

Tissue Engineering of the Intervertebral Disc's Annulus Fibrosus: A Scaffold-Based Review Study

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Abstract Tissue engineering as a high technology solution for treating disc's problem has been the focus of some researches recently; however, the upcoming successful results in this area depends on understanding the complexities of biology and engineering interface. Whereas the major responsibility of the nucleus pulposus is to provide a sustainable hydrated environment within the disc, the function of the annulus fibrosus (AF) is more mechanical, facilitating joint mobility and preventing radial bulging by confining of the central part, which makes the AF reconstruction important. Although the body of knowledge regarding the AF tissue engineering has grown rapidly, the opportunities to improve current understanding of how artificial scaffolds are able to mimic the AF concentric structure—including inter-lamellar matrix and cross-bridges—addressed unresolved research questions. The aim of this literature review was to collect and discuss, from the international scientific literature, information about tissue engineering of the AF based on scaffold fabrication and material properties, useful for developing new strategies in disc tissue engineering. The key parameter of this research was understanding if role of cross-bridges and inter-lamellar matrix has been considered on tissue engineering of the AF.

Keywords Annulus fibrosus · Tissue engineering · Biomechanical properties · Biochemical properties · Intervertebral disc

1 Introduction

Low back pain (LBP) is one of the major health problems in western countries that greatly affects the quality of patients' life and has been the subject of several clinical research studies. The relationship between LBP and disc degeneration [1–6], herniation [7, 8], nutrition [9–12] and other external factors, such as mechanical loading larger than physiological limits were well documented [13–21]. Recently, tissue engineering as a high technology solution for treating disc's problem has been the focus of research

for several studies; in fact, it has been suggested as the next step for finding a solution [18, 22].

To achieve a better insight of the tissue engineering applications for the treatment of the annulus degeneration, an accurate understanding of the local chemical and mechanical environment (sometimes called mechano-chemical mutual impacts) around the disc cells in the annulus as well as its anatomy and physiology is required.

As it was presented in Fig. 1—including the AF and NP structure in different magnifications, a herniated disc, and two different tissue engineered scaffolds—at the macro scale (mm or tissue range), the AF forms the outer boundary of the disc, consisting of almost concentric collagen-matrix composite rings [1].

The cartilaginous extracellular matrix of the annulus contains collagen fibers whose orientation imparts material anisotropy [23]. At the micro scale, the annulus has a

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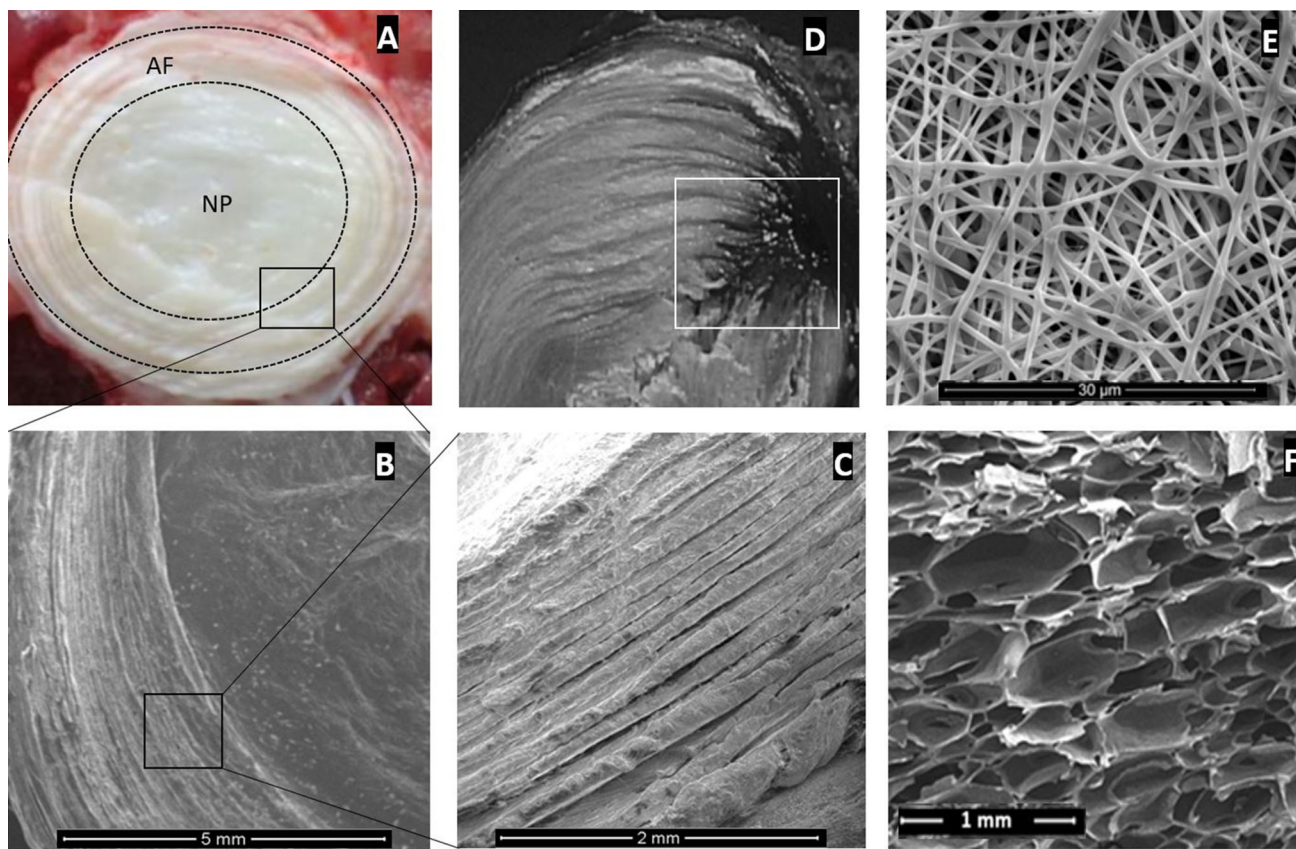


Fig. 1 The AF and NP structure of an intervertebral disc in different magnifications (A, B, C), *black squares* show the magnified region. *White square* shows the NP extrusion through the AF (D), the place

complex structure, with few mesenchyme cells embedded in an extracellular matrix [24] that synthesis collagen and aggregating proteoglycans [25]. The matrix composition and organization alter over time, with the cellular repair response being inadequate; as a result, the degraded matrix would no longer be able to carry the external loads effectively, leading to onset of disc degeneration [3]. Disc degeneration consists of a complex interaction of mechanical [17, 26], biological [27] and chemical [28] changes within the IVD, where the AF and NP boundary becomes less distinct as the NP losses its gel-like property and develops fibrotic changes. Disorganization of the AF and inter-lamellar matrix (ILM) including collagen and elastin disorders and irregularities of annular lamellae are other symptoms of degeneration [29].

Herniation occurs subsequent to ILM failure and affected the integrity and adhesion between annular layers [30]. The pattern of herniation clearly demonstrates that the posterior part of the AF is more vulnerable to structural failure due to weak inter-lamellar cohesion [31], low annular wall thickness [32] and a relatively large number of incomplete lamellae in adjacent layers. Based on abrupt changes in the disruption pattern from intra- to inter-

needs to be carefully focused for the AF treatment. Images “E” and “F” show two different scaffolds (E: nano-fibres and F: silk-base porous structure) that may be used for the torn AF re-construction

lamellar, the distinct lack of inter-lamellar connectivity caused concentric tears by mechanical factors rather than degeneration [33].

A fundamental understanding of ILM structure-function behavior is important for determining the complex loading conditions under which the AF is at risk for delamination and subsequent tissue disruption that may lead to degeneration. Therefore after herniation, clinical treatments should aim to strengthen and repair the AF to confine the NP, as well as target its rehydration and regrowth [34, 35]. Current treatments (invasive or conservative) for degenerated disc are not able to restore its original function and structure [36]. Tissue engineering techniques have emerged as a promising therapeutic approach, by totally or partially replacing the degenerated disc with scaffold-cells implants [36–40].

Based on the literature review it was found that scaffold surface modification [41–43], cell and cellular activities [24, 40, 44–59], cell bioengineering tips including cellular migration, cell’s biology and phenotype, cell and matrix metabolism and scaffold free constructions as a common areas between engineers and biologists [24, 44, 48, 51, 52, 59], cellular and intercellular mechanics [24, 45, 49, 54–56],

preparation methods for cell culture [46, 53, 56–58] and cell–extracellular matrix interaction [40, 47, 50, 53, 56] has been focused by researches in disc’s tissue engineer activities. In some other studies scaffold structure [60–71], material [72–86], design [87–97] and its biomechanical properties [98–105] mainly considered as an important factor toward the AF tissue engineering. Some researchers presented hybrid systems whose function was not only tissue engineering but also delivery of bioactive agents for degeneration or its consequent (i.e. pain) treatment [106, 107]. Clinical application of tissue engineering in laboratory scale and their challenges was another point of view [22, 23, 27, 39, 108–115].

The requirements, achievements and challenges in the AF tissue engineering had indicated before; however, some gaps were found in this rapidly emerging field of research. The main important question that previous reviews have not answered yet is how fabrication, biomaterial selection and mechano-chemical testing of the artificial AF scaffold mimic the integrity of adjacent lamellae of the AF. Therefore the main reason of this review study was to find out whether the role of inter-lamellar matrix has had noticed on selecting biomaterials and fabrication processes or had influenced the mechano-chemical evaluation methods. The aim of this study was firstly review the most relevant journal articles published in the international scientific literature about tissue engineering of the AF based on scaffold fabrication and material properties and the second to find out what sort of activities performed to help artificial scaffold mimicking lamellae’s integrity. This review could become a comprehensive resource for researchers working in the field of the AF scaffold fabrication and evaluation.

The literature review was undertaken through an online search using the databases of Web of Science Core, PubMed (NLM) and Science Direct; articles had to be written in English language, published in peer-reviewed journals. The search terms used were as follows: “annulus fibrosus” AND/OR “intervertebral disc” AND “tissue engineering”, covering the papers published in the period from the early 1980s to 2015.

2 Scaffold fabrication methods

Tissue engineering scaffold fabrication of the AF has been challenging due to its complex structure. Electrospinning, bio-printing, stereo-lithography and producing porous scaffolds based on chemical processing has been introduced as some of the methods for the AF tissue engineered scaffold preparation. Nano-fibrous scaffolds usually were fabricated by electrospinning and wet spinning [36, 82] in many studies, where mandrel rotational speeds and applied

voltages varied related to the materials’ specifications (see Table 1).

Some presented scaffold fabrication methodologies like 3-dimensional bio-printing and micro-sterolithography (μ SL) allowed prefabrication of anatomically relevant scaffolds in a layer-by-layer process [42, 80]. These methods were patient-based CAD modelling process and obtained dimensions from MRI or CT scans [116]. In order to successfully apply these methods, light penetration depth control and residual strains in different layers [117–119]. Using a winding machine with custom-made modifications in rotation and sliding assemblies, some researchers fabricated silk based scaffolds. In this method the angle between slide and rotation directions which manipulate the direction and maintain the angle of winding fibers, respectively, had been mentioned as two important parameters that formed the scaffold structure [60].

Contracted AF(collagen)-NP(alginate) method was used to prepare a scaffold for the whole disc replacement. This method conducted by the NP and AF dimension measurement and simple molding method. Accurate measurement and preparing too many solutions with different concentrations had to be considered [25, 87]. This method is based on generating collagen fibril structures by contracting collagen gels with different boundary conditions [87].

It was reported that porous silk scaffold that prepared by chemical processing of cocoons could be used as the AF tissue engineering. Protein extraction (Sercin), Lyophilization and special structure induction (mostly β -sheet) were the main steps in the method [62, 73, 78, 79, 107, 120–124]. The ability of silk fibers to be used as a substitution for collagen is almost new subject in tissue engineering researches [125–127]. The silk-based nano-fibrous scaffolds prepared under an all-aqueous process in ambient conditions introduced simple but highly appreciated method to the future use of these material systems in tissue studies [128–130].

Demineralized bone particle (DBP) gels had been used as an injectable scaffold to substitute intervertebral discs. DBP gel-shaped scaffold were fabricated with different percentages of DBP powder and acetic acid, including pepsin [83]. Photochemical crosslinking collagen scaffold, which cured by laser irradiation [88], riboflavin induced collagen, which was known as high density collagen gels [89], Genipin-crosslinked fibrin as an injectable adhesive [95] were introduced as intra injectable scaffold, as well.

Alginate and poly lactic acid base scaffolds (sometimes called shape-memory scaffolds), fabricated by following the sequences of crosslinking process (carbodiimide chemistry), unreacted chemical reagents removal, freeze-drying process and cell seeding [19, 74, 82, 90, 96].

Table 1 Electrospinning conditions in different studies

Material	Mandrel speed	Applied voltage (KV)	References
Polyurethane	1200 rpm	20	[51]
	1000 rpm	5–10	[77]
	1250 rpm	18	[104]
	–	15	[125]
	–	12	[126]
Polyurethane/collagen/chitosan	4000 rpm	18	[127]
Poly(ϵ -caprolactone)	10 m/s	13	[106]
	500–2000 rpm	12	[83]
	300 rpm	8	[98]
	30,000 rpm, 11 m/s	23	[50]
	10 m/s	13	[73]
	10 m/s	13	[99, 100]
	7500 rpm	12	[47, 111]
Poly(L-lactic acid)	–	16	[84, 123]
	–	15	[74]
Collagen/chitosan/poly(ethylene oxide)	10 m/s	5–30	[124]

The fabrication of poly lactic acid foams containing bio-glass scaffolds were made using the thermal induced phase separation (TIPS) process [75]. It was claimed that TIPS process supports fabrication of foam like scaffold with tailored porosity appropriate to the tissue concerned [131]. TIPS consisted of a sequences of polymer and bioglass-solvent mixture preparation, Lyophilization process, freezing and vacuum drying processes [132].

Freeze drying was a simple method used by some researches to convert the collagen-GAG slurries into porous scaffold [66] or at the end of the solvent casting/salt leaching technique [68, 81] or film casting [85]. Usage of this method of fabrication was limited to lab activities (i.e. proliferative and biosynthesis activity of the AF cells) due to poor mechanical properties.

3 Biomaterials selection

Selecting materials for fabricating scaffold is still a big concern due to the AF complex structure and physico-chemical properties. Efforts to produce AF tissue *in vitro* have involved various materials.

Silks which were introduced as protein polymers differ widely in composition (amino acid sequence and type), properties and structure depending on source and the most bio-oriented ones forms from the silkworm and spiders [120, 125]. Silkworm (*B.mori* silk) fibers are composed of core filament protein (fibroin) consist of highly organized crystalline region and the gum-like protein (sericin) that surrounded the fibers. New approaches for utilizing silk fibers in the AF scaffold led to recombinant silk-collagen fibers fabrication. Linking silk's repeating sequences to a

collagen domain preserved silk's useful properties, while adding selective bioactive functions [125]. Silk fibroin-chitosan hybrid had been evaluated as a tissue engineered scaffold, where biomechanical and biochemical correlation showed some mechanical properties enhancement [126]. Based on silk biocompatibility and mechanical properties most of researches had focused on its application on scaffold construction and impact of scaffold characteristics on cell response rather than presenting new generation of silk base materials [62, 63, 73, 122, 124, 128, 129, 133].

Polyurethanes (PU) with bioactive, biocompatible and biodegradable characteristics have been considered as a substitution for the degenerated or damaged AF [97]. The possibility of managing biodegradation process has made this material a good candidate for the AF scaffold [134]. Aliphatic ester linkages [135, 136], environmental stress cracking [137], temperature and humidity [134], enzyme presence in biological environment [138] and calcification [139] were the sources of the degradation that altering PU structure. Surface modification of PU nanofibers with anionic oligomers [97, 140], coating nanofibers of polyurethane with fibronectin [141] and addition of collagen to PU nanofibers during electrospinning process [142] were valuable efforts on presenting new biomaterials for the AF tissue engineering purposes.

Poly(D, L-lactic acid), its copolymer with poly(glycolic acid) [81] and its combination with bioglass (usually in filled-composite foam form) being considered as tissue engineered scaffold [131]. Collagenous scaffold that crosslinked *in situ* with riboflavin at 468 nm (1400 KWcm⁻²) for 40 seconds were injectable and have been used to repair defects made to the AF [25, 101]. In the presence of ammonia as a crosslinking agent, argon laser at

514 nm and 0.2 W for 100 seconds was used for collagen gelation [88]. Collagen fibers usually harvested from animal tail tendon and got ready to used subsequent to some chemical and physical procedures [87, 143]. Collagen-chitosan composite blended with polyethylene oxide (PEO) was used in some researches [144]. Chitosan compatibility with glycosaminoglycan structure held superior biological properties at relatively low cost, where PEO affected fabricating process as a plasticizer [144, 145]. Collagen coated silicon membrane was used as a temporary scaffold enable researchers to develop tensile pre-strained AF cells for tissue regeneration study *in vitro* [103]. Poly caprolactone, was widely used as tissue engineered scaffold, alone [100] or as a blend [70]. In one research Poly (polycaprolactone triol malate) introduced as the AF's biocompatible scaffold whose degradation capability and mechanical properties were tailor-made in accordance with pre-polymerization process [69].

4 Scaffold biomechanical properties

Silk is environmentally stable due to the structural crystallinity, protein hydrophobicity and the extensive hydrogen bonding; however its unique biomechanical properties have arose from the nanoscale features and conformational polymorphism originated from oriented β -sheet crystals [62, 146], stress alignment of the chains and properties of crystalline-amorphous interface [120]. With regards to two weeks cell culture on Silk fibroin-chitosan hybrid's scaffold it was shown that scaffold's compressive modulus was positively correlated with collagen and Glycosaminoglycan (GAG) content [126]. Mechanical study of cross linked silk fibroin fibers with chondroitin sulphate (CS) which assessed in compression mode implied on about 9-fold increase in modulus compared with non-cross-linked construct and 1.4-fold higher by the week four after cell culture for cross-linked structure [60]. Not surprisingly, crosslinking with CS increased CS-silk construct stiffness approximately twice higher than silk construct [60].

It was shown that biodegradation affected mechanical properties of polyurethane scaffold, initial modulus followed by initial wetting decreased by about 5 times, ultimate stress (MPa) decreased from 6 to 2 during four weeks wetting [97]. Surface modification of PU nanofibers with anionic oligomers (AO) affected mechanical properties of scaffold [97] as well as its physic-chemical properties [140]. Also, relation between surface modifications with matrix proteins, provided molecular and topographical cues that permit the AF cells to orient parallel to scaffold fibers [41]. Addition of AO increased scaffold's surface polar characteristic, while reducing contact angle ($\approx 40\%$) affected cell attachment, positively [97, 140, 147]. On the

other hand coating PU nanofibers with fibronectin (a matrix protein) resulted in greater collagen synthesis and accumulation indicated that cell shape and adhesion to scaffold appeared to be correlated to matrix production [141].

Study the effect of collagen addition on PU nanofibers during electrospinning process showed that scaffold mechanical properties depended on the collagen percentages, whose increment resulted in modulus, tensile strength and breaking strain decrease [142]. Tensile strengths ranged from 2 MPa to 13 MPa and breaking strains from 160 to 280%. Electrospun PU had a tensile strength of 13 ± 4 MPa and a breaking strain of $220\pm 80\%$. Incorporation of collagen led to significant decrease in tensile strength as well as reduction in modulus; however, no meaningful correlation between collagen content and breaking strain were observed [148].

Some studies focused on PU nanofibers alignment's effects on scaffold properties [41, 84, 97, 140, 148] and cell migration [149]. It was observed that most cells migrated along the fiber orientation direction on the uni-directionally aligned fibers and could travel among different fibers and change movement directions back and forth [133, 149]. Also it was proved that applying tension to PU scaffold during cell culture affect cell proliferation, alignment and morphology that suggested tensile strain was required to generate properly formed AF tissue [84]. Electrospun PU scaffolds with random and aligned nanofibers presented different mechanical properties, where aligned structures were found to have higher tensile strength and modulus ($\sigma=14\pm 1$ compare to 1.9 ± 0.4 (MPa) and $E=46\pm 3$ compare to 2.1 ± 0.2 (MPa)) prior to degradation [97]. Also the tensile strength of the aligned nano-fibrous scaffold showed significant differences between parallel (14 ± 0.6 MPa) and perpendicular (5.1 ± 1 MPa) directions; moreover comparison of strain at break between scaffold of parallel and perpendicular alignment showed an even sharper difference (60 ± 15.5 and 8 ± 1) [142].

Comparing PCL and PU mechanical and biochemical properties demonstrated that structural orientation had significant impact on properties improvement. It was shown that oriented PU scaffolds had higher yield strength and in contrast PCL oriented ones were stiffer [70]. Mechanical properties of the AF scaffold's materials listed in Table 2.

5 Biochemical analysis

Investigating structural and mechanical properties' effects on deposition and orientation of matrix [60], cell culture, proliferation and interaction with the structural elements and gene related evaluation [48] are the most major compartments of the AF's scaffold biochemical studies. Major

Table 2 Selected mechanical properties of the AF scaffolds' candidate

Material	UTS (MPa)	% Strain at break	E (GPa)	References
Silk ^a	610–690	4–16	15–17	[132, 62]
Collagen	0.9–7.4	24–68	0.0018–0.046	[132, 145]
PLA ^b	28–50	2–6	1.2–3	[132]
PCL ^c	–	7–10	0.055–0.06	[77, 98]
PU ^c	–	26–31	0.020–0.030	[77]

^a From silkworm, individual filament following sericin extraction

^b 50,000 < Mw < 300,000

^c nanofiber

factors governing biocompatibility and immunogenicity of the AF scaffolds' biomaterials including molecular aspect (Size and shape), architectural properties (morphology, orientation, surface topography, porosity), surface chemistry and implantation site have been extensively overlooked through many studies as they are in common with other tissue engineering applications [127]. Therefore adaptive and innate immune response of silk biomaterials in different conditions and environment were explored [120, 121, 127].

In-vitro culture of human nasal chondrocytes on engineered fibers with alternating angled orientation demonstrated cell alignment with fibers that showed deposition of an orientation collagenous matrix, mimicking the organization and arrangement of native AF [60]. It was discussed that a scaffold with proper action have to able to produced

extracellular matrix rich in GAG and collagen after implantation [25]. The influence of boundary geometry and composition of scaffold containing collagen gel on cell alignment was studied and shown that in the regions where cells were in tight proximity, collagen fibers were rearranged to form larger bundles on lines between cells. The more concentration of collagen with in the injectable scaffold, the more development of circumferential collagen fibril and cellular alignment [87].

Biochemical and histological analysis shown that coupling the silk scaffold with peptides affected cell morphology, however; no contribution was seen in cell formation and attachment as the surface treatment seemed to reduce scaffold porosity's size and amount where porous silk is an appropriate scaffold on which to grow the AF cells [62].

Table 3 Protocols, assays and equipment in the annulus biochemical evaluation activities based on scaffold engineering

Equipment	Studied item and references
Scanning electron microscopy (SEM)	Scaffold morphology [20, 51, 53, 68, 82, 127, 134, 136, 139, 140], pore size measurement [81, 136], cell morphology [82, 97, 127]
Atomic force microscopy (AFM)	
Transmission electron microscopy (TEM)	
Contact angle measurement	Hydrophobicity [139]
β-Scintillation counter (β-liquid scintillation counter)	Collagen synthesis quantification [51, 70, 106], proteoglycan quantification [70, 106], GAG content analysis [49]
Confocal microscopy imaging follow by staining	Collagen and fibronectin synthesis and organization by AF cells [51], histology [27, 60], cell appearance and viability [86, 140]
Second harmonic generation (SHG) and two-photon excited fluorescence (TPEF) microscopy	Cell—scaffold interaction imaging [94]
Spinner flask (bioreactor)	Dynamic AF cell culture [80, 100]
ATR-FTIR analysis	Scaffold bonding and content [35, 86, 124, 127, 140]
Assays, protocols and standards	Studied item and references
Analysis of pro-inflammatory signal transduction pathways	Inflammation [139]
Quantitative ELISA assays	Cytokine release analysis [139], Silk binding assays [137]
Quantitative real time PCR	Gene expression [139]
Dye binding (Hoechst 33,258) assay followed by fluorometric analysis	Annulus fibrosis cell attachment [51], DNA content of the tissues determination [53, 80, 81, 83]
Live-dead assay	AF cell viability [81, 86, 127, 138, 140]
Dimethylmethylene blue assay	Glycosaminoglycan content [17, 53, 70, 83], proteoglycan content [53, 70]

Improvement of tissue formation probability within the silk scaffold in different cell culture methods (dynamic and static) along with their effect on cell diffusion into larger pore size of scaffold had been studied. Significantly more matrix generation in dynamic cell culture with specific scaffold's pore size (600 μm pore diameter demonstrated more cell uniformity distribution and greatest amount of collagen I) result in better AF tissue formation and uniform spatial cell distribution [73, 93].

Insoluble cross-linked membranes of chitosan nanofibers diameter with the range of 150 – 650 nm were investigated to be very similar to the AF's natural ECM both in composition and structure and exhibited excellent biocompatibility property and proper degradation rate, *in vivo* [144]. It was shown that *in situ* photo chemically crosslinking of collagen within the injectable scaffold survived physiologically relevant compression and torsion loading. Also it was proved that effectively reduced leakage and osteophyte formation in an animal study [88].

In some studies relation between development of the AF cells with simultaneous mechanical loading during cell culture process studied and it was shown that various bilateral tensile strain duration need to be optimized in order to lead cell-matrix interaction enhancement for IVD tissue engineering [13, 48, 103].

Table 3 indicates biochemical analysis related equipment or different assays usually had been used in different research activities.

6 Conclusion

Introducing advanced technologies that developed methods to characterize and measure different properties of the AF in multi-scale levels, provided the opportunities to improve current understanding and addressed unresolved research questions. Research in tissue engineering of the AF has grown rapidly during the past decade. Nonetheless, one main unsolved point is about the role of the inter-lamellar space between adjacent lamellae, for which the question “how should the reconstructed lamellae be interconnected?” remains still unanswered. Unfortunately, the researcher in the area have not considered the ILM's impact on the integrity of the AF structure. It was shown that this potentially important subject hadn't put into consideration neither in selecting biomaterials and fabrication method nor in scaffold evaluation process.

On the other hand using hydrogels confined to the NP reconstruction or substitution rather than the AF tissue engineering. However selecting hydrogels with proper mechanical properties as the AF scaffold may result in preventing tissue dehydration that postpone degeneration process.

It seems that in spite of successful development in fabrication methods, more progress have to be achieved to help researchers make integrated scaffold with the properties that mimic the AF inter-connectivity. Further research is needed to introduce scaffolds that mimic the compositional structure and viscoelastic properties of the AF.

Compliance with ethical standards

Conflict of interest The author has no commercial relationship that may lead to a conflict of interest.

Ethical statement There are no animal experiments carried out for this article.

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