Characterization of Biomaterials Intended for Use in the Nucleus Pulposus of Degenerated Intervertebral Discs

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Review article

Characterization of biomaterials intended for use in the nucleus pulposus of degenerated intervertebral discs

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ABSTRACT

Biomaterials for regeneration of the intervertebral disc must meet complex requirements conforming to biological, mechanical and clinical demands. Currently no consensus on their characterization exists. It is crucial to identify parameters and their method of characterization for accurate assessment of their potential efficacy, keeping in mind the translation towards clinical application. This review systematically analyses the characterization techniques of biomaterial systems that have been used for nucleus pulposus (NP) restoration and regeneration. Substantial differences in the approach towards assessment became evident, hindering comparisons between different materials with respect to their suitability for NP restoration and regeneration. We have analysed the current approaches and identified parameters necessary for adequate biomaterial characterization, with the clinical goal of functional restoration and biological regeneration of the NP in mind. Further, we provide guidelines and goals for their measurement.

Statement of significance

Biomaterials intended for restoration of regeneration of the nucleus pulposus within the intervertebral disc must meet biological, biomechanical and clinical demands. Many materials have been investigated, but a lack of consensus on which parameters to evaluate leads to difficulties in comparing materials as well as mostly partial characterization of the materials in question. A gap between current methodology and clinically relevant and meaningful characterization is prevalent. In this article, we identify necessary methods and their implementation for complete biomaterial characterization in the context of clinical applicability. This will allow for a more unified approach to NP-biomaterials research within the field as a whole and enable comparative analysis of novel materials yet to be developed.

1. Introduction

Low back pain affects millions of people worldwide, leading to a high economic burden. Often, this pain originates in the intervertebral disc (IVD) of the spine due to disc degeneration [1,2]. IVDs allow for stability and flexibility of the spine in multiple degrees-of-freedom while providing resistance to axial compression and a centre of rotation in the functional spine of vertebrates.

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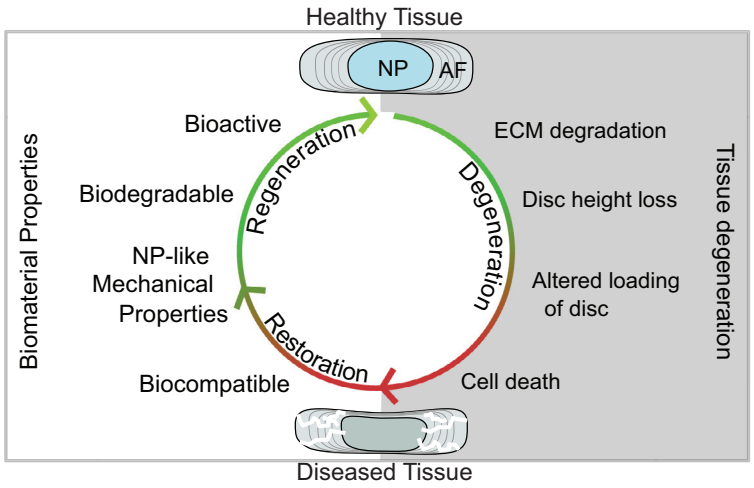

Fig. 1. Potential biomaterial features influence treatment goals after tissue degeneration within the NP.

Within the IVD, the ligamentous annulus fibrosus (AF) confines the gel-like nucleus pulposus (NP) in the centre, while the cartilaginous endplates (CEP) connect the IVD to the adjacent vertebrae on the cranial and caudal surfaces. The NP is an isotropic, fibre reinforced, gelatinous, water-rich and swollen core providing compressive resistance as well as a centre of rotation for movement [3–7]. Prior to adolescence, large vacuolated notochordal cells (NCs) can be found in the human NP, which maintain the NP [8,9] and mostly disappear with adolescence, being replaced by small nucleus pulposus cells [10,11] diminishing the regenerative capacity of the IVD. Disc degeneration slowly sets in, often culminating in neck and low back pain in adults many years later during the normal ageing processes, or at an accelerated rate in some individuals [12].

Degeneration of the IVD is a complex and multifactorial: genetic, molecular, cellular and mechanical process all lead to an imbalance between production and degradation of the ECM, initially within the NP [13,14]. Increased catabolic processes decrease the content of glycosaminoglycans (GAGs) and therefore the swelling capacity of the NP. The pressure within the IVD drops and thus leads to altered mechanical loading of the disc as a whole, tissue injury, vascularization and neoinnervation with infiltration of immune cells can then occur [4,15–20]. Additionally, calcification of the CEP inhibits nutrients from entering the IVD as well as waste removal [21,22]. With build-up of waste products like lactic acid, the pH decreases [23], which reduces cell viability and accelerates disease progression [24]. The painful symptoms of degenerative disc disease (DDD) are largely an end result of the altered extracellular matrix composition: altered mechanical loading [25], invasion of nociceptive nerves and production of neurotropic agents [20,26–29] and/or painful mechanical loading of adjacent structures due to reduced disc height or herniation [7].

Currently, few treatment options are clinically available for DDD. These focus on pain management, removal of extruded disc tissue, and IVD replacement as a whole or spinal fusion [3,30]. Commercially available IVD or NP replacement materials are often made from metal [31,32] or non-degradable polymer blends [33,34]. These restore motion segment mobility but do not regenerate the tissue, while exhibiting a broad range of potential adverse effects (possible degradation of neighbouring vertebrae [35], restricted range of motion [36], and more [37]). Therefore, it is critical to develop new strategies that promote IVD regeneration, have a viable translation to the clinic and improve the quality of life of the patient. As early IVD degeneration occurs mostly in the nucleus, the NP represents a promising target when considering potential therapies. Therefore, tissue engineering with NP bio-instructive materials presents an alternative to current treatments. Biologically compatible materials to restore the damaged tissue will not only be useful to restore NP functionally by restoring disc height, but also as a delivery vehicle for cells and/or biomolecules for re-establishing a healthy tissue. This distinction between NP restoration and regeneration also imposes different key requirements on the biomaterials in question (Fig. 1).

Although several clinical trials have already explored injectable biomaterials for NP restoration [38], it is crucial to accurately assess biomaterials for their efficacy *in vitro* and *ex vivo* prior to clinical trials. However, comparing between various biomaterials for NP restoration is hindered by differing biomaterial-characterization approaches. ISO 10,993–1 provides standards for evaluating biocompatibility of medical devices and ISO18192–2 includes recommendations for mechanical testing for spinal nucleus prostheses. These provide an overview and the legal framework but need modifications when testing e.g. biologically derived biomaterials for tissue regeneration. [39] For further standards that can serve as templates we refer to ASTM-E111–17 (Young's modulus), ASTM-F2346–18 (Artificial Discs), ASTM-F2423–11 (Total Disc Prostheses), ASTM-D2990–17 (Mechanical Testing of Plastics), ASTM-F2789–10 (Mechanical and Functional Characterization of Nucleus Devices) and ISO 10,993–9 (Biological Evaluation of Medical Devices). Identifying key principles in research for biomaterials is necessary for a patient-orientated advancement, understanding of clinical treatment of degenerated NP and reducing the number of potentially failed *in vivo* and in-patient studies.

Here, we discuss necessary considerations and evaluation parameters for potential biomaterials from conceptual design to *in vivo* study. We provide resources for more information and highlight new approaches to known challenges. Finally, we suggest guidelines for the mechanical, biological and *in vivo* characterization of biomaterials for IVD regeneration.

2. Methods

PubMed and Web of Science were searched for publications on NP restoration materials using the following keywords in the abstract or title: ((Intervertebral disc OR nucleus pulposus OR intradiscal) OR (injectable AND disc) OR disc OR (injectable AND nucleus pulposus) OR nucleus pulposus regeneration OR nucleus pulposus repair OR nucleus pulposus replacement OR nucleus pulposus substitute OR nucleus pulposus tissue engineering OR nucleus pulposus tissue regeneration) AND (biomaterial OR hydrogel OR scaffold NOT annulus NOT anulus NOT spinal

Table 1
Matrix composition of human nucleus pulposus tissue. Adapted from Antoniou *et al.* (2004).

Thompson grade of lumbar NP tissue	H_2O (%) [55]	GAG (µg/mg dry weight) [55]	Collagen (µg/mg dry weight) [55]	Elastin:Collagen ratio [45]	Protein (µg/mg dry weight) [55]
1	-/-	-/-	-/-	0.08 ± 0.02	-/-
2	82.7 ± 4.2	622 ± 152	18 ± 9		286 ± 67
3	78.7 ± 4.5	384 ± 207	29 ± 15	0.28 ± 0.05	434 ± 196
4	76.3 ± 5.0	230 ± 204	27 ± 9		596 ± 248
5	75.0 ± 1.4	86 ± 42	24 ± 1		501 ± 174

Table 2
Human NP mechanical properties are affected by degeneration state of the tissue. Adapted from Johannessen *et al.* (2005), Yang *et al.* (1998) and Iatridis *et al.* (1997). All measurements were taken at ambient temperature.

IVD Thompson grade	Swelling pressure (MPa) [47]	Effective aggregate modulus (MPa) [47]	Bulk modulus (MPa) [56]	Magnitude of complex modulus (kPa) at 1 rad/s, 10% strain [46]
1	0.139 ± 0.029	1.01 ± 0.43	1720	≈5 ± 4
2				≈17 ± 10ol
3	0.037 ± 0.038	0.44 ± 0.19	-/-	
4				≈22 ± 10
5				

fusion NOT lumbar fusion NOT cage NOT whole disc). Publications featuring computational, cell-only, whole-disc, spinal fusion, annulus fibrosus-only approaches and from conference proceedings were further excluded. Ten further papers were additionally found during publication review. Thus, 270 publications in total were identified, and their methodology analysed for this review.

3. Results

3.1. The human np biomechanical environment

In the NP, notochord-derived NP cells (NPCs) are found [40,41] in low numbers (≈4 × 10^3 cells/mm^3) [42], and the extracellular matrix (ECM) is composed mostly of water-attracting GAGs. A hallmark is a high GAG:hydroxyproline ratio around 27:1 and a collagen-II:collagen-I ratio of roughly 6:1 in healthy NPs [43,44], while degeneration is associated with increase in elastin:collagen ratio (Table 1) [45]. The decrease in GAGs in the NP during degeneration leads to lowered swelling pressure of the disc and consequently lower aggregate and instantaneous shear modulus (Table 2) [46,47]. Within the normal disc, osmolarities of 450–550 mOsm have been reported, in addition to an oxygen concentration of ≈2.6–13% *in vivo* and a pH of ≈7 in discs with no visible pathological changes. [23,48] The range of motion in a healthy lumbar spine has been reported to be ca. 50° in flexion, 20° in extension and ca. 30° in right or left lateral flexion [49,50]. In the lumbar spine 9° of axial rotation were observed [51]. Pressures in the spine are posture dependant, and have been quantified by Wilke *et al.* (1999) [52] (Table 3). Strains within the human IVD range between ≈7% (non-degenerate disc) to ≈11% (degenerate disc) [53]. Natural loading frequencies were reported at about 4–5 Hz (25–35 rad/s) [54].

Table 3
Pressures recorded intradiscally *in vivo* in human subjects during various routine postures and exercises (adapted from Wilke *et al.* (1999)).

Posture	Pressure (MPa)
Lying	0.10 – 0.12
Standing	0.5
Sitting (no backrest)	0.46 – 0.55
Walking	0.53 – 0.65
Lifting 20 kg (rounded back)	2.3
Lifting 20 kg (straight back)	1.7

3.2. Current approaches to biomaterial characterization

Most biomaterials developed for IVD regeneration have been tested *in vitro* (~60%) for their cytotoxicity and effect on gene expression in 3D cell cultures, whereas only 30% actually conducted *in vivo* studies, implanting the biomaterial in question into quadrupeds (Fig. 3). Biomaterials for NP restoration should ideally address both, mechanical and biological, aspects of IVD restoration/regeneration, while also considering the applicability and translation into a clinical setting from the beginning (Table 4). Consequently, a broad range of parameters must be considered during development and related to their effect on the material efficacy and their performance over time within the disc (Fig. 5). A range of different materials have been investigated (Fig. 2). While

Table 4
Purposes and characteristics of biomaterials used for nucleus pulposus restoration and regeneration. IVD: intervertebral disc, NP: nucleus pulposus, DOF: degrees of freedom, ECM: extracellular matrix.

Purpose		Characteristics
Mechanical	- Anatomical restoration (IVD height) - Biomechanical restoration (flexibility in multiple DOF)	- Swelling properties similar to NP - Material & mechanical characteristics similar to NP
Biological	- Inhibition of degenerative processes - Inhibition of catabolic factors and cytokines - Prevention of nerve and blood vessel ingrowth - Stimulation of regeneration	- Reservoir for cells, proteins and/or growth factors - Allow tissue remodelling
Clinical	- Pain relief - Minimally invasive - Does not migrate/extrude - Economically and translationally viable?	- Non-immunogenic - Integration into NP ECM

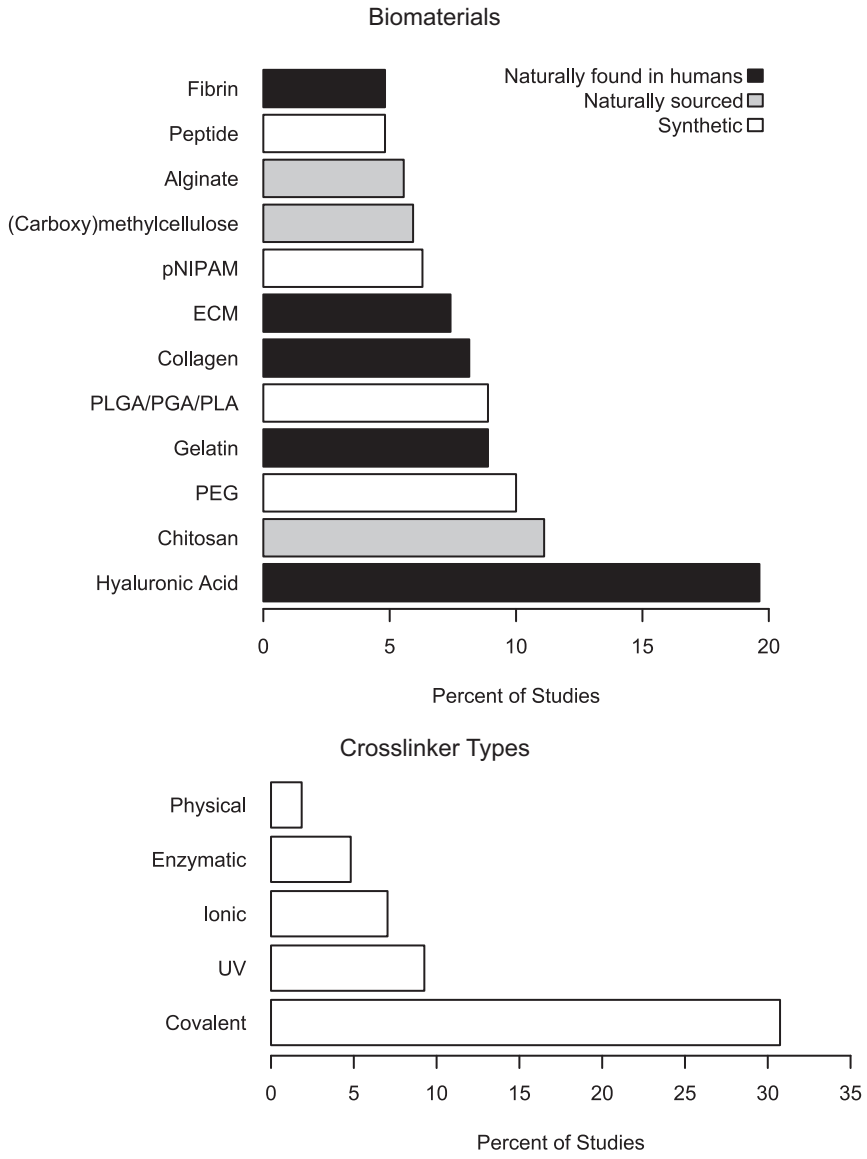

Fig. 2. Biomaterials used in ≥5% of studies (top) and crosslinking methods (bottom) considered for nucleus pulposus restoration. ECM = extracellular matrix, pNIPAM = Poly(N-isopropylacrylamide), PEG = polyethylene glycol, PLGA = poly(lactic-co-glycolic acid), PGA = poly(glycolic acid), PLA = poly(lactic acid, UV=ultraviolet light).

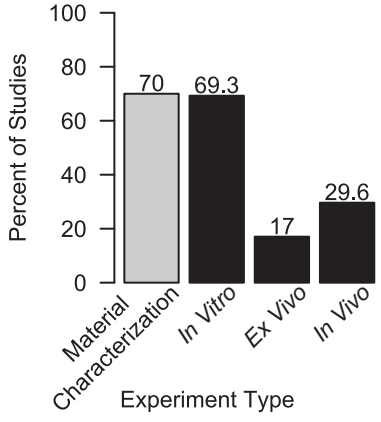

Fig. 3. Experiment types conducted in studies published. *In vivo* refers to any application of the biomaterial in question within an animal (subcutaneous/intradiscal/intramuscular implantation). Total number of preclinical studies: 270.

different materials require different characterization methods, certain parameters are critical to understand the suitability for *in vivo* applications (Table 6). Similarly, identifying an appropriate target patient population influences therapeutic strategy, material development and characterization. While injectable materials on their own may be applicable to symptomatic early to moderate disc degeneration or prophylactically used in segments adjacent to total disc replacement or fusion, they could also be used post-herniation where a combined NP and AF approach or whole IVD would be needed. In such situations preformed materials replacing the whole IVD may reduce the risk of re-herniation compared to injected materials without annulus repair [57].

To bridge the gap between mechanical and biological requirements of biomaterials, mostly naturally-derived hydrogels have been explored (Fig. 2). Many ECM-components can be used as they can form networks with immense swelling capacity, but disadvantages are their weak mechanical properties, difficult processability and risk of disease transmission. Synthetic biomaterials allow tailoring of their biomechanical properties, and are easy to manufacture and process. Both classes can be degradable or

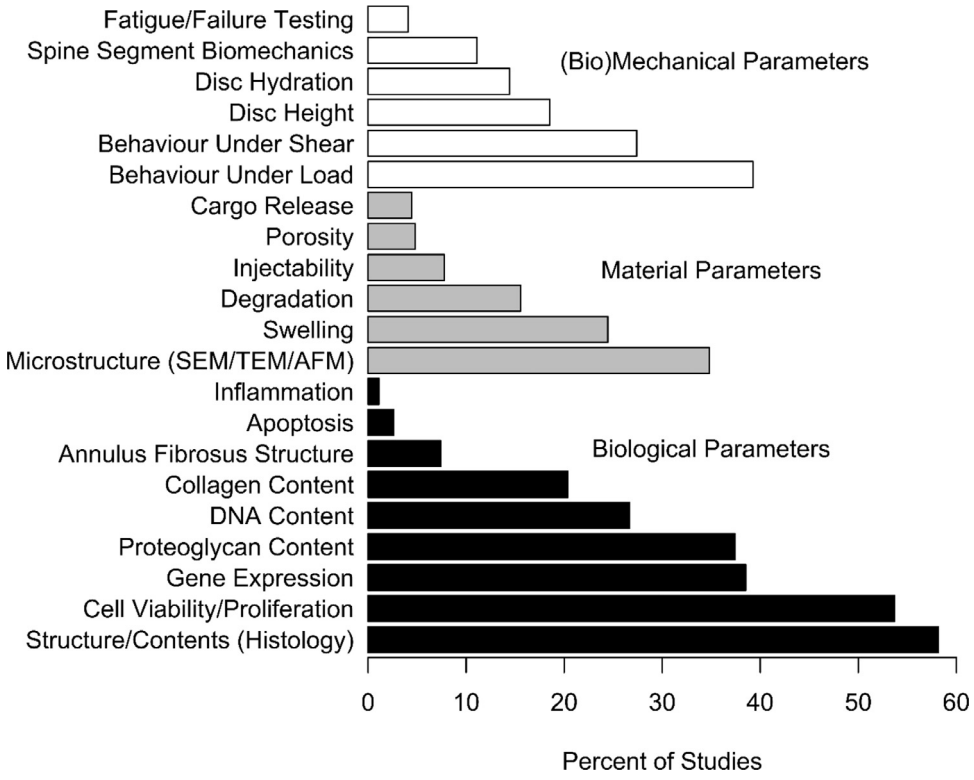

Fig. 4. Parameters investigated in nucleus pulposus restoration and regeneration studies. AFM = atomic force microscopy, DMA = dynamic mechanical analysis, ECM = extracellular matrix, MRI = magnetic resonance imaging, SEM = scanning electron microscopy, TEM = transmission electron microscopy, AFM = atomic force microscopy.

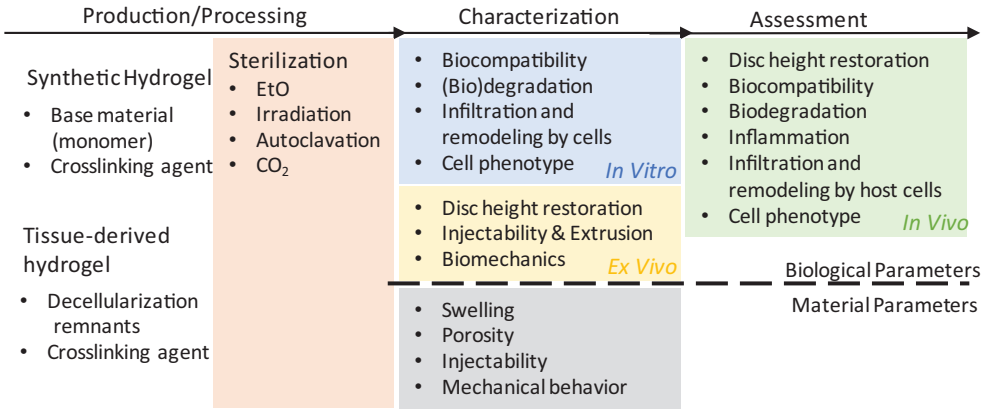

Fig. 5. Parameters of biomaterial systems that need to be evaluated for nucleus pulposus restoration & regeneration.

non-degradable: non-degradable polymers allow the fabrication of materials with higher mechanical performance however they can lack biological cues, potentially affecting cell infiltration and tissue integration and can illicit immune responses [58,59]. On the other hand, some non-degradable biomaterials have been developed which enable cellular migration, are biocompatible and do not illicit an immune response [38,60,61]. Degradable biomaterials can be remodelled by the cells and the growing tissue, however the degradation rate must meet the tissue reformation rate.

To date, research groups mostly consider the immediate beneficial biological effects at the cell and tissue level exerted by the biomaterial, i.e. cell viability, gene expression and ECM deposition (Fig. 4). In contrast to this, inflammatory markers, cell senescence and apoptosis are seldom considered. Subcutaneous implantation in small animal models is often performed but does not provide information about the suitability of the material in its clinical setting of the degenerated IVD. AF or CEP rupture can lead to influx of inflammatory cells, which is especially pertinent to ECM-derived materials, or cell-delivery approaches. Investigations into the functional restoration of the NP *in vivo* are performed in quadrupeds and therefore examination of the biomechanical performance of the material is only partly transferable to the conditions found in the human spine [62–65]. Similarly, applying a biomaterial intradiscally warrants an inspection of damage done to the AF, and maintenance of the intradiscal pressure. Other aspects such as biomaterial compatibility with GMP standard sterilization methods are often neglected.

From a mechanical point of view, mostly unconfined compression tests and rheology are performed to understand the biomaterial's behaviour under physiologically relevant stresses. While swelling behaviour is also often considered, the porosity is seldom investigated, although this parameter influences the swelling behaviour and interaction of cells with the material. Degradation of the biomaterial over time is only examined in some stud-

ies. Injectability for later application to the patient, particularly in biomaterials serving also as cell-carriers, is neglected, and rather approximated by rheological data.

Due to the lack of methodological consensus, much partial biomechanical data on various materials has been published, which still might profit from further experiments. In the following sections we discuss material evaluation parameters, resources for in-depth analyses of material choice and testing, as well as appropriate techniques to answer research questions.

3.3. Sterilization methods and their potential effect on biomaterials

Every biomaterial intended for use in a clinical setting must be sterilized according to GMP practices without rendering its mechanical properties and bioactivity unsuitable for its intended purpose. Assessing the success of sterilization usually is done by confirming a reduction in microbial load by a factor of 10^6 and details on organisms to be used have been published by the FDA [66,67]. Additionally, various testing methods for bacterial endotoxins have been established in the past years which is interesting especially for materials of animal origin due to potential septicaemia. [68] Current FDA-approved sterilization methods include dry heat, autoclaving, ethylene oxide (EO), and gamma-irradiation [67]. Several studies demonstrated that biomaterial properties change using these techniques: ethylene oxide was shown to influence mechanical properties as well as ultrastructure of decellularized porcine bladder matrix [69], while γ-irradiation of human dermis leads to protein denaturation and degradation [70]. Similarly, synthetic materials may be affected by γ-radiation (initiating additional crosslinking or unspecific scission) [71]. Dry heat/autoclaving often exceeds the polymers' melting point [71] in addition to denaturing proteins [72]. Residual levels of chemical sterilants like ethylene oxide on the biomaterial could also impair cell viability and/or phenotype. They therefore need to be quantified and completely removed prior to utilization in cell culture or patients [73,74]. Peracetic acid has been often discussed as a less harmful sterilant compared to the FDA-recognized ones, with a required immersion into liquid peracetic acid that allows for complete (exterior & interior) sterilization. As NP replacement strategies rely in part on the material's swelling, immersion-based approaches seem counterproductive prior to material application into the disc. Further, while peracetic acid is commonly referred to as less damaging, it seems to impair tissue remodelling post-implantation [75].

One novel approach for sterilization utilizes supercritical CO_2 to eliminate microbial contamination within the sample while retaining biomaterial properties [74,76–78] and thus could represent a gentle processing method for biomaterials which require it. Future research efforts thus need to identify suitable sterilization methods for NP replacement materials. Alternatively, aseptic production might provide a route to sterile biomaterial production for materials sensitive to the aforementioned sterilization techniques, albeit with significantly higher costs.

3.4. Characterization of mechanical and material properties

Ideally, biomaterials need to restore the disc's height and biomechanical properties mimicking the NP's material properties and withstanding physiological loading conditions. As such, measuring the swelling capacity is needed to judge the necessary volume of material for intradiscal application. This can be easily measured by immersing the biomaterial into PBS at 37°C and weighing it at predetermined timepoints covering at least a 30 day period or longer, depending on the material [79]. Important here is adjusting the buffer to an osmolarity mimicking that of the (degenerated) disc [80]. The porosity of the material not only influences the water and nutrient uptake, but also potential infiltration of NP cells or migration of co-administered cells [81]. Pore-sizes of 150–300 µm have been recommended for fibrocartilaginous tissues [82], and scaffolds with this pore size have been successfully employed *in vitro* for NP tissue engineering [83,84]. Importantly, SEM sample preparation should not affect the pore size, and snap-freezing samples in liquid nitrogen is an advised preparation method [85]. The degree of crystallinity of a polymeric hydrogel also determines many of its mechanical and biological properties like stiffness and biodegradability and should therefore be evaluated *via* e.g. differential scanning calorimetry [79,86–88].

Complementing this information with data from uniaxial confined compression testing (mimicking its placement in the IVD) will result in a detailed understanding of the biomaterial's response to mechanical deformations and stresses. The FDA guidance document 1637 and ASTM standard F2423–11 provide guidelines for conducting axial compression tests on total disc prosthesis which also pertain to NP replacement strategies (Table 5). Additionally, the sample should be tested in a bovine serum solution of 20 g/l protein content at 37°C. Experiments should be run for 10 million cycles on >6 samples to establish validity of the material. Strains and loading rates should reflect or exceed physiological values (see above). Dynamic and diurnal loading with loads, strains and frequencies that best mimic the *in vivo* situation are recommended (e.g. based on ASTM F2789 & F2423–11). Fatigue tests should be run in load and displacement control for 10 million cycles on >6 samples to establish validity and safety of the material. 10 million cycles reflect approx. 80 years of activities, and thus, a material withstanding such a number of cycles is currently considered to be safe for long-lasting use.

An *ex vivo* approach can be used to investigate the suitability of the material under study. In the context of mechanical testing, many parameters might influence the performance of the material apart from the material's properties themselves, including e.g. material storage and preconditioning [89–91]. Using cadaveric animal/human motion segments allows for cyclic compression as well as torsional testing. Ideally, a setup providing six degrees of freedom provides insights into the resulting range of motion and its restoration to the previously stated values [89]. Additionally, the Poisson's ratio informs about material's expansion and subsequent the load distribution from NP to the AF, and can be obtained from unconfined compression experiments or digital image correlation [92,93]. Further, rheological measurement inform about the material's response to shear, strain and temperature, which again should resemble or exceed physiological values (see Section 3.1). Temperature measurements should cover a range of at least 20°C - 37°C but may be extended in either direction in case of thermoresponsive materials.

A powerful mechanical testing method is unconfined and confined dynamic mechanical analysis, where many of the described measurements can be obtained [94].

Table 5
Test regimen for compression/shear testing recommended by the FDA and ASTM. ROM = Range of motion.

Test	Samples	Cycles	Frequency (Hz)	ROM
Static and dynamic compression testing	All ≥6	10,000,000	All 1 – 2	-/-
Compression shear testing				Max. allowed for by machine, > physiological ROM
Static and dynamic compression testing		Until failure		-/-

3.5. Assessing cytocompatibility and bioactivity in vitro

Preliminary assessment of the material *in vitro* can already provide insight into the applicability of the biomaterial *in vivo*. Effects of production/processing, subsequent sterilization and degradation onto cells can easily be observed employing a cell viability assay. Additionally, cell infiltration into the biomaterial, cell phenotype, and deposition of ECM by the cultured cells are of major interest.

Residual presence of antigens, pathogens and pyrogens for tissue-derived decellularized scaffolds must be assessed to prevent hyperacute rejection, long-term chronic inflammation after implantation and disease transmission. Most studies focus on remaining cellular debris [95] and DNA (due to transmittance of e.g. endogenous viruses [96,97]) using chemical or enzymatic solutions [74]. Currently, few studies examine the removal of the Galα1–3-galβ1-(3)4GlcNAc-R epitope (α-Gal) (Fig. 4) [98,99], although humans naturally express antibodies against α-Gal [100]. It plays a diverse role in host response to the biomaterial, ranging from hyperacute graft rejection in humans [98,100–102] to faster remodelling due to macrophage infiltration into the tissue [103,104]. Elucidating its effect in NP regeneration is therefore critical. The α-Gal specific M86 antibody has been successfully used in immunohistochemical detection and quantitative enzyme-linked immunosorbent assays (ELISAs) have been developed with this antibody [105–108]. Complement activation studies should also be considered, as not only the total amount of α-Gal, but also the density of the antigen within the tissue plays a role in eliciting an immune response [109].

Controlling biomaterial degradation rates particularly in a degenerate disc with elevated matrix degrading enzymes may be problematic [110]. Degradation products must not be harmful for the surrounding environment or compromise the healing process. Frequently used synthetic polymers for IVD regeneration are made from poly(lactic-co-glycolic acid) or poly(glycolic acid) [84,111]. Despite suitable mechanical properties, their degradation leads to the release of acidic monomers into the diseased IVD [112]. This potentially lowers the pH in the disc beyond its already low value, enhancing the degradation processes. Masking this decrease in pH requires addition of basic compounds into the biomaterial (e.g. sodium bicarbonate) [113], the effect of which on IVD tissue is not known. In contrast to this, hydrogels employing e.g. polyethylene glycol (PEG) compromise cellular remodelling within the NP after implantation as PEG degrades very slowly under the conditions found in the IVD [59] and contains no natural binding sites for cells. A similar situation is true for agarose and alginate, as both cannot be degraded enzymatically in mammals [114–116].

In vitro degradation studies are often carried out in PBS over varying time periods, often adding enzymes to the buffer to more accurately reflect *in vivo* conditions [117–135]. However, the amount of e.g. collagenase units used is arbitrary, since the number of active protease units within the healthy/degenerated IVD has only been reported for lysozyme to the best of our knowledge [136] and also depends on the degeneration state of the disc. quantification of ECM degrading enzymes within the NP of degenerated discs is necessary, not only to enable appropriate *in vitro* biomaterial degradation studies, but also for establishing more accurate IVD degeneration models for *in vivo* studies. Relating the levels of e.g. matrix metalloproteinases to grading scores for IVDs would enable more differentiated *in vitro*/*ex vivo* approaches targeting individual stages of DDD. For hydrolytically degradable gels, *in vitro* degradation timeframes don't necessarily reflect *in vivo* conditions [137]. Intradiscal implantation is necessary for accurate measurements of degradation properties for these materials.

One strategy to improve biomaterial stability and mechanical properties is by crosslinking of the polymer [138]. A comprehensive review on crosslinking methods for biomaterial applications has been published by Reddy *et al*., discussing the various agents employed in hydrogel crosslinking [139]. In some studies glutaraldehyde is used for crosslinking of materials [140–142] despite its known cytotoxic effects [143]. Ionic crosslinking of natural polymers, e.g. alginate scaffolds in $CaSO_4$/$CaCl_2$-solutions [117,118,144–147] might present a viable alternative, however the increased calcium-ion concentration in the disc might alter mechanotransduction of AF cells, cell survival of NP cells and lead to CEP degeneration [148–150]. Enzymatic crosslinking using microbial transglutaminase has yielded promising results for peptide-based hydrogels but demands enzyme-removal before *in vivo* application [151–153]. For all crosslinking methods cell attachment and matrix remodelling depend on the crosslinking-degree, and an optimum between biomaterial stiffness and cytocompatibility must be achieved [154,155].

Ultimately, screening for leachable monomers of the biomaterial, crosslinking agent, as well as other reagents used needs to be considered before *in vivo* assessment. Monitoring the degradation process can be done by assessing the average molecular weight (MW), and the distribution of MWs within the sample. Especially with slowly degrading materials, timeframes covering several months might be necessary to evaluate the biomaterial's degradation behaviour and the resulting biocompatibility in presence of degraded material. Leachable components from the material should be extracted in polar and non-polar solvents and subsequently identified *via* e.g. HPLC-MS [156]. Cell density in the NP has been reported at $\approx 4 \times 10^3$ cells/mm^3 ($\approx$200 cells/mm^2) and should be aimed for when trying to mimic conditions found in the NP in e.g. MTT/Alamar Blue-based viability assays to study the effect of the biomaterial on NP cells in a 2D/3D environment [157].

NP cells express a variety of markers including BCAM, CD24, integrin α6, α3, and β4, keratin 8 and 19, laminin α5, Forkhead Box F1, Paired Box 1, N-cadherin, and vimentin, which can be checked to get a detailed response profile to the biomaterial over time [158,159]. The timeframe for *in vitro* cell culture depends on the timeframe for biomaterial degradation obtained by previous immersion experiments in PBS (see above). Notably, only few studies currently perform their *in vitro* experiments under hypoxic conditions, although oxygen levels were measured at $\approx$2.6–13% *in vivo* and low availability of oxygen has been shown to increase ECM production by bovine NP cells in culture [48,160]. When using a biomaterial as a carrier for cells, growth factors, or NSAIDs further aspects such as type, concentration and release of bioactive molecules/survival of cells have to be considered and have been reviewed elsewhere [161–167].

Cell infiltration into the material can be obtained by observing a cross-section of the biomaterial after incubation with cells for 7–30 days *via* histology [168]. Several approaches for cell seeding and assessment of cell infiltration have been published so far, the easiest of which is to apply cells on top of the material in question. More complex approaches utilizing transwell setups could be employed to investigate e.g. cell immune recruitment. Evaluation is typically done *via* light microscopy, but can be complemented by electron microscopy [169,170]. Histological stains employing hematoxylin/eosin, Alcian Blue and/or Picrosirius Red/Masson's Trichrome can quickly convey information about the cell infiltration, GAGs and collagen, respectively, on synthetic scaffolds. More specific immunohistochemical methods detecting species-specific ECM molecules might be necessary to differentiate cell-derived matrix deposition in biomaterials obtained through tissue decellularization from the biomaterial itself for example by utilizing species-specific antibodies. Infiltration and response of macrophages can be investigated again *via* immunohistochemistry, or flow cytometry/ELISAs/quantitative polymerase chain reaction (qPCR) [171,172]. Macrophages of both type M1 and M2

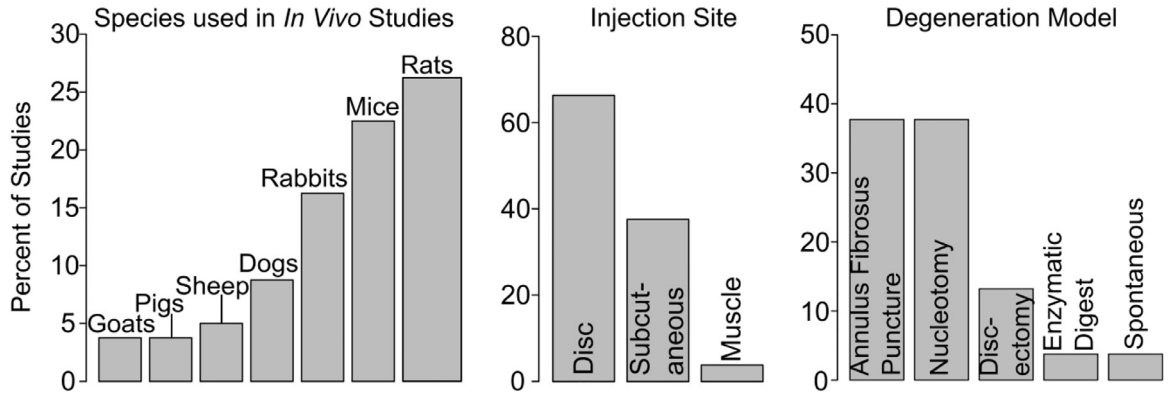

Fig. 6. Current methodologies for *in vivo* studies investigating potential biomaterials for nucleus pulposus restoration/regeneration.

are found in the IVD, and their incidence has been correlated to IVD degenerative state [173]. However while M1 macrophages are generally detrimental to tissues leading to increased cytokine levels, which in the disc would trigger further degeneration, M2 macrophages are considered reparative and could be beneficial following initial biomaterial implantations. Macrophage response to biomaterial application in the NP can therefore be investigated utilizing these subtypes and their relative amounts. Assuming later stages of IVD degeneration with AF and CEP tears and increased vascularization, potential macrophage recruitment and polarization can be investigated using M0 macrophages. Crosstalk between IVD resident cells and macrophages in degeneration presents a further avenue in IVD degeneration model research [174], and should also be considered in biomaterial-mediated cell-delivery approaches. This is particularly important to consider in relation to the catabolic phenotype of native degenerate IVD cells which could drive macrophages to the M1 phenotype. Analogue to the study of Silva *et al.*, (progenitor) cells can be cultured within the biomaterial with macrophages exerting indirect influence from a transwell insert or co-culture and should be further enhanced with co-cultures of IVD cells from degenerate human IVDs in the biomaterial [174]. ECM composition after cell-/macrophage-mediated regeneration should ideally resemble the healthy, pre-degenerate state, i.e. exhibiting >600 µg/mg dry weight GAGs, and ≈20 µg/mg dry weight collagen with >85% being collagen type II [43]. Cell morphology ideally should resemble the rounded NP cell [175].

3.6. *Biocompatibility assessment* in vivo

Biocompatibility and functional *in vivo* studies are paramount to assess the suitability of the biomaterial for IVD regeneration (Fig. 6). The persistence of notochordal cells (NCs) within the NP in certain animal species (e.g. rodents, porcine and some canines) should be taken into account when choosing an animal species for a study: NCs are thought to secrete a distinct set of soluble stimulating factors leading to increased ECM production by NPCs [176–179] and thereby are not representative of the human disease environment and confound any effect of the implanted biomaterial alone. Thorpe *et al.* (2018) recommend murine and leporine animals for initial safety studies, while larger mammals such as sheep, goats, alpacas and (non-)chondrodystrophic dogs provide information on the clinical efficacy of the material in question [38]. Subcutaneous implantation could provoke an immune response not occurring within the intended setting of the biomaterial in the IVD, and thus to premature exclusion of an otherwise suitable material. Implantation into the IVD mimics the intended use and allows for determination of several disc associated parameters (e.g. retention of disc height, fibrous encapsulation of implant or cell infiltration, ECM production, remodelling of implant etc.).

Complicating the comparison of *in vivo* study results are the various methods used to induce disc degeneration within the study animals, including AF puncture/incision [180–186], nucleotomy [187–192], discectomy [118,193,194] and enzymatic degradation [195], next to spontaneous degeneration models [196]. Comprehensive reviews of animal disc degeneration models and their limitations have been compiled by Lotz *et al.* (2004) and Alini *et al.* (2008) and offer great insight into this topic [62,64]. In annular puncture models and enzymatic digestion a gradual decrease in NP volume and pressure is observed, and the adaption of the disc cells to these processes mimics the stress- and fibrotic response observed in human degenerated discs. Especially in enzymatically induced disc degeneration, the effects of the enzyme(s) and avenues for their inactivation need to be studied *ex vivo* prior to usage *in vivo*. The differing specificity for ECM-component cleavage of e.g. trypsin compared to hyaluronidase and their natural occurrence in mammals further influence their applicability in degeneration models as discussed by Fusellier *et al.* [197]. As enzyme amount and degeneration grade have not been correlated yet in DDD, mimicking the conditions found in the NP accurately is rather difficult and may lead to an altered IVD degeneration progression compared to the naturally occurring pathophysiology. Especially for regenerative approaches application of too high enzyme concentrations may lead to a diminished observed ECM-production by resident/applied cells, as there's no way to inactivate enzymes once injected so far.

Nucleotomy with minimal trauma to the AF results in IVD degeneration and may be appropriate to use for the evaluation of NP augmentation strategies only after sufficient time of repair of the injured AF. In this regard, testing NP augmentation immediately after nucleotomy to induce disc degeneration would be not advisable due to the sudden alteration in disc physiology and biomechanics. Discectomy as a control *in vivo* is only needed when examining a whole-disc-replacement approach.

Another problem arises due to the application of biomaterials or cells into the NP *via* injection or insertion. This may damage the AF in the process, often inducing degenerative processes itself later on [198–200]. Various sealing methods for the AF have been explored with often unsatisfactory results, i.e. incomplete sealing of the NP and extrusion of the biomaterial into the AF [201]. Especially when applying progenitor cells into the NP, extrusion of the cells into surrounding tissues can lead to unwanted tissue-formation outside the NP/AF resulting in e.g. osteophyte formation [200,202]. Due to its central location within the IVD, biomaterial application into the NP always requires sealing and repair of the surrounding penetrated tissue. Consequently, minimizing the damage to these tissues is a prerequisite for clinical success, and testing the injectability of the material through a needle into the tissue and evaluating whether the material's properties are affected

is paramount and should assume a prominent role in coming research efforts. Elliott *et al.* found that a needle diameter <25% of the disc height reduces the damage occurring *via* annular puncture to a minimum and thus should be considered when testing the biomaterial *in vivo*/*ex vivo* [203]. Within this context, to our knowledge the effect of the needle tip design on the AF during injection has not been explored as a possible avenue of minimizing trauma. Whether an AF sealant after biomaterial injection is successful can be investigated by testing extrusion of the material into the injection canal under multiple degrees of freedom with physiological cyclic loading. Preformed materials for implantation into the NP may require a larger AF defect to be made than injectable ones. Recently, high-density collagen patches have been explored for annulus repair with promising results, potentially enabling use of preformed sponges/gels for NP augmentation [204–206]. Therefore, further research is necessary as especially moderate degeneration might profit from the combined approach. Incorporation of radiopaque substances into the biomaterial is important for controlled application and tracing in the patient, but the choice of substance (iodine-/metal-based [207,208]) and their interaction with NP tissue and cells has not been studied extensively so far, and needs to be elucidated in coming research efforts.

An important aspect for *in vivo* studies is the observed timeframe. Periods of two to sixteen weeks postoperatively have been considered in experiments thus far. Comparing the results of these studies consequently is very difficult. Acute inflammation shows symptoms rapidly within the first hours to days after implantation, with chronic inflammation potentially setting in afterwards [209]. To accurately assess the biocompatibility of an implanted material, considering a timeframe that covers the material's *in vivo* degradation is essential to observe potential immune reactions to degradation products. Similarly, implant retention within the IVD, stability of disc height, and biomaterial remodelling in combination with ECM deposition within the NP over these time periods contribute to a better understanding of the material efficacy. Determining the timeframe needed however is difficult and depends on the material in question and is further impeded by the lack of consensus for *in vitro* degradation methodology.

In conjunction with *in vivo* studies, several grading parameters have been established to facilitate comparison between degenerative states of IVDs, and their (dis)advantages have been discussed in detail by Kettler *et al.* [210]. They recommend the use of seven grading schemes related to the lumbar spine. Magnetic resonance imaging (MRI) presents a powerful non-invasive technique for evaluating several degeneration- and therapy-associated parameters. Not only is it possible to assess the disc height but additionally, the Pfirrmann scale allows for determination of the degeneration state taking into account T2 weighted images that convey information about the hydration level of the NP [211]. Often these two parameters are also combined into an "MRI-index" by multiplying the NP pixel area or volume with the signal intensity [183,212]. An increase in hydration initially however could simply be a result of injection of the material and thus demonstrating maintenance of increased signal intensity over time is essential.

Assessing the degeneration state of the tissue histologically using macroscopic Thompson's or microscopic Boos' scores provides information on the pre- and post-intervention state of the tissue. These are acquired with ≥2 trained investigators rating histological sections from the sagittal plane made from spinal motion segments [175,213]. Importantly, clinically relevant outcome parameters (e.g. pain) are rarely evaluated in the experimental studies conducted thus far [38,214], while guidelines within this context are largely lacking and would have the ability of linking the experimental work to the clinical situation.

4. Conclusion

Functional NP restoration and regeneration using biomaterials presents a promising approach towards IVD regeneration. Evaluating the biomaterials within the context of clinical applicability and efficacy however presents a challenge itself. Data on the molecular, cell and tissue level must be gathered and supplemented by experiments determining the material's mechanical properties, needed for restoration of the disc's or motion segment's biomechanical behaviour. Further regeneration to a healthier state of the NP is also possible with a material which supports matrix deposition and embedded bioactive components (Fig. 1). As more studies are published, working towards a more unified set of characterization experiments will allow for more streamlined identification of viable NP restoration and regeneration biomaterials as well as strategies for testing them in a clinical setting. Recommended assessment methods and further resources can be found in Table 6.

Table 6
Parameters to evaluate for potential NP restoration biomaterials, and corresponding assessment methods and further reading.

	Characterization parameter	Technique	Approach
Material properties	**Viscosity, stiffness**	Rheometry	Oscillation rate 0.1–100 rad/s, ≈10% strain, 37°C Strain sweep from 0.1–100% strain at ≈30 rad/s, 37°C Temperature sweep covering 20°C - 37°C at ≈10% strain, ≈30 rad/s (Sample drying prevented by e.g. PBS + FBS[1] application around sample)
	Stress/relaxation behaviour, aggregate modulus	Compression testing	Confined compression at 37°C in PBS+FBS, 10^7 cycles/until material failure in *ex vivo* setup
	Poisson ratio	Compression testing	Unconfined compression or digital image correlation (refer to [93]) at 37°C
	Pore size	SEM	Lyophilization as sample preparation technique after freezing in liquid N_2 to minimize physical influence on poresize measured. Alternatively: environmental SEM.
	Porosity	SEM	Sectioning of scaffold. ImageJ with jPOR macro for analysis. Refer to Loh *et al.* [81]
	Crosslinking degree	Crosslinker-dependant	E.g. commercial 2,4,6-Trinitrobenzenesulfonic acid (TNBS)/ninhydrin kit to measure amount of free amine residues
	Crystallinity	Differential scanning calorimetry	Performed on dried samples, temperature ramp of 10°C/min under N_2 flow
	Swelling degree	Gravimetric measurement of weight increase	Immersion in PBS + FBS over time, weight measurements at predetermined timepoints. *Swelling degree* $= \frac{m_{wet}-m_{dry}}{m_{dry}} * 100\%$

(*continued on next page*)

Table 6 (*continued*)

	Characterization parameter	Technique	Approach
Sterilization	**Efficacy**	Quantify microbial load	Refer to FDA guidelines [66,67]
	Effect on material properties	See above	See above
Biological response[2]	**(Bio)degradation**	No current accepted standard	Immerse in PBS ± enzymes/implant intradiscally for ≥30 days, monitor release of degradation products by e.g. HPLC-MS, NMR, BCA assay or similar, depending on biomaterial components
			Additional measurement of degradation in acidic milieu
	Cell compatibility[3]	Viability	3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) assay
		Function	Gene expression, matrix deposition (1,9-Dimethyl-Methylene Blue (DMMB), Hydroxyproline assay (HYP))
		Morphology	Microscopy
		Infiltration	Microscopy of haematoxylin & eosin staining
		Histological evaluation	Microscopy of alcian blue- and picrosirius red/Masson's trichrome-stained sections to visualize GAGs and collagen, respectively
	Tissue compatibility	Fibrous encapsulation	Microscopy
		Cell infiltration	(Fluorescence) Microscopy *via* haematoxylin&eosin/4′,6-diamidino-2-phenylindole (DAPI)
		Non-neurogenic effect	Culture material in presence of neural cells (refer to [215])
		Remodelling	Microscopy on immunohistochemistry, matrix deposition (DMMB, HYP assay)
	Blood compatibility	Complement activation assay[4]	Commercial ELISA
		Non-angiogenic effect	Culture material in presence of human umbilical vein endothelial cells (refer to [215])
	Immunological compatibility	α-Gal-epitope quantification (where applicable)	ELISA
		Macrophage polarization	FACS/qPCR/ELISA/immunostaining
		Immune cell infiltration	Microscopy using cell tracking dye
	***In Vivo* study design**	Intradiscal implantation of material	Rabbit or larger mammal
		Degeneration model Timeframe	Annular puncture/enzymatic digestion/spontaneous(/nucleotomy) Study timeframe > *in vitro* degradation timeframe where possible
		Morphological evaluation	Thompson score (macroscopy), Boos scores (histology), Pfirrmann score (MRI), $MRI\ index = NP\ area\ or\ volume * T2\ pixel\ signal\ intensity$
	***In Vivo* toxicity**	Full blood count analysis	Full blood count *via* FACS/hemocytometer
		Quantify endotoxin & pyrogen levels	Commercially available Limulus Amebocyte Lysate reagent test or alternatives
		Key organs' appearance	Organ morphology investigated by pathologist
		Liver and kidney function testing	Investigate Kidney function *via* serum creatinine [216] ELISA
			Investigate liver function *via* serum concentrations of alanine aminotransferase/glutamate dehydrogenase ELISA and/or mtDNA/nuclear DNA fragments [217] *via* DNA isolation & gel electrophoresis
Injectability	**Injection into cadaveric motion segment**	Cell viability within material during/after injection	MTT assay, Calcein-AM/Propidium iodide staining
		Injection pressure	Measure manual pressure applied to syringe
		Gauge size necessary for application	Must be as small as possible, <25% of disc height
		Incorporation of radiopaque substances [218]	µCT/MRI scan
Biomechanics[5]	**Range of motion**	Measure maximum angle	Measure maximum angle from photo/video/software in *ex vivo* spinal setup
		Stiffness	Determine neutral and elastic zone stiffness (refer to [219,220])
	Mechanical restoration	Disc height index [221]	Measure disc & vertebral body height from photo/video. $Disc\ Height\ Index = \frac{2(DH1+DH2+DH3)}{(LB1+LB2+LB3)+(UB1+UB2+UB3)}$ DH: disc height, LB: lower vertebral body height, UB: upper vertebral body height
		Stiffness	Determine functional spine unit (FSU) stiffness under uniaxial loading, and under dynamic and diurnal loading
	Biomaterial failure	Fatigue testing	Same conditions as confined compression testing, loading cycles until failure
		Material extrusion	Human FSU with implanted/injected stained material bent within the human ROM and checked visually for expulsion of material. Bending outside of human ROM can report on critical angle/load for expulsion to happen. Material can also be radiolabeled and then checked in CT during the above-mentioned tests

[1] In this context refers to PBS + FBS with a final protein concentration of 20 g/l in the combined solution with osmolarity matching that of the (diseased) NP.
[2] Refer to ISO10993, ASTM guideline F2150–13, FDA document 1637.
[3] Optimally in hypoxic conditions ($O_2 \leq 13\%$).
[4] See also Peakman *et al.* [222].
[5] Refer to Newell *et al.* [89].

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests

KI has a pending patent application WO 2017/121,736 A1, Notochordal Cell Matrix as a Stimulant for Intervertebral Disc Regeneration, and is independently contracted by and a non-voting shareholder in NC Biomatrix BV. CLM is an inventor on a patent for an injectable biomaterial. CLV is also an inventor on a patent for an injectable biomaterial.

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