differentiation of human bone marrow derived mesenchymal stem cells. Biomacromolecules **15**: 1185-1193.

Yang SH, Wu CC, Shih TT, Chen PQ, Lin FH (2008) Three-dimensional culture of human nucleus pulposus cells in fibrin clot: comparisons on cellular proliferation and matrix synthesis with cells in alginate. Artif Organs **32**: 70-73.

Yildirim S, Borer ME, Wenk E, Meinel L, Lacroix C (2010) Development of silk fibroin-based beads for immobilized cell fermentations. J Microencapsul **27**: 1-9.

Zeiter S, der Werf MV, Ito K (2009) The fate of bovine bone marrow stromal cells in hydrogels: a comparison to nucleus pulposus cells and articular chondrocytes. J Tissue Eng Regen Med **3**: 310-320.

Zeng Y, Chen C, Liu W, Fu Q, Han Z, Li Y, Feng S, Li X, Qi C, Wu J, Wang D, Corbett C, Chan BP, Ruan D, Du Y (2015a) Injectable microcryogels reinforced alginate encapsulation of mesenchymal stromal cells for leak-proof delivery and alleviation of canine disc degeneration. Biomaterials **59**: 53-65.

Zeng S, Liu L, Shi Y, Qiu J, Fang W, Rong M, Guo Z, Gao W (2015b) Characterization of silk fibroin/chitosan 3D porous scaffold and *in vitro* cytology. PLoS ONE **10**: e0128658.

Zeng C, Yang Q, Zhu M, Du L, Zhang J, Ma X, Xu B, Wang L (2014) Silk fibroin porous scaffolds for nucleus pulposus tissue engineering. Mater Sci Eng C Mater Biol Appl **37**: 232-240.

Zhang X, Baughman CB, Kaplan DL (2008) *In vitro* evaluation of electrospun silk fibroin scaffolds for vascular cell growth. Biomaterials **29**: 2217-2227.

Zhang X, Reagan MR, Kaplan DL (2009) Electrospun silk biomaterial scaffolds for regenerative medicine. Adv Drug Deliv Rev **61**: 988-1006.

Zhang W, Zhu C, Ye D, Xu L, Zhang X, Wu Q, Zhang X, Kaplan DL, Jiang X (2014) Porous silk scaffolds for delivery of growth factors and stem cells to enhance bone regeneration. PLoS ONE **9**: e102371.

Zhao H, Heusler E, Jones G, Li L, Werner V, Germershaus O, Ritzer J, Luehmann T, Meinel L (2014) Decoration of silk fibroin by click chemistry for biomedical application. J Struct Biol **186**: 420-430.

Zhou J, Cao C, Ma X, Hu L, Chen L, Wang C (2010) *In vitro* and *in vivo* degradation behavior of aqueous-

derived electrospun silk fibroin scaffolds. Polymer Degradation and Stability **95**: 1679-1685.

Zhu B, Li W, Lewis RV, Segre CU, Wang R (2015) E-spun composite fibers of collagen and dragline silk protein: fiber mechanics, biocompatibility, and application in stem cell differentiation. Biomacromolecules **16**: 202-213.

Zhu J, Zhang Y, Shao H, Hu X (2008) Electrospinning and rheology of regenerated *Bombyx mori* silk fibroin aqueous solutions: The effects of pH and concentration. Polymer **49**: 2880-2885.

Zigler J, Delamarter R, Spivak JM, Linovitz RJ, Danielson GO, Haider TT, Cammisa F, Zuchermann J, Balderston R, Kitchel S, Foley K, Watkins R, Bradford D, Yue J, Yuan H, Herkowitz H, Geiger D, Bendo J, Peppers T, Sachs B, Girardi F, Kropf M, Goldstein J (2007) Results of the prospective, randomized, multicenter Food and Drug Administration investigational device exemption study of the ProDisc-L total disc replacement versus circumferential fusion for the treatment of 1-level degenerative disc disease. Spine **32**: 1155-1162.

Web References

1. Studer T, Fortunato G, Gadhari N, Frauchiger D, Rossi R, Gantenbein-Ritter B (2014) Tailoring silk based nano fibres for human intervertebral disc repair. Proceedings of eCM XV conference – Cartilage & Disc: Repair and Regeneration, Davos, June 16-18, page 70: http://www.ecmconferences.org/abstracts/2014/Collection4/c4.html

2. Frauchiger DA, Chan SCW, Benneker LM, Gantenbein B (2015) Silk membrane-fleece in combination with genipin-enhanced fibrin hydrogel for annulus fibrosus repair. Proceedings of 21th annual meeting of the Swiss Society of Biomaterials and Regenerative Medicine, Lausanne, June 6-10, Page 40: http://www.ecmconferences.org/abstracts/2015/Collection4/c4.html

Editor's notes: Replies to questions raised by reviewers were incorporated into the text of the paper, so there is no Discussion with Reviewers section.

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