Lucas Dall Agnol

ADESIVO À BASE DE POLIURETANO PARA REPARAÇÃO PÓS-CIRÚRGICA DO DISCO INTERVERTEBRAL

Dissertação apresentada à Universidade de Caxias do Sul, para obtenção do Título de Mestre em Ciências da Saúde.

Caxias do Sul
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Co-Orientador: Prof. Dr. Sidnei Moura e Silva

Caxias do Sul

2018

Dados Internacionais de Catalogação na Publicação (CIP)
Universidade de Caxias do Sul
Sistema de Bibliotecas UCS - Processamento Técnico

D144a Dall Agnol, Lucas
Adesivo à base de poliuretano para reparação pós-cirúrgica do disco intervertebral / Lucas Dall Agnol. – 2017.
76 f. : il. ; 30 cm

Dissertação (Mestrado) - Universidade de Caxias do Sul, Programa de Pós-Graduação em Ciências da Saúde, 2017.
Orientação: Otávio Bianchi.
Coorientação: Sidnei Moura e Silva.

1. Materiais biomédicos. 2. Poliuretano. 4. Deslocamento do disco intervertebral - Cirurgia. I. Bianchi, Otávio, orient. II. Silva, Sidnei Moura e, coorient. III. Título.

CDU 2. ed.: 616-7

Catalogação na fonte elaborada pela(o) bibliotecária(o)
Paula Fernanda Fedatto Leal - CRB 10/2291

UNIVERSIDADE DE CAXIAS DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

COORDENADOR DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

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ADESIVO À BASE DE POLIURETANO PARA REPARAÇÃO PÓS-CIRÚRGICA DO DISCO INTERVERTEBRAL

Lucas Dall Agnol

Dissertação de Mestrado submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Ciências da Saúde da Universidade de Caxias do Sul, como parte dos requisitos necessários para a obtenção do título de Mestre em Ciências da Saúde, Linha de Pesquisa: Engenharia e Terapia Celular.

Aprovado em 05 de julho de 2018.

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Dedicatória

Dedico esta obra a minha família e aos meus amigos, que de muitas formas me incentivaram e ajudaram para que fosse possível a concretização deste trabalho.

Agradecimentos

Ao meu orientador Dr. Otavio Bianchi pelos ensinamentos, paciência e confiança, mas sobre tudo pela amizade ao longo desses anos, muito obrigado.

A minha grande amiga Dr. Fernanda Trindade Dias pela orientação, pelos conselhos pessoais e profissionais e acima de tudo pela nossa parceria e amizade.

Aos queridos amigos do Grupo de Compósitos e Polímeros Avançados, agradeço pela amizade, carinho e pelos bons momentos com café e troca de ideias.

Ao Dr. Asdrubal Falavigna, Dr. Sidnei Moura e Silva e à Dr. Natália Fontana Nicoletti pelas valorosas orientações, pelo incentivo e pela amizade.

A todos os professores das disciplinas que cursei durante o mestrado e à todas as pessoas do meu convívio que acreditaram e contribuíram, mesmo que indiretamente, para a conclusão deste curso.

Ao Laboratório de Polímeros (LPOL) da UCS e seus técnicos pela forma competente como conduziram as análises térmicas.

Por fim, agradeço a CAPES pela bolsa de mestrado.

Sumário

1 INTRODUÇÃO

Durante as últimas décadas, a dor lombar se tornou uma patologia endêmica, gerando elevados custos, afastando as pessoas do mercado de trabalho e de suas atividades diárias. Cerca de 90% das doenças da coluna estão localizadas no disco intervertebral (DIV) e necessitam de investimentos em tratamentos que proporcionem uma melhora significativa do paciente. A degeneração do DIV é uma consequência do envelhecimento devido a fatores genéticos, nutricionais e biomecânicos, requerendo intervenção cirúrgica na maioria dos casos clínicos (1, 2). O DIV é uma estrutura com baixa vascularização composta por três regiões morfologicamente distintas: núcleo pulposo central (NP), rico em colágeno tipo II e proteoglicanos; anel fibroso (AF), rico em colágeno altamente organizado e elastina; placas endoteliais cartilaginosas, constituídas por finas camadas de cartilagem hialina que separam o disco das vértebras adjacentes (3, 4). O confinamento do NP pelo AF permite que o disco intervertebral forneça flexibilidade e suporte de cargas de compressão, além de auxiliar na dissipação de cargas mecânicas e choques prejudiciais à coluna vertebral (5). O colapso estrutural destes tecidos faz com que partes do NP possam migrar para a região externa defeituosa do AF, comprimindo os nervos espinhais adjacentes. A hérnia e a inflamação resultantes deste evento são a principal causa de dor lombar crônica (6).

Os tratamentos para a hérnia de disco variam de fisioterapia e uso de medicamentos (analgésicos e anti-inflamatórios) a procedimentos cirúrgicos altamente invasivos, cuja técnica mais utilizada é a discectomia. Este procedimento remove a porção degenerada do disco por meio de uma pequena incisão no AF, diminuindo a dor do paciente (4). Entretanto, a incisão feita no AF permanece danificada, o que pode provocar alterações na biomecânica e no ambiente biológico do DIV, além de aumentar os riscos de re-herniação com degenerações subsequentes em todo o disco (7). Isto pode ser observado em estudos que demonstraram que entre os pacientes submetidos à discectomia, há um índice de 14% de reoperação e 2,3% em duas ou mais reoperações (8). Portanto, o desenvolvimento de técnicas que restaurem a integridade do AF é urgente e indispensável.

Atualmente, o uso de bioimplantes na correção cirúrgica de hérnias é previsto e recomendado. Das próteses reportadas na literatura, aquelas que prometem selar mecanicamente a região comprometida do AF e restaurar a fisiologia do DIV merecem ser profundamente estudadas. Os biomateriais selantes, que compreendem desde hidrogéis a dispositivos mecânicos, podem ajudar na restauração da pressurização e na prevenção da infiltração de citocinas pró-inflamatórias no interior do DIV (9). Ainda não existem opções de selante clinicamente disponíveis para o reparo do AF (10). Todas as soluções propostas até o momento apresentam alguma limitação associada à incompatibilidade biológica ou mecânica (11, 12). Em comparação aos implantes biológicos rígidos, o uso de um selante injetável para restaurar o AF parece ser uma opção bastante promissora. Um selante injetável não requer ancoragem no AF ou no corpo vertebral, além de poder ser aplicado percutaneamente (13). Quando combinados com biomateriais fibrosos ou outros materiais compostos, estes adesivos selantes também serão capazes de recuperar defeitos maiores no AF (9).

Alguns aspectos físico-químicos, biológicos e mecânicos devem ser considerados para que estes selantes apresentem viabilidade clínica. Em relação às características físico-químicas, exige-se a compatibilidade fisiológica e uma boa integração adesivo-tecido. O implante injetável deve solidificar rapidamente *in situ* quando em contato com os tecidos circundantes. Quanto aos aspectos biológicos, estes adesivos devem ser esterilizáveis e biocompatíveis, não provocando qualquer resposta citotóxica para seus componentes ou produtos de degradação a longo prazo. Mecanicamente, os adesivos necessitam resistir aos estímulos naturalmente experimentados pelo DIV. Para a restauração biomecânica do disco, são recomendados implantes com valores de 0,5−5 MPa, 0,3 MPa e 30 MPa para os módulos de compressão, cisalhamento e tração, respectivamente (14). Além disso, estes materiais devem resistir a forças adesivas de 0,2 MPa e a pressões intradiscais de ~2,3 MPa (15, 16).

Adesivos a base de cianoacrilatos (Dermabond, SurgiSeal, LiquiBand, Histoacryl), de glutaraldeído-albumina (Bioglue) e de cola de fibrina (BIOSTAT BIOLOGX®, Tisseel®, Evicel™ e Crosseal™) estão comercialmente disponíveis, mas não são adequados para o reparo do AF (9). Biomateriais a base de uretano têm sido considerados uma alternativa promissora devido à biocompatibilidade e

taxa de degradação controlada deste polímero, o que permite o desenvolvimento de produtos com propriedades mecânicas sintonizáveis (17-20). Embora a literatura reporte as vantagens de um polímero biodegradável na reparação pós-cirúrgica do AF, sendo esta uma região avascularizada e sem potencial regenerativo, o correto seria a implantação de um material que se tornasse parte integrante do organismo, sem a necessidade de ser removido ou degradado. A maior parte dos adesivos teciduais desenvolvidos até o momento sofrem degradação em aproximadamente duas semanas. Adesivos a base de poliuretano aderem rapidamente ao tecido biológico, não liberam calor em excesso durante a síntese, são estáveis à temperatura corporal e resistentes à umidade, não apresentam potencial carcinogênico e podem ser esterilizados (21). Além de apresentarem excelente processabilidade e características físico-químicas, estes materiais são capazes de favorecer a proliferação celular (17-20). Quando este polímero possui terminações isocianato, reage com compostos com hidrogênio ativo (como álcoois, amino, água, ureia e grupos ácidos) presentes nas moléculas biológicas, formando ligações covalentes e promovendo a adesão do tecido (20).

O estudo em questão propõe o desenvolvimento de um adesivo biológico à base de PU capaz de restabelecer a qualidade de vida de milhões de indivíduos que sofrem de dores lombares devido ao desgaste dos DIV’s. Acredita-se que estes materiais possam contribuir terapeuticamente para o restabelecimento da biofuncionalidade do disco e, consequentemente, para a redução do número de incidências de re-herniação.

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3 POLIURETANO COMO ESTRATÉGIA PARA REPARAÇÃO E REGENERAÇÃO DO ANEL FIBROSO: UMA REVISÃO SISTEMÁTICA

Manuscrito submetido ao periódico:

Regenerative Medicine

POLYURETHANE AS A STRATEGY FOR ANNULUS FIBROSUS REPAIR AND REGENERATION: A SYSTEMATIC REVIEW

REGENERATIVE MEDICINE

Running Head: Polyurethane as a strategy for annulus fibrosus restoration

Polyurethane as a strategy for annulus fibrosus repair and regeneration: A systematic review

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Abstract

Background: Disc herniation is a spine disease that leads to suffering and disability. Discectomy is a Janus-faced approach that relieves pain symptoms and motor deficit but became the intervertebral discs predisposed to herniation and degenerative process. This systematic review discussed the appropriate mechanical and biological requirements for a polyurethane-based biomaterial to be used in annular disc repair. **Methods:** Search strategy was performed in three online databases (PubMed, Web of Science and SCOPUS) and the MeSH dictionary was used to define the relevant terms. The variables included: main mechanical properties of the polyurethane-based materials; biological findings, tissue and cell type employed; type of study and model used and follow-up. The range was limited to articles published from January 2000 to December 2017 in English-language. **Results:** In the search

carried out 82 articles were assessed. From these, 39 were screened for eligibility and 21 were excluded by not meeting the search criteria. A total of 18 articles underwent a full-text analysis, and 16 studies were included in the review. **Conclusion:** This review pointed out the recent progress regarding the use of polyurethane as a device for spinal disc annulus repair. The suitable physiomechanical properties of this polymer arise as an engineered solution to re-establish the microenvironment and biomechanical features of the intervertebral disc.

Keywords: polyurethane, biomaterial, scaffold, annulus fibrosus, intervertebral disc, herniation, adhesive, sealant, tissue engineering.

Introduction

In recent decades, low back pain has been the most prevalent chronic condition, representing high direct and indirect costs to the national healthcare system.[1] Clinical studies have shown that approximately 90% of spinal diseases are due to the intervertebral disc (IVD).[2] The IVD has three different components: central gelatinous nucleus pulposus (NP), rich in type II collagen and proteoglycan; the annulus fibrosus (AF), rich in collagen and elastin; and the thin layers of hyaline cartilage that form the cartilaginous end plates, bounded above and below to the adjacent vertebral bodies.[3] The confinement of the NP by the AF allows the IVD to provide flexibility and support for compression loads that could damage the spine.[4] The structural injury and collapse of the IVD allows for the dislocation of the NP through defective parts of the AF and compresses the adjacent spinal nerves. Disc herniation with consequent inflammation and degeneration is the primary cause of chronic low back pain.[5] Spontaneous regression of disc herniation in patients who undergo conservative treatment occurs in up to 70% of cases.[6] Surgical interventions such as discectomy, spinal fusion, and total disc arthroplasty are performed after the failure of analgesic, anti-

inflammatory, and physiotherapy treatments. Regardless of the type of surgery performed, biomechanical changes and adjacent IVD degeneration are typically observed.[7]

Current studies aim to develop devices to mimic the properties of NP,[8] but attention should be focused on restoring the AF to confine the NP and maintain the intradiscal pressure during loading.[9] Attempts have been made using synthetic or natural polymers for AF restoration.[10] Biomaterials are commercially available for annuloplasty,[11] but preliminary results demonstrated that these devices did not promote AF healing in the long term. The simplest strategy is to develop three-dimensional matrices that are activated by sowing cells.[12] Urethane-based biomaterials have been the most promising polymers,[10] because they are capable of cellular adhesion, and cell proliferation and promote controlled degradation kinetics, in addition to having high tenacity, hardness, chemical resistance, flexibility, biocompatibility, and excellent processability.[13,14] When these materials present isocyanate terminations, they react with active hydrogen compounds present in biological molecules, such as, alcohols, amino, water, urea, and acid groups, resulting in the formation of covalent bonds with the tissue.[15] Polyurethane (PU) nets and tissue adhesion occurs after contact with the surrounding fluids.[16]

Despite promising results in the short-term, no publications were found demonstrating the long-term efficacy and safety of the existing annulus closure techniques.[11,12] Methods to restore the integrity of the AF are still necessary. The present study performed a systematic review of the PubMed, Web of Science and Scopus databases to analyze the publications of PU-based materials to obtain AF recovery.

Systematic Review Methodology

The systematic review was conducted following the methodological guidelines outlined by the Transparent Reporting of Systematic Reviews and Meta-Analyses (PRISMA)[17] and

focused on the advances in tissue engineering related to the design and applications of PU-based biomaterials in the treatment of AF degeneration.

Studies were selected using the PubMed.gov (http://www.ncbi.nlm.nih.gov/pubmed), Web of Science (https://webofknowledge.com) and Scopus (www.scopus.com) online databases. The dictionary MeSH (Medical Subject Heading Terms) used to define the terms were: ((polyurethane OR urethane OR isocyanate OR urethane scaffold) AND (annulus fibrosus OR annulus closure OR intervertebral disc OR intervertebral disk OR disc repair OR disc herniation OR annulus repair) AND (tissue engineering OR tissue adhesive OR tissue sealant OR bioadhesive OR adhesion OR sealant OR glue OR hydrogel)). The results were limited to English-language articles that were published from January 2000 to December 2017.

The results found in the three databases were compared, and duplicate records were removed. The exclusion criteria were review articles, restoration studies of NP or studies that did not involve the use of PU biomaterial for repair of the AF. The identified articles had their titles and abstracts assessed independently by two reviewers (LDA and FTGD) to screen their allocation in the systematic review. In cases of disagreement, a third independent reviewer (NFN) addressed the article in question and pursued further discussion with the reviewers to reach a consensus.

The variables analyzed in the eligible articles were: main mechanical properties of the PU-based materials, biological findings, type of study, model used, tissue and cell type employed, system used to obtain AF-derived stem cells (AFSCs)/mesenchymal stem cells (MSCs) and follow-up.

Results of Data Collection

Study selection proceeded in compliance with the requirements of the PRISM flow diagram, which illustrates the number of studies that have been identified, included, and excluded, as well as the reason for exclusion (**FIGURE 1**). The search strategy developed from the PubMed, Web of Science and Scopus databases identified a total of 82 articles. From those 82 papers, 43 were excluded for duplicity. Thirty-nine articles were screened and 21 were excluded for not meeting the search criteria (three papers were reviews, and 18 studies were related to other applications). The 18 resulting articles underwent full-text analysis, and only 16 studies were included in the study.

FIGURE 1

The 16 studies were published in the last 7 years, which shows the recent application of polymeric biomaterials to repair AF. Eleven of the 16 papers studied the mechanical behavior of scaffolds.[18-28] Seven articles[19,21,22,25-28] reported the tensile properties of electrospun PU scaffolds, three of these[22,26,28] being associated with superficial polymer modification with anionic dihydroxyl oligomers (ADO). In one study, the elastic behavior of a PU additive manufacturing device was evaluated by compressive and shear testing.[24] The adhesive and biomechanical properties of isocyanate-terminated glues for annulus repair were analyzed in two papers.[18,23] One of them evaluated AF interlamellar, and AF-NP interfacial adhesive properties, and *in vitro* compressive behavior.[20] Among the 11 studies that investigated the mechanical properties of PU-based materials, only three assessed the behavior of scaffolds seeded with cells.[20,22,28] Concerning the biological behavior, the tension applied to the cells causes differential effects on shape, phenotype, extracellular matrix (ECM) synthesis, and proliferation.[22,28]

In vitro assays to evaluate the biological properties of PU biomaterials were reported in 12 of the 16 articles.[19,20,22,24-32] Cell viability, attachment, and proliferation were the most

considered biological parameters of interest to assess PU biocompatibility. [24-27,30,32] An important matter revolved around cell alignment and morphology and the capability of transforming growth factor-b3-mediated bone marrow stem cells (BMSC)/AFSC to achieve a similar phenotype to that of native disc cells under appropriate stimuli.[19,27,30,31] The technique of *ex vivo* biofunctional assessment of AF exposed to PU-based biomaterials was less commonly observed. Bovine and caprine IVD segments were chosen due to their anatomic and biomechanical proximity to the human spine.

Five articles studied the biomaterial adherence and biomechanical restoration of the disc. [18,20,21,23,33] Only two articles evaluated *in vivo* the inflammatory response of PU.[20,27] Sixteen articles focused on clinical translation of the elastomeric PU polymers for disc correction and restoration. Mechanical and biologic properties were divided into two tables according to the type of approach (**TABLE 1 and TABLE 2**).

TABLE 1 and TABLE 2

Discussion of Results

Morphology studies have demonstrated that different cell phenotypes with distinct ECM compositions mold the IVD tissue.[32,34] Cells within the AF are highly oriented and parallel to the lamellar collagen fibers.[35] Due to interspecies and age variation, however, very little is known about the differentiated states in the transient zone between inner AF (IAF) and outer AF (OAF) cells that coexist within the AF, despite the critical importance of this tissue in maintaining disc functionality.

The maintenance of the IVD architecture and morphology are biologically demanding and intensive processes for the AF cells. The regeneration potential of the AF is somewhat limited because of its precarious nutritional pathway.[11,36] The IVD's ability to support load is controlled by the balance between the applied load, the swelling pressure, and the mechanical

(stress-strain) response of the collagen-proteoglycan matrix.[37] The AF structure exhibits a complex mechanical behavior, like compression to bending, flexion, extension, and torsion, which is nonlinear, anisotropic (direction dependent), and viscoelastic. [22,25,26,38-40] The mechanical properties of AF vary with sample orientation (axial, circumferential, or radial), structure (single lamella or multilamellar), tissue location (anterior, postero-lateral, inner, or outer) and biochemical composition (degree of hydration and chemical-electrical behavior of the tissue).[26,37,41,42] The high water content in the AF contributes to its viscous-poro-elastic-properties, resulting in a significant creep response. Intradiscal pressure measurements range from 0.06 MPa to 0.24 MPa, with an average of 0.15 MPa.[43] *In vivo* range of motion (ROM) measurements of human lumbar IVDs of 6–13º, 1–5º, 2.9–11º and 2–3º in flexion, extension, lateral bending, and torsion, respectively, have been reported.[44] Spine movements are mainly sustained by a combination of collagen fiber stretch and inter-lamellar shearing at the OAF lamellae.[38,45]

The elasticity of AF tissue varies significantly along the radial direction.[27] The elastic modulus obtained for single lamellae AF has been reported to range from 60 MPa (IAF) up to 140 MPa (OAF). [25,37,42,46,47] The OAF has higher elastic modulus and is stiffer than IAF.[27,37] The stiffness values of the outer and IAF regions are 13 MPa and 4.8 MPa, respectively.[48] This stiffness difference reflects the role of inter-lamellar matrix connectivity.[49] Extensive numerical simulations are used by researchers to investigate the IVD's response to different loads.[48] These studies mostly model the physiological disk by assuming a homogeneous stiffness distribution, which is not true. For *in situ* motion segment stiffness, IVD values range from 0.44–2.42 kN/mm for axial stiffness, 0.47 kN/mm and 0.58 kN/mm for posterior and anterior shear stiffness, respectively, and 3.18 ± 0.89 N for 6º axial rotation at 0.5 Hz cyclic loading. In lateral bending, the stiffness ranges from 4.21 to 10.04 Nm/deg.[42] Tensile *moduli* of single lamellae from the outer and inner portions of AF tissue are around 64.8 MPa

and 31.2 MPa, respectively.[42] Testing multiple lamella specimens along the predominant fiber direction of one family of fibers obtained a mean tensile modulus in the annulus ranging from 210 to 645 MPa.[50] This range is comparable to the tensile properties of other collagenous soft tissues.[51,52]

The AF compressive properties are necessary for distributing vertical loads as well as confining the NP.[42] The compressive modulus for AF tissue is reported in the literature as being 0.12 ± 0.13 MPa to 0.56 ± 0.21 MPa.[53-55] Shear properties of the AF are important for controlling and limiting motion between vertebrae during bending and twisting of the spine.[42] The complex shear modulus (|G*|) has a range of 0.10–0.28 MPa, and the phase shift angle between stress and strain (δ) has a range of 9–35º, depending on the frequency and shear strain amplitude.[56]

The mechanical property of the scaffold plays a significant role in cellular behavior.[27,57] Long *et. al.* (2016)[42] suggested that promising biomaterials for AF repair must have high tensile failure strain (65%) and adhesion strength (0.2 MPa), to advance to *in situ* and *in vivo* validation tests. Values of approximately 1 MPa, 0.3 MPa, and 30 MPa for compressive, shear, and tensile *moduli*, respectively, would also be desirable. It has been stated that Young's compressive modulus of IVD scaffolds should range from 0.5 to 5 MPa, and the ultimate strength should be at least 8-10 MPa.[55] Dynamic and static compression tests are recommended for IVD constructs, as the forces on the spine are primarily compressive. So far, the various strategies proposed for AF replacement have failed to replicate the AF multi-scale structural hierarchy.[27,38] Approaches to engineering AF tissue using a single-phase material or single cell type to construct scaffolds will likely fail.[37]

The re-herniation that occurs in 5%-15% of spinal decompression surgeries[58] highlights the restoration of disc structure as essential for AF and NP functionality. An ideal composite biomaterial for clinical treatment of IVD disorders must be able to restore AF

tensile strength and keep the NP from extruding to achieve original disc biofunctionality. Due to the AF complex-oriented lamellar structure, the best biomaterial properties for regeneration of AF that most closely mimics the native tissue have not yet been discovered. Existing AF tissue regeneration approaches have focused attention on biodegradable polymeric scaffolds such as those created from synthetic poly(ε-caprolactone) (PCL)[38] or poly (1,8-octanediol malate)[35] or based on natural polymer sources such as collagen and hyaluronan,[59] chitosan[60] or porous silk.[61] Current approaches to AF repair are limited and do not reestablish the disc structural integrity. The degradation of polylactide, polyglycolide, and their copolymers, for example, generates acidic products that can overwhelm the tissue-buffering and cell-regulating capacities, affecting biocompatibility adversely. A slow degradation of the scaffold material better matches the regeneration rhythm of AF tissue, considering its avascular nature and slow self-repair capability.[27]

The biomaterial influences the types of molecules which are synthesized and the microenvironment can profoundly affect cell metabolism and phenotype. [28,30] PUs are possible candidates for AF tissue engineering as they are biodegradable and biocompatible, breaking down into carbon dioxide and water as final degradation products.[62] Carbon dioxide is a component of the body's dominant carbonic-acid-bicarbonate buffering system and generates mildly acidic conditions. PU elastomers are susceptible to degradation by AF cells' hydrolytic enzymes, such as esterases, but are resistant to degradation when hard-segment content or crystallinity is higher.[22,26,63] PU scaffolds exhibit *in vitro* hydrolytic degradation rates over an 8-week period ranging from 15% to 45%.[64] This period is sufficient for cell proliferation and cell–material integration during the healing process.[65] By varying the composition of the monomer units and the size of the blocks of the different monomers within the PU chain, the materials' properties can be tailored. None of the PU-based biomaterials included in this review evidenced any cytotoxicity from its degradation products.

Scaffolds made of PU are also mechanically robust, elastic, and support cell adhesion and proliferation, allowing ECM production and preserving native IVD cell alignment and morphology.[22] This polymer can be electrospun into aligned nanofibers, which simulate collagen fiber orientation in a single lamella of the AF.[66] Additionally, the PU scaffolds allow chemical surface modification by anionic dihydroxyl oligomers (ADO), which increases surface polar chemistry and improves AF cell attachment and ECM retention on the scaffold.[22,26,32]

Fiber diameter and orientation can also be vital parameters to determine AF cell phenotype stimulation.[27] PU-fiber scaffolds promoted AF proliferation with a trend toward an upregulation of GAG and ECM for oriented relative to nonoriented scaffolds. Aligned scaffolds are highly dependent on the strength of the fiber orientation, whereas random structures have more anisotropic properties and are physically dependent on fiber assembly.[25,26] Yeganegi *et. al*. (2010)[26] found higher tensile strength and initial modulus for aligned PU scaffolds ($\sigma = 14 \pm 1$ MPa and $E = 46 \pm 3$ MPa) when compared to random structures ($\sigma = 1.9 \pm 0.4$ MPa and $E = 2.1 \pm 0.2$ MPa). Also, the tensile strength of an aligned nanofibrous scaffold showed significant differences between parallel (14 ± 0.6 MPa) and perpendicular directions (5 ± 0.95 MPa).[67] Oriented PU electrospun scaffolds exhibited higher yield strain values (and consequently better resistance to stretch) compared with other scaffolds, an advantageous feature in a dynamic mechanical environment.[21,25]

Pirvu and coworkers (2015)[21] developed a combined mechanical and biological repair in a whole organ AF defect model under dynamic load. Designed poly(trimethylene carbonate) (PMTC) scaffolds seeded with MSCs implants combined with the sealed PU membrane restored the disc height of annulotomized discs, preventing disc re-herniation and improving disc biomechanics, with regulatory effects on anabolic and catabolic mechanisms in host disc cells. After 14 days under repetitive loading cycles, the sutured PU membrane

retained the PTMC scaffold within the AF defect. The ultimate strength of the PU membrane was 53.0 ± 2.0 MPa; the yield strength was 4.9 ± 1.4 MPa and the elongation at break was 593.8 ± 57.7%. The PU membrane had attractive design characteristics for AF repair, but when used as part of a combined repair strategy.[25,33]

Whatley *et. al.* (2011)[24] fabricated an elastic PU scaffold by using an additive manufacturing technique to mimic the IVD native shape and structure. The values for the compressive storage moduli of the scaffolds (350 kPa) were within the range previously reported in the literature for native IVD tissue (220–800 kPa). [53,54,68] Dynamic viscoelastic results proved the elastic nature of PU scaffolds. The storage modulus (G′ = 56.7 kPa) and dynamic modulus (G* = 57 kPa) were higher than those reported for native IVD tissues (G′ = 5.8 kPa and G* = 7.4 kPa).[69] The loss modulus (G″ = 6.5 kPa), however, was almost identical to the reported value of 5.2 kPa.[40,69] No cytotoxicity from scaffold degradation products affected cell viability.

Blanquer *et. al.* (2012)[18] developed an AF closure device comprising a diisocyanate resorbable glue based on polyethylene glycol–PTMC triblock copolymers. The adhesive strength upon bonding the porous membranes to the scaffolds varied from 20 kPa to 50 kPa using the different PEG-(TMCx-NCO)$_2$ glue types. Dermabond, a commercial cyanoacrylate tissue glue used for comparison, gave a bonding strength of 80 kPa. Fibrin glue, on the other hand, failed at a stress of only 10 kPa. Although Dermabond and fibrin glues are commercially available, significant drawbacks associated with these materials are pointed out in the literature. Isocyanate-terminated glues appear as promising materials to be used as tissue adhesives, since they show good adhesion properties and limited cytotoxicity to human AF cells.[70] Vergroesen *et. al.* (2015)[23] biomechanically tested an isocyanate-terminated glue for annulus closure using a nondegenerate goat IVD. The glue fulfilled the criteria for endurance and medical application, showing accurate material properties for input into AF

with easy deliverable, viscous and fluid properties to abide in place. Strength testing demonstrated that the glue restored the ultimate strength, work to failure, and yield strength/ultimate strength ratio to 79%, 75%, and 119% of native values, respectively. Additionally, the glue strength was maintained after 864,000 load cycles, indicating that the glue did not detach. The tissue-forming implant ensured the functional biomechanical properties of the disc and obtained an overall reduction in the risk of herniation.

The surface modification of PU scaffolds with anionic dihydroxyl oligomers (ADO) had no significant effects on mechanical properties and cytotoxic character nor material biostability.[26] The addition of ADO affected the scaffold's surface polar characteristic,[26,32,71] thereby increasing AF cell adhesion and collagen production.[32] Importantly, the study conducted by Iu *et. al.*, (2014)[28] using IAF and OAF harvested from bovine caudal spines, showed that PU-ADO scaffolds could maintain AF cell phenotype features. IAF cells accumulate more versican and type II collagen than OAF cells and both accumulated different ECMs to those in the native disc environment. The relation of surface modification with matrix proteins provided molecular and topographical conditions that allowed AF cells to orient parallel to scaffold fibers.[29] In this way, Attia *et. al.* (2011)[29] linked PU-ADO with extracellular matrix proteins, such as fibronectin, appear to be involved in better cell attachment. Specific to AF, fibronectin pre-coated PU-ADO played a pivotal role in AF cells, influencing them to spread and elongated/oriented parallel to fibers like AF-native tissue through α5β1 integrin upregulation. A relatively linear biodegradation rate of 0.56 ± 0.05 mg/week was observed for ADO-modified PU scaffolds.[26] Turner *et. al*. (2014) highlighted that AF cells are affected both functionally and morphologically by tensile forces.[22] After 7 days' culture, tension led to a significant increase in elastic modulus in the direction parallel to the scaffold fibers of PU-ADO samples. On the relaxed scaffold, cells grew and

synthesized more collagen type I and expressed TGFβ-1, yet these cells were not well parallel aligned as could be seen on those seeded in the monotonic strained scaffolds.[27]

Autologous AF cells and stem cells (SCs) are reported for AF tissue engineering.[72] Iu *et. al.* (2014)[28] investigated the effect of mechanical loading on the differentiated states of inner and outer AF cells. Both IAF and OAF cells attached to PU scaffolds and accumulated ECM which increased the biomaterial's tensile strength by 14 days of culture. PU scaffolds cultured with IAF and OAF cells exhibited significantly higher values of elastic modulus and ultimate tensile strength ($E = 25 \pm 12.5$ MPa; $\sigma = 6.2 \pm 3.5$ MPa for scaffolds with IAF cells and $E = 23.7 \pm 8.7$ MPa; $\sigma = 6.0 \pm 3.0$ MPa for scaffolds with OAF cells) than those cultured without cells ($E = 18 \pm 8.7$ MPa; $\sigma = 4.2 \pm 2.0$ MPa). No significant differences in mechanical properties were observed for PU scaffolds cultured with IAF cells or OAF cells.

Iu *et al*. (2017)[20] fabricated a two-step *in vitro* IVD coculture model engineered from outer AF and NP tissues. Multilamellated AF-like tissue were preformed by PU nanofibrous and a porous bone substitute material (calcium polyphosphate - CPP). Multilayered PU scaffold presented lamellar adhesion after 2/3 weeks of culture, and its interlamellar shear stress was 0.03 ± 0.005 N/mm, significantly lower than that of native bovine AF tissues (0.6 ± 0.07 N/mm). The AF-NP interfacial shear strength in the biphasic construct was 96 ± 16 kPa after 2 weeks of coculture, a magnitude lower than that of the native disc (487 ± 14 kPa). The *in vitro* IVD model showed compression-bearing mechanical behavior, with $64.3\% \pm 0.007$ of hysteresis and a compressive modulus of 17 ± 0.007 kPa, in comparison to the bovine native IVD, which had a hysteresis and a compressive modulus of $56.7\% \pm 6.5$ and 7.5 ± 3.2 kPa, respectively. An additional set of experiments generated under identical *in vitro* conditions[30] revealed that media supplementation with insulin-transferrin-selenium (ITS) and proline favored DNA contents accumulation of IAF and OAF tissues and cell proliferation. However, it was observed that the use of ITS and proline made IAF cells lose the typical phenotype.

Insofar, supplementation based on the addition of dexamethasone and sodium pyruvate restored IAF phenotype given the presence of both type II collagen and aggrecan in the ECM; it also allowed the maintenance of the OAF-phenotype. Dexamethasone increased mitochondrial membrane potential of multilamellated IAF and OAF tissues in the presence of sodium pyruvate, signaling a relevant association between small metabolites in modulating IAF and OAF phenotypes. This multilamellated AF-construct made by PU appeared well integrated and adhered when implanted at a bone substitute in a bovine caudal spine.[20] The implanted AF/PU tissue remained stable during the post-operative period, but no reparative response to the disc height and function was discussed. Collagen type I and II accumulation at the interface between AF and NP tissues probably served as a biological ‘‘glue’’, contributing to an efficient implant integration and strong adherence. Importantly, no inflammatory response was noted by this IVD construct implantation on the spinal site trough 1-month follow-up.

As the availability of functional autologous AF cells is highly limited, MSCs transplantation becomes an attractive alternative.[19,21] An increment of 50% of the compression *moduli* value was observed for 6 weeks with bovine chondrocyte culture in PTMC-based resins.[73] The shear modulus for PCL scaffolds seeded with bovine MSCs significantly increased after 12 weeks.[57] Mechanical equivalence with the AF tissue was reached by Nerurkar *et. al.* (2009)[38] after 10 weeks of *in vitro* culture. The mechanical properties continue to rise for longer culture times until the scaffold reaches its maximum capacity to hold cells; by this time, the values are expected to decrease in parallel to the scaffold’s material deterioration.[73] Exposure to aqueous medium disrupts the material’s intermolecular structure and results in a reduction of its mechanical properties.[26]

The elasticity of the cell culture substrate significantly affects and directs SCs differentiation.[74] Four poly(ether carbonate urethane)-urea (PECUU) scaffolds were

fabricated with different elastic modulus to mimic the stiffness gradient of AF tissue.[19] For low-modulus PECUU materials, collagen-I gene expression in both AFSCs and BMSCs was downregulated, and collagen-II and aggrecan expression was fairly high, resembling that of IAF cells. For stiff PECUU scaffolds, however, AFSCs and BMSCs preferentially differentiated into cells with OAF characteristics. Such differentiation-associated scaffold stiffness has also been seen in other types of SCs.[75,76] Zhu *et. al.* (2016)[27] used AFSCs to achieve diversified cell differentiation on a series of PECUU scaffolds with different elasticities. By just adjusting the molecular weight of the soft segment, PECUUs materials were synthesized with elastic modulus ranging from 2.5 MPa to 13.4 MPa, very close to that of native AF tissue. Tissue response and *in vivo* degradation behaviors of PECUU membranes were achieved by subcutaneous implantation using a rat model. The implants were harvest at different time points up to 20 weeks and prepared for histological evaluation. Although the implantation site was not specific to the spine microenvironment, no inflammatory process nor rejection was observed through the follow-up.

The scaffold used for AFSCs approaches should mimic the microstructure of the native tissue and can be crucial to differentiating an AF-like cell profile. The AFSCs phenotype varies in response to the PU scaffold properties and by mimicking the microstructure of outer or inner AF tissue, may direct AFSCs to differentiate to an OAF or IAF cell phenotype.[19,27,28,30,31] The elastic modulus of PECUUs did not appear to change during degradation, meaning that they may consistently deliver similar mechanical signals to the cells during *in vitro* cell culture or *in vivo* implantation. Finally, the possibility that BMSCs or MSCs can differentiate into AF-like cells with gene expression profiles similar to that of AFSCs brings excellent prospects for their use. Assuming that harvesting AFSCs from healthy IVDs through a non-invasive approach is quite challenging, and intradiscal

procedures are not advisable, it could be feasible to obtain SC from other popular sources in large quantity without traumatic operations.

Conclusion

Degeneration of the IVD is a part of normal aging, involving genetic, nutritional, and mechanical factors, which might explain why this complex and multifactorial disease is difficult to halt or reverse. To date, no tissue engineered solution had proven to be effective in repairing the AF structure or reversing the degeneration process. In fact, no indication of clinical trials is available so far. Nevertheless, several studies are being conducted to develop scaffolds with properties that mimic the AF inter-connectivity focused on compositional structure and viscoelastic properties. This review paper summarized recent progress regarding the use of PU material as an AF restoration approach. The suitable physiomechanical properties of this polymer in terms of biocompatibility, bioactivity, mechanical properties, and hydrolytic resistance were pointed out. Most of the studies are performed under *in vitro* conditions lacking the native disc architecture and microenvironment. Caprine and bovine IVD were the most used in *ex vivo* models because they show similar biology as well as anatomic and biomechanical similarities to humans.

For patients with an early diagnosis of IVD disease, the closure of AF tears is of vital importance, and the regenerative therapy chosen will depend on the extent of the AF that has been compromised.[77-79] The PU-based biomaterials would have applicability at mild stages of disc degeneration, when structural changes in AF are more significant (mainly the disc height reduction).[80] These biomaterials must be capable of inducing the proliferation of residing disc cells.[79] The lack of *in vivo* experiments and the need for developing an implant fixation strategy to prevent its later migration are the greatest insufficiencies of the scaffold approach.[80,81] Furthermore, there are concerns regarding the long-term consequences of implanting inert artificial materials into disc space, which is subject to age-related biological

changes.[78] Care must be taken when linking results from *ex vivo* studies or translating animal in results to clinical use. For a more in-depth investigation into the use of PU scaffolds at risk of wear and failure in AF restoration, preclinical studies should be conducted to acquire a better understanding of PU-based biomaterials and how they should be handled to achieve their desired performance.

Future Perspective

Many difficulties must be overcome for the goal of using tissue engineering to repair damaged IVD. These challenges include: how to surgically introduce the implant material without further compromising the existing disc structure; how to ensure that implanted cells thrive in environments where damage may arise not solely from injury but also due to factors such as poor disc nutrition, vascular in-growth, apoptosis, and aging. However, the only other possible treatments for severely damaged discs are transplantation, prosthetic implantation, or gene therapy, which is still in an early stage and which may be combined with tissue engineering scaffolds. Therefore, a tissue engineering treatment for the damaged IVD remains a worthwhile long-term goal. Advances in cell biology and tissue engineering have shed new light on the field of biological treatments to induce disc regeneration. However, preclinical studies have not yet achieved the expected efficacy of injected MSCs to restore disc functionality. First, the procedure of intradiscally inject MSCs itself can contribute to further degeneration; beyond that, the fate of the cells once implanted into the disc space and their mechanism of action has yet to be solved.

A promising alternative to suppress AF fissure growth at mild stages of IVD degeneration is by occluding it using a sealant. Injectable biological therapies with appropriate viscosity and stiffness, and *in situ* welding techniques should be explored.[77,82] As the adhesion of photocrosslinked materials to the AF tissue still needs to be improved, self-

curing formulations emerge as a solution in this regard. These polymerized constructs should conform precisely to the disc defect[78] and be supplemented by biomolecules with the ability to secret an appropriate ECM into the allograft disc space.[80]

Although there is an obvious need to fix a patch or barrier, the scientific literature on the use of glues to close the AF is scarce. Only two works in this review addressed the use of PU-based glues for annulus repair [18,23]; moreover, these annulus implants were biodegradable. The use of natural polymers raises concerns regarding batch variation and immunogenic risk.[77] Besides, biodegradable polymers are rapidly degraded (~6 weeks) by hydrolysis of the ester bonds. Due to the avascularity and slow healing potential of the AF, removal of the acidic degradation products of these polymers is not facilitated. An acidic environment contributes to both ECM degradation and AF cell damage.[78] Synthetic polymeric implants, in contrast, have the advantage of synthesis control, bioactivity tailoring ability, large-scale production, mechanical properties and controllable degradation rates.[77] The development of an injectable implant that provides immediate closing of the AF defect, at the same time allowing generation of a functional tissue, will be of relevance in future regenerative applications.

Executive summary

AF regeneration strategies must be considered in relieving the symptoms of pain and motor deficit caused by disc herniation

- Structural injury to the IVD allows the dislocation of the nucleus pulposus (NP) through defective parts of the annulus fibrosus (AF), compressing the adjacent spinal nerves and leading to pain and disability. Although the literature offers NP-engineered solutions, studies should also focus on restoring the AF to confine the NP and re-establish the internal environment and the biomechanical features of IVD.

Urethane-based (PU) biomaterials are a promising strategy to obtain AF recovery

- PU biomaterials are controlled degraded and can promote cell proliferation and tissue adhesion by contact with the surrounding fluids. These materials have high tenacity, hardness, chemical resistance, flexibility, biocompatibility, and excellent processability.

Sixteen studies related to the application of PU-based materials in AF restoration were selected from a search of PubMed, Web of Science and Scopus databases

- The 16 studies were published in the last 7 years. Eleven of these sixteen papers evaluated the mechanical behavior of scaffolds. The tensile, elastic, biomechanical, and adhesive characteristics of PU biomaterials were the main properties analyzed. The *in vitro* assays of PU scaffolds were observed in 12 of the 16 articles selected. Cell viability, attachment, and proliferation were the most frequently considered issues in assessing PU biocompatibility. The technique of *ex vivo* biofunctional assessment of AF exposed to PU-based biomaterials was less commonly observed. Only two articles evaluated *in vivo* the inflammatory response of PU.
- PU-based scaffolds proved to be mechanically robust, elastic and capable of supporting cell proliferation, allowing ECM production and preserving native IVD cell alignment and morphology. The mechanical properties were intrinsically related to the cellular behavior.

Conclusions

- To date, no tissue-engineered solution had proven to be completely effective in repairing the AF structure or reversing the IVD degeneration process. Nevertheless, several studies are being conducted to develop scaffolds with properties that mimic AF inter-connectivity focused on compositional structure and mechanical properties.
- The lack of *in vivo* experiments and the need to develop an implant fixation strategy to prevent later implant migration are the greatest insufficiencies of the scaffold approach.
- PU-based biomaterials emerge as a promising alternative to suppress AF fissure growth at mild stages of IVD degeneration. An injectable and *in situ* curing PU sealant capable of closing the AF defect, at the same time allowing generation of functional tissue, may arise as a new option for IVD restoration.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors acknowledge financial support from the Brazilian Agency *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)* through Master's scholarship to Lucas Dall Agnol and a post-doctoral fellowship to Fernanda Dias.

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Figure captions:

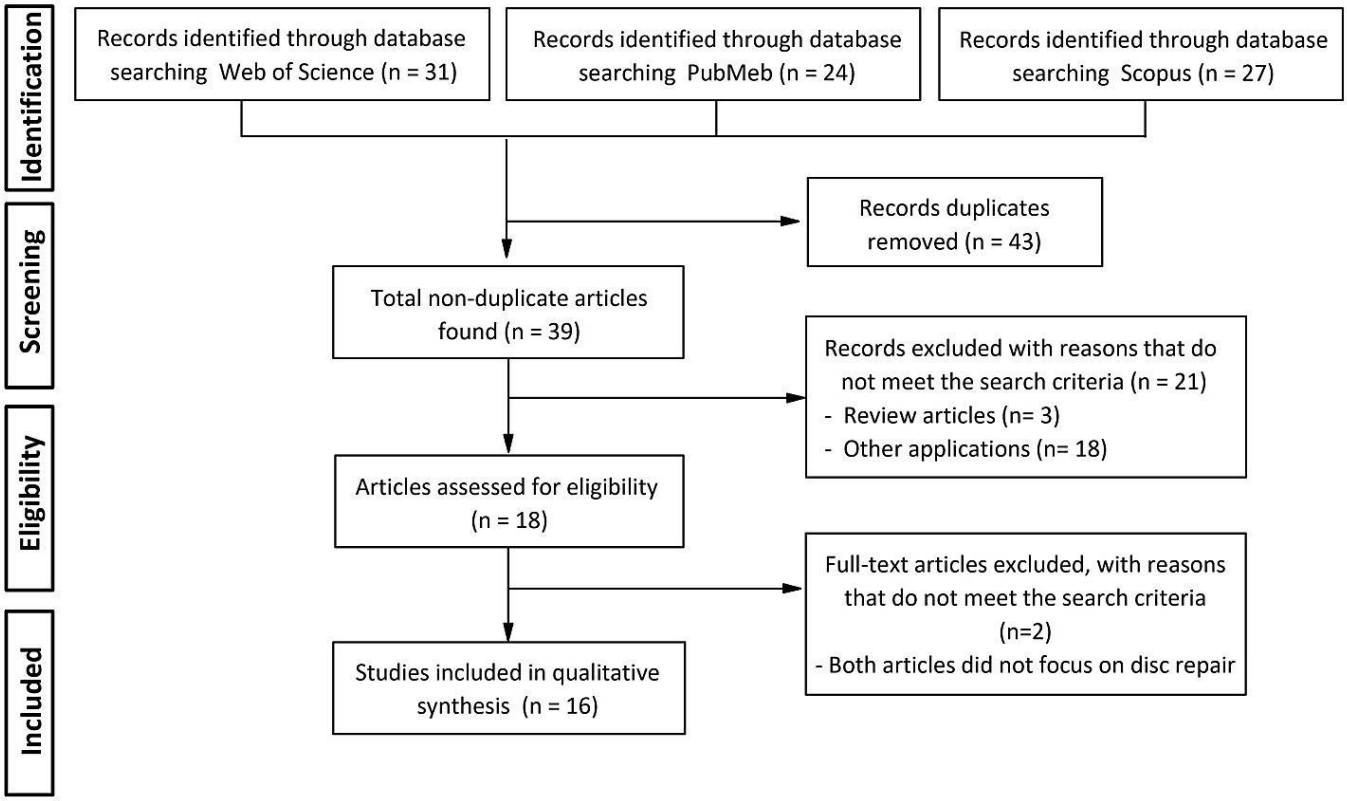

FIGURE 1 – Systematic review flowchart, including inclusion and exclusion criteria.

TABLE 1: Mechanical properties of the polyurethane-based materials

			Tensile Properties				Compressive Properties	Adhesive Properties
System	**PU Composition**	**Mechanical Testing Conditions**	**Elastic Modulus (MPa)**	**Ultimate Strength (MPa)**	**Yield Strength (MPa)**	**Elongation at Break (%)/Work to failure (MPa*mm)**	**Compressive Modulus (linear and dynamic) and Compressive Strain (%)**	**Adhesive Strength (kPa)**
Electrospun PU scaffolds superficially modified with ADO [26]*	HDI:PCL:BDO (3:2:1) ADO (0.5 wt.%)	Tensile (Instron 8501) (50 N load cell; 10 mm/min strain rate; 6 mm x 30 mm x 106 µm specimens)	46 ± 3.0 (aligned PU) 42 ± 4.5 (aligned PU-ADO) 2.1 ± 0.2 (random PU)	14 ± 1.0 (aligned PU) 13 ± 0.8 (aligned PU-ADO) 1.9 ± 0.4 (random PU)	-	-	-	-
PU scaffold fabricated by additive manufacturing [24]*	LDI and PCL	Compression (DMA Q800) (1mm/min rate; 50% strain; 5.75 mm x 2 mm specimens) Dynamic compression (DMA Q800) (0.008 Hz; 65% strain) Dynamic shear (AR G2 dynamic rheometer) (1.5% shear strain; 0.05 -1.05 Hz; 0.05 Hz interval)	-	-	-	-	45.4 ± 5,6 kPa (Initial compressive storage modulus) 350 ± 19.6 kPa (Compressive modulus of the linear region) Compressive dynamic shear moduli (1 Hz): 57 ± 23.7 kPa (15% strain); 97 ± 15.2 kPa (30% strain); 135 ± 12.6 kPa (45% strain) Compressive storage shear moduli (1 Hz): 56.7 ± 23.7 kPa (15% strain); 96.5 ± 15.2 kPa (30% strain); 134 ± 12.4 kPa (45% strain)	-
Diisocyanate adhesive based on PEG-PTMC triblock copolymers [18]*	PEG:TMC = 4:1, 6:1, 8:1 NCO:OH = 2.05	Adhesive properties (Zwick Z020 Universal Tensile Tester) (ASTM F2255-05; 500 N load cell; 25 mm grip-to-grip separation; 10 mm/min rate)	-	-	-	-	-	20 - 50 kPa [for the different PEG-$(TMCx\text{-}NCO)_2$ glues] 80 kPa (for Dermabond commercial tissue glue) 10 kPa (fibrin glue)
Electrospun PU scaffolds superficially modified with ADO [28]*	PHECD + HMDI + BDO ADO (0.5 wt.%)	Tensile (Instron 8501)(50 N load cell; 10 mm/min strain rate; 8 mm x 2 mm x 170 µm specimens)	18 ± 8.7 MPa (PU-ADO) 25 ± 12.5 MPa (PU-ADO with IAF cells; 14 days culture) 23,7 ± 8.7 MPa (PU-ADO with OAF cells; 14 days culture)	4.2 ± 2.0 MPa (PU-ADO) 6.2 ± 3.5 MPa (PU-ADO with IAF cells; 14 days culture) 6.0 ± 3.0 MPa (PU-ADO with OAF cells; 14 days culture)	-	-	-	-
Electrospun PU scaffolds superficially modified with ADO [22]*	PHECD + HDI + DBDA ADO (0.5 wt.%)	Tensile (Instron 8501) (50 N load cell; 10 mm/min strain rate; 8 mm x 0.38 mm^2 sectional area)	2.9 ± 1.1 (no-strained PU-ADO) 7.9 ± 1.1 (PU-ADO strained for 7 days culture) 6.2 ± 1.6 (relaxed PU-ADO) 6.8 ± 1.1 (PU-ADO + AF cells strained for 7 days culture) 4.5 ± 1.1 (relaxed PU-ADO + AF cells) Relaxed: tension removed after 24 h	-	-	-	-	-

Electrospun PU scaffold [25]*	PCL + ISO + HDI	Tensile (DMA equipment) (0.1%/s or 0.5%/s strain rate; 1 Hz; 5 mm x 35 mm specimens)	47.0 ± 1.1 MPa (0.1%/s) and 53.3 ± 10.3 MPa (0.5%/s) (aligned PU)	-	Yield strain: 26.8 ± 1.2 % (0.1%/s) and 31.9 ± 4.2 % (0.5%/s) Yield stress: 4.6 ± 0.4 MPa (0.1%/s) and 7.1 ± 0.7 MPa (0.5%/s) (aligned PU)	-	-	-
Electrospun PU membrane [21]*	HDMI:PCL:ISO 1:0.32:0.64 molar ratio	Tensile (Instron 4302) (100 N load cell; 10 mm/min strain rate)		53.0 ± 2.0 MPa	4.9 ± 1.4 MPa	593.8 ± 57.7%	-	-
Electrospun PECUU scaffold [19]*	PEO-PPO-PEO + TMC + HDI	Nanoindentation test (Nanoindenter G200) (10, 5.62, 3.16, 1.78 and 1 Hz frequencies; 50 nm oscillation amplitude)	13.4 ± 1.7 MPa (PECUU-1) 6.4 ± 0.5 MPa (PECUU-2) 5.1 ± 0.2 MPa (PECUU-3) 2.5 ± 0.2 MPa (PECUU-4)	-	-	-	-	-
PEG-TMC copolymers functionalized with HDI [23]*	PEG + TMC + HDI	Biomechanical Tess: → Ultimate strength (Instron 8872) (5º left lateral flexion; compression at 2mm/min) → Endurance [sinusoidal load alternating in magnitude every 30 min (40-60 N and 80-180 N), followed by 8 hours of low dynamic load (40-60 N)]	-	Pre-endurance Test: 9.8 ± 6.1 MPa Post-endurance test: 7.4 ± 2.2 MPa	Pre-endurance Test: 6.9 ± 3.2 MPa Post-endurance test: 5.5 ± 1.7 MPa	Pre-endurance Test: 6.3 ± 6.3 MPa*mm Post-endurance test: 3.8 ± 2.4 MPa*mm	-	-
Electrospun PECUU scaffold [27]*	PEO-PPO-PEO + TMC + HDI	Tensile (Instron E10000) (15 mm/min strain rate; 12 mm x 3.4 mm specimens)	13.4 ± 1.7 MPa (0 PTMC length) 6.4 ± 0.5 MPa (1070 PTMC length) 5.1 ± 0.2 MPa (1730 PTMC length) 2.5 ± 0.2 MPa (1270 PTMC length)	8.2 ± 0.8 MPa (0 PTMC length) 11.9 ± 1.1 MPa (1070 PTMC length) 16.1 ± 1.2 MPa (1730 PTMC length) 2.0 ± 0.2 MPa (1270 PTMC length)	-	329 ± 62% PECUU-1 (0 PTMC length) 1544 ± 234% PECUU-2 (1070 PTMC length) 2146 ± 88% (1730 PTMC length) 465 ± 97% (1270 PTMC length)	-	-
Electrospun PU scaffolds superficially modified with ADO and coated with fibronectin [20]*	PHECD + HDMI + BDO ADO (0.15 wt.%) + fibronectin	AF interlamellar shear strength (Instron 8501) (Peel test - 10 mm/min strain rate); AF-NP interfacial shear strength (Instron 4301) (Pushout test - 0.5mm/min loaded rate) Dynamic compressive deformation (MACH-I tester) (0.1 Hz frequency, 0-10% strain rate, 20 cycles)	-	-	-	-	Compressive modulus: 17 ± 0.007 kPa Hysteresis: 64.3% ± 0.007	AF interlamellar shear strength: 0.03 ± 0.005 N/mm AF-NP interfacial shear strength: 96 ± 16 kPa

ADO: anionic dihydroxyl oligomers; AF: annulus fibrosus; BDO: butane diol; DBDA: dibutyltin dilaurate; DMA: dynamic mechanical analysis; HDI: hexane diisocyanate; HDMI: 1,6-hexamethylene diisocyanate; IAF: inner annulus fibrosus; ISO: 1,4,3,6-dianhydro-D-sorbitol; LDI: lysine diisocyanate; NCO: isocyanate group; NP: nucleus pulposus; OAF: outer annulus fibrosus; OH: hydroxyl group; PCL: poly(ε-caprolactone) diol; PECUU: poly(ether carbonate urethane)-urea; PEG: polyethylene glycol; PTMC: poly(trimethylene carbonate); PEO: poly(ethylene oxide); PHECD: poly(1,6-hexyl 1,2-ethyl carbonate)diol; PPO: polypropylene oxide; PU: polyurethane; TMC: trimethylene carbonate.

TABLE 2: Biologic properties of the polyurethane-based materials

System	PU Composition	Environment	Model	Donor tissue/ Cell types	Outcomes measures	Main Biological Findings
Electrospun PU scaffolds superficially modified with ADO [32]*	PCN + HDI + DBDA - ADO (0.5 wt.%)	*In vitro*	-	Bovine AF cells	Cell attachment (DNA content) and morphology (SEM); proteoglycan and collagen quantification.	Biomaterial surface polarity contribute to AF cell attachment and collagen accumulation.
Electrospun PU scaffolds superficially modified with ADO [26]*	HDI:PCL:BDO (3:2:1) ADO (0.5 wt.%)	*In vitro*	-	Bovine AF cells	MTT and live/dead viability assay	No significant cytotoxic effects from either non-soluble or soluble degradation products.
Electrospun PU scaffolds superficially modified with ADO [29]*	PCN + HDI + BDO - ADO (0.5 wt%)	*In vitro*	-	Bovine AF cells	Cell attachment (DNA content and SEM); Collagen synthesis; expression of integrin, ECM, collagen and fibronectin (IF and CLSM)	Better response of Fn pre-coated PU-ADO scaffold: well cell attachment; cells elongated and oriented parallel to fibers like AF-native tissue; increase collagen synthesis and actin amount; upregulated α5β1 integrin expression.
PU scaffold fabricated by additive manufacturing [24]*	LDI and PCL	*In vitro*	-	Bovine IVD cells	Cell proliferation, viability and cytocompatibility (alamarBlue® assay); morphology (DAPI-SEM)	Good biocompatibility; increase cell attachment and proliferation; increase IVD native cells alignment; Scaffold degradation no affect cell viability.
Diisocyanate adhesive based on PEG-PTMC triblock copolymers [18]*	PEG:TMC = 4:1, 6:1, 8:1 NCO:OH = 2.05	*Ex vivo*	Tensile lap-shear tests	AF tissue	Adhesive properties (shear stress at break)	Partial adhesive strength values.
Electrospun PU scaffolds superficially modified with ADO [28]*	PHECD + HDI + BDO - ADO (0.5 wt.%)	*In vitro*	-	Bovine OAF and IAF cells	DNA content and cell viability; RT-qPCR, IH, WB of collagen and proteoglycan; tensile testing	IAF and OAF cells increase attachment and proliferation; spread ECM. PU-ADO scaffolds are able to keep AF cell phenotype: IAF cells increase collagen I and II, aggrecan and versican; OAF cells increase collagen I and aggrecan only.
Electrospun PU scaffolds superficially modified with ADO [22]*	PHECD + HDMI + DBDA - ADO (0.5 wt.%)	*In vitro*	-	Bovine AF cells	DNA content and proliferation; collagen synthesis; morphology (DAPI and SEM); gene expression; tensile testing	Tension affects AF cells alignment and morphology; relaxed scaffold increase cell proliferation and collagen I (no aligned); monotonic strained scaffold cells and collagen I well parallel aligned.
Electrospun PU scaffold [25]*	PCL + ISO + HDI	*In vitro*	-	Bovine AF cells	Biochemical analyses (DNA, GAG and collagen content); RT-qPCR; Histology	Increase AF cells proliferation, GAG and ECM retention on PU orientated scaffold; fiber diameter and orientation can be the main parameters to influencing AF cells phenotype stimulation.
Electrospun PU membrane [21]*	HDMI:PCL:ISO 1:0.32:0.64 molar ratio	*Ex vivo*	MSCs-implanted into bovine caudal IVD	Human bone marrow derived MSCs / bovine IVD	Biochemical analysis (DNA, GAG and OHP content); qRT-PCR; Safranin-O/Fast Green staining (proteoglycan and collagen deposition)	Restore disc height and hinder herniation of NP tissue into AF defect; MSCs increase anabolic and decrease catabolic gene expression in host disc cells; positively modulation of cell phenotype of native disc tissue.
Electrospun PECUU scaffold [19]*	PEO-PPO-PEO + TMC + HDI	*In vitro*	-	Rabbit AFSCs and BMSCs	MTS assay; morphology (SEM); RT-qPCR; CTFM	BMSCs are able to differentiate in AF-like cells with gene expression profile similar to AFSCs; increase collagen II and aggrecan in tBMSCs cultured in PECUU; Stiffness PECUU scaffold

						turn tBMSCs differentiation (soft PECUU tBMSCs into IAF cells; stiff PECUU tBMSCs to OAF cells).
PEG-TMC copolymers functionalized with HDI [23]*	PEG + TMC + HDI	*Ex vivo*	Strength and endurance test	Goat IVD	Biomechanical test	Glue fluently and viscous enough to abide in place; Partial restoration of AF integrity and yield strength/ultimate strength ratio of IVD; Overall reduction in the risk of herniation.
Electrospun PECUU scaffold [31]*	PEO-PPO-PEO + TMC + HDI	*In vitro*	-	Rabbit AFSCs	MTS assay; morphology (SEM); RT-qPCR, biochemical analysis and CTFM	Aligned PECUU scaffold improve AFSCs shape and increase collagen-I and GAGs but decrease CTFM; mimic OAF tissue direct AFSCs to an OAF cell phenotype.
Electrospun PECUU scaffold [27]*	PEO-PPO-PEO + TMC + HDI	*In vitro*	-	Rabbit AFSCs	MTS assay, RT-qPCR, biochemical analysis, CTFM	Well proliferation and viability; increase collagen I; decrease collagen II and aggrecan; CTFM of AFSCs increased on PCUU-1 and -4.
		In vivo	Subcutaneous implantation	Rats	Histology	No significant inflammatory response.
FibGen hydrogel adhesive, PTMC scaffold and a PU membrane [33]*	(Composition of PU membrane not specified)	*Ex vivo*	AF bone defect	Bovine segments (bone-IVD-bone)	Biomechanical analysis; Histology	FibGen easily deliverable during surgical procedures and well adhered to the AF tissue; partial restore of biomechanical disc; failure by cracking FibGen and fissuring native disc tissue.
Electrospun PU scaffolds superficially modified with ADO and coated with fibronectin [20]*	PHECD + HDMI + BDO ADO (0.15 wt.%) + fibronectin	*In vitro*	-	Bovine NP and AF cells	Biochemical analysis (DNA, GAG and collagen content); Histology and Immunostaining.	The NP and AF cell-seeded on PU scaffolds created an IVD-like tissue similar to native disc (NP rich in collagen II and aggrecan; AF collagen I).
		In vivo	Spinal unit implant into bovine caudal defect	Bovine	Histology	1-month follow-up showed spinal implant integrated to bovine IVD; fibrous adherence between the native and implanted AF was note, without inflammatory response.
Electrospun PU scaffolds superficially modified with ADO and coated with fibronectin [30]*	PHECD + HDMI + BDO ADO (0.15 wt.%) + fibronectin	*In vitro*	-	Bovine OAF and IAF cells	Biochemical analysis (DNA, GAG and collagen content); mitochondrial membrane potential (MitoTracker Red); Histology and Immunostaining.	The multillamelar PU scaffold contributed to IAF and OAF phenotype; ITS and proline increased IAF and OAF cell proliferation; dexamethasone plus sodium pyruvate induced mitochondrial membrane potential and type II collagen and aggrecan (IAF) or type I collagen (OAF) accumulation.

ADO: anionic dihydroxyl oligomers; AF: annulus fibrosus; AFSCs: AF derived stem cells; BDO: butane diol; BMSCs: transforming growth factor-b3-mediated bone marrow stem cells; CLSM: confocal laser scanning microscopy; CTFM: cell traction force microscopy; DAPI: (4',6-diamidino-2-phenylindole); DBDA: dibutyltin dilaurate; ECM: extracellular matrix; FibGen: genipin-crosslinked fibrin gel; FN: fibronectin; GAG: collagen-glycosaminoglycan; HDI: hexane diisocyanate; HDMI: 1,6-hexamethylene diisocyanate; IAF: inner annulus fibrosus; IF: immunofluorescence; IH: immunohistochemistry; ISO: 1,4,3,6-dianhydro-D-sorbitol; ITS: insulin-transferrin-selenium; IVD: intervertebral disc; LDI: lysine diisocyanate; MSCs: mesenchymal stem cells; MTS: CellTiter 96® AQueous One Solution Cell Proliferation Assay; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCO: isocyanate group; NP: nucleus pulposus; OAF: outer annulus fibrosus; OH: hydroxy group; OHP: orthohydroxyproline; PCL: poly(ε-caprolactone) diol; PCN: poly(1,6-hexyl 1,2-ethyl carbonate) diol; PECUU: poly(ether carbonate urethane)-urea; PEG: polyethylene glycol; PEO: poly(ethylene oxide); PHECD: poly(1,6-hexyl 1,2-ethyl carbonate)diol; PPO: polypropylene oxide; PTMC: poly(trimethylene carbonate); PU: polyurethane; RT-qPCR: real time quantitative Reverse transcription polymerase chain reaction; SEM: scanning electron microscopy; TMC: trimethylene carbonate; WB: Western Blot.

4 ADESIVOS DE TECIDO À BASE DE POLIURETANO PARA REPARO DO ANEL FIBROSO: RESTAURAÇÃO MECÂNICA E CITOTOXICIDADE

Manuscrito submetido ao periódico:

Materials Science and Engineering: C

POLYURETHANE TISSUE ADHESIVES FOR ANNULUS FIBROSUS REPAIR: MECHANICAL RESTORATION AND CYTOTOXICITY

Materials Science and Engineering: C – Materials for Biological Application

POLYURETHANE TISSUE ADHESIVES FOR ANNULUS FIBROSUS REPAIR: MECHANICAL RESTORATION AND CYTOTOXICITY

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Abstract

Lower-back pain caused by degenerative intervertebral disc (IVD) is a significant reason for long-term disability for millions of people. An incision made in the annulus fibrosus (AF) during the microdiscectomy of the IVD remains open and must be sealed to avoid reherniation and the subsequent degeneration of the disc. In this study, we developed an injectable and *in situ*-polymerizable polyurethane adhesive as an AF repair strategy following microdiscectomy. The challenge was to create a long-term implant since the AF tissue is not regenerative, and is capable of meeting the performance requirements: strong wet adhesion, reactivity with collagen tissue, low toxicity, minimal swelling, and mechanical behavior similar to that of native AF. To evaluate the urethane-based adhesive with respect to further applications, this work investigated (1) the synthesis and chemical structure of the prepolymers, (2) their compressive behavior, (3) their preparation time using a kinetic approach, (4) the adhesiveness between polyurethane (PU) and collagen through tensile tests, (5) material wettability and swelling behaviors, and (6) the cell viability assay and adhesion by scanning electron microscopy. One of the adhesives presented a compressive modulus of ~ 3.1 MPa, the closest of the IVD outer region although still superior. This same adhesive adhered covalently to the gelatin films exhibited 18-day stability under moisture and required a minimum preparation time of 10 h at 60 ºC before use. When formulated with an excess of reactive -NCO groups, this adhesive was cytotoxic in the first 24 h, but positively impacted cell proliferation after 48 h. The current findings provide preliminary evidence of the ability

of this adhesive to act as an AF closure device, although additional tests and adaptations are necessary.

Keywords: urethane adhesive; annulus fibrosus repair; injectable sealant; kinetic predictions; mechanical viability; reactivity with gelatin.

1. Introduction

Lumbar discectomy is a spinal surgery that plays a vital part in the relief of pain symptoms and motor deficit caused by intervertebral disc (IVD) herniation regardless of is often linked to postoperative complications [1]. Re-herniation events are an unfavorable evolution of discectomy appearing in 5−15% spinal decompression surgeries that highlight the disc structure restoration is essential for annulus fibrosus (AF) and nucleus pulposus (NP) functionality [2, 3]. These structures have specific mechanical characteristics and exhibit viscoelastic behavior to allow and control the movement of the functional vertebral bodies [4]. However, IVD itself is structurally avascular and have a precarious nutritional pathway [5], which makes the regenerative potential of the AF and NP tissues somewhat limited. During the structural collapse of the IVD, parts of the NP can move to defective outer parts of the AF, compressing the adjacent spinal nerves and causing inflammation. The degenerate portion of the disc can be removed through a small surgical incision (3−4 mm in diameter) in the AF, but the unrepaired defect can create changes in biomechanics and the microenvironment of IVD [6]. Therefore, sealing the compromised AF can help to restore the physiological function of the herniated IVD and prevent painful conditions for the patient [6, 7].

Non-injectable AF regenerative approaches include sutures that do not restore intradiscal pressure [8] and plugs that have a risk of NP extrusion [9]. Injectable AF repair solutions seem to be more promising than rigid implants since they do not require anchoring to the vertebral body, besides easily handling [10, 11]; nonetheless, all materials developed so far showed mechanical limitation or biological incompatibility [12]. Mechanical resistance and biodegradability were the two structural requirements for AF engineered materials in most of the regeneration devices reported in the literature [9, 12]. An AF repair device should be mechanically designed to provide flexibility, maintain intradiscal pressure, and withstand the daily loads experienced by the IVD [13]. The material biodegradability, however, is a

controversial requirement since it does not always meet the specificity of the application. Considering the avascular nature and slow self-repair capability of the AF microenvironment, a scaffold material with a low rate of degradation is more technically feasible [5]. Also, spine devices will remain *in situ* for extended periods of time and long-term chemical stability should be guaranteed.

For an AF engineered adhesive or sealant to be a promise of clinical delivery, it must be injectable and polymerizable under physiological conditions and present strong wet adhesion, cytocompatibility, minimal swelling, a mechanical modulus comparable to the native tissue, and long shelf life [14]. Initial attempts to develop tissue adhesives involved the use of cyanoacrylates, fibrin, albumin-glutaraldehyde, epoxy resins, methacrylate-based systems, but these materials were inappropriate due to their low bonding strength, degradability or high infection rates [6]. Among semisynthetic tissue adhesives, urethane-based ones have called the attention of researchers by their mechanical robustness, controlled degradation, and cell affinity. Also, these materials provide adherence to the biological tissue through covalent bonds [15]. The wide variety of polyurethane (PU) components and processing conditions, allow tailoring the adhesive formulations for the designed use [16]. Unfortunately, only biodegradable PU sealants are thoroughly discussed in the literature [14]. PU adhesives based on the functionalization of isocyanate with oxidized dextran [17], and polyether/polyester copolymers [18-20] were developed, but none proved to be suitable for AF repair. Contributions from specialists in biomaterials, biological and clinical areas are necessary to create a construct which will, upon implantation, provide immediate closure of the defect and maintain the mechanical properties of the disc. Only an interdisciplinary approach can address the highly complex problem of providing an intra-operative procedure which could lead to reduced re-herniation of repaired AF tissue and decrease long-term pain for patients [11].

This work aims to develop a PU-based adhesive capable of sealing small AF injuries from discectomy surgical procedures. Specifically, this study comprises the production of an injectable and *in situ* polymerizable sealant and the validation of its performance through the following design properties: tunable mechanics, preparation time, strong adherence to collagen, minimum swelling, low toxicity, high cell affinity, and adhesion. The researchers believe that these materials present interesting characteristics and will contribute to the newly emerged tissue adhesive technology.

2. Materials and methods

2.1. Materials

The polyols Eternacoll PH50 (500 g/mol), PH100 (1000 g/mol) and PH200 (2000 g/mol) were used as the aliphatic polycarbonate diol (PCD) and supplied by UBE Corporation Europe (Spain). The monomer 1,6-hexamethylene diisocyanate (HDI, 99+% purity) was purchased from Vencorex Chemicals (France). The commercial gelatin was supplied by Gelnex (Brazil). VERO (kidney epithelial cells African green monkey) and NIH/3T3 (murine fibroblast) cell lines were purchased from The American Type Culture Collection (ATCC-Rockville, Maryland, USA). All solvents were of analytical grade, and all other chemicals were used as received.

2.2. Synthesis and structure of urethane-based prepolymers

The urethane-based prepolymers were produced by the 'one-shot' method (in the absence of organic solvents) from reactions of the monomers 1,6-hexamethylene diisocyanate (HDI) and polycarbonate diol (PCD) with different molar masses (500, 1000, and 2000 g/mol). The reaction was performed by stirring the PCD and HDI monomers in a round-bottomed flask under a nitrogen atmosphere. The product was kept in an oven at 60 ºC under vacuum for at least 24 h. The prepolymers were formulated from 1.0–5.0 NCO/OH molar ratios according to Table 1. Only samples produced with an excess of isocyanate groups (–NCO) will exhibit adhesive characteristics. Fourier Transform Infrared spectroscopy in attenuated total reflectance mode (ATR-FTIR) confirmed the chemical structure of the prepolymers. The ATR-FTIR measurements were performed in a Perkin Elmer Impact 400 spectrometer from 4000−400 cm^{-1}, with 32 scans and 4 cm^{-1} resolution (diamond crystal at 45º). The free –NCO content was quantified through titration using dibutylamine according to ASTM D2572.

Table 1. Description of the polyurethane samples formulated

Sample	NCO/OH molar ratio	PCD molar mass (g/mol)
U500-1	1	500
U1000-1	1	1000
U2000-1	1	2000
U2000-1.2	1.2	2000
U2000-2	2	2000
U2000-3	3	2000
U2000-5	5	2000

2.3. Dynamical mechanical viability

The viscoelastic response of the fully polymerized PU samples establishes an important rule in the definition if the synthesized materials can support conditions similar to the native IVD. The dynamic-mechanical properties of U500-1, U1000-1 and U2000-1 samples were evaluated using a DMA 242C (Netzsch, Germany) in compression mode. Specimens with cylindrical shapes (15 mm diameter and 4 mm thickness) were tested. The measurements were performed at 37 °C in a linear viscoelastic region, under constant strain amplitude (± 30 μm) and 0.1–10 Hz frequency range. The experiments were repeated three times for each condition.

2.4. Polymerization reaction: kinetics parameters and rheology

Since the adhesive is intended to be applied in therapy, it becomes essential to know its (i) preparation time and (ii) final viscosity when the entire limiting reagent (–OH groups from PCD) of polymerization is consumed. The adhesive preparation time was simulated by monitoring the polymerization kinetics of samples U500-1, U1000-1, and U2000-1 using differential scanning calorimetry (DSC). The DSC experiments were performed with ~10 mg of sample in a DSC-50 Shimadzu under nitrogen atmosphere (50 mL/min). The materials were sealed in aluminum crucibles and heated from 25 to 180 ºC under nonisothermal conditions, using four different heating rates (5, 10, 15 and 20 ºC/min). The kinetic parameters of the reactions were computed using 'Netzsch Thermokinetics: A Software Modulus for the Kinetic Analysis of Thermal Measurements' [21]. The activation energy ($E_{\alpha(T)}$) values were determined through integral and differential model-free isoconversional methods. The Flynn-Wall-Ozawa (FWO) [22], Friedman (FR) [23] and Kissinger-Akahira-Sunose (KAS) [24] methods were used to establish the $E_{\alpha(T)}$ dependence with the conversion degree, α(T). The corresponding kinetic parameters were evaluated by a 'Multivariate Non-linear Regression' program, which uses a hybrid Marquardt-Levenberg approach. The best kinetic model was chosen by the least squares and *F-test* method. The kinetics model equations employed by the software are shown in supplementary files (Table S1).

The rheological profiles of the U2000-1 and U2000-2 materials were investigated in an Anton Paar MCR301 rheometer coupled with plate-plate geometry (diameter 25 mm, gap 1 mm). The isothermal experiments were performed within the linear viscoelastic regime (small stress 50 Pa) under the dynamic oscillation mode using 1.0 Hz frequency at 37.5 and 60 ºC.

2.5. PU- gelatin adhesion strength

The adhesion strength tests determine how strongly the sealant adheres to collagen and can be characterized by its resistance to traction when glued between two gelatin films [25, 26]. The adhesiveness of PU-based prepolymers was evaluated by tensile strength tests using a Universal Testing Machine Emic DL2000. The U2000-1.2, U2000-2, U2000-3 and U2000-5 samples were applied to the tip of gelatin films (7 cm × 2 cm) using a spatula, covering an area of approximately 4 cm^2. The gelatin films were prepared by a solution casting method (10 g of gelatin dissolved in 100 ml of distilled water) at room temperature. The tips of the bonded films were then pressed together at 37.5 °C for 36 h to allow attachment and polymerization of the adhesive to the substrate. The system was subjected to traction using 12 mm of grip-to-grip separation and 20 mm/min of crosshead speed. The possible reactions and intermolecular interactions between gelatin and PU materials were investigated by ATR-FTIR. The surface of the gelatin films in contact with the adhesive was immersed in dimethyl sulfoxide (DMSO) solvent to remove the urethane phase. The solvent treatment only extracted the non-bonded PU materials. Thus, the functional groups (urea and amide) formed by the reaction between the PU and gelatin were identified by means of ATR-FTIR.

2.6. Physico-chemical properties

The surface free energy of the PU-based adhesives and gelatin films was determined by the Owens–Wendt method, which is based on contact angle measurements conducted with certain standard liquids [27]. The wettability behavior of the solid specimens provides a better understanding of adhesion phenomenon and biocompatibility [28]. The contact angle measurements were carried out in an SEO® Phoenix100 (Korea) instrument and four probe liquids were employed at 23 ± 2 ºC: distilled water (γ_L^P = 51.0 mJ/m^2; γ_L^D = 21.8 mJ/m^2; γ_L = 72.8 mJ/m^2), glycerin (γ_L^P = 29.7 mJ/m^2; γ_L^D = 33.6 mJ/m^2; γ_L = 63.3 mJ/m^2), dimethyl sulfoxide (γ_L^P = 8.0 mJ/m^2; γ_L^D = 36.0 mJ/m^2; γ_L = 44.0 mJ/m^2) and hexadecane (γ_L^P = 0.0 mJ/m^2; γ_L^D = 27.6 mJ/m^2; γ_L = 27.6 mJ/m^2); where γ_L^P, γ_L^D and γ_L represent the polar component, the dispersive component and the surface free energy of the liquids, respectively [29]**.** The sessile drop method was adopted using 2 μL drops. The contact angle was measured at least ten times at different sites on the surface for the consideration of the average value.

Information about the swelling behavior of the adhesive is needed to prevent any damage to the surrounding tissues due to a volume variation. The U2000-1 sample was primarily dried until constant weight at 60 °C under vacuum conditions (W_s being the weight of the dry

sample) to assess its water sorption capacity. The dried sample was then placed in a container with a saturated solution of pentahydrated copper sulfate and weighted at different times through the mass gain until reaching a maximum weight (W_d). The water uptake (WU) was calculated using Eq. (1) [30].

$$\mathrm{WU} = \left(\frac{W_s - W_d}{W_d}\right) x\ 100\% \quad (1)$$

Since the adhesives containing isocyanate end groups present the ability to react with air moisture, it is crucial to determine how their reactivity will influence the manipulation or storage events. The stability of the –NCO groups was evaluated by maintaining the U2000-2 sample in a sealed container under a water saturated atmosphere. The ATR-FTIR band at 2260 cm^{-1} was monitored at different intervals until the –NCO groups were no longer detected, attesting the end of the reactivity of the material.

2.7. *In vitro* cytotoxicity and cell adhesion

For adhesives to be used *in vivo*, its presence should not elicit any deleterious effect on cells functions. To assess cell viability, the VERO and NIH/3T3 cell lines were evaluated by their metabolically active mitochondria using an MTT assay. The cells were cultured in Dulbecco's modified eagle medium with 10wt.% fetal bovine serum (FBS) at a temperature of 37 °C, minimum relative humidity of 95%, and an atmosphere of 5wt.% CO_2 in air. The PU-based materials were previously sterilized by UV light for 30 min. The cell lines were incubated with U2000-1 and U2000-2 samples by elution methods (3 cm^2/mL), as recommended by ISO 10993 (2009). The results are expressed as the percentage of cell viability in relation to the positive control. The MTT experiments were performed three times in triplicate. To verify cell adhesion, the materials were held in the deep 24-well plate, and the cells were seeded on the materials at the density of $15–20\times10^3$ cells per well, and cultured for 24, 48, and 72 h. Cell morphology and adhesion were assessed by Field Emission Scanning Electron microscopy (FEG-SEM) in a Mira 3 Tescan (Czech Republic) microscope. Before FEG-SEM evaluation, the materials were fixed with 2.5% glutaraldehyde, sequentially dehydrated in increasing concentrations of ethanol (50, 70, 85, 95 and 100%) and then coated with gold using a plasma sputtering apparatus. Data from *in vitro* cytotocixity experiments were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc

test, using Graph-Pad Software (San Diego, USA). Results are reported as the mean ± standard deviation (SEM). $P < 0.05$ was indicative of statistical significance.

3. Results and discussion

3.1. Urethane-based prepolymers production and characterization

The prepolymers were produced from PCD and HDI monomers using an excess of –NCO groups ($1 \leq NCO/OH \leq 5$) to ensure that part of them was free to react with biological tissue (see Scheme S1 in supplementary files). The prepolymer structure was qualitatively examined by ATR-FTIR technique (see Fig. S2 in supplementary files). The formation of the adhesive was confirmed by the decrease in intensity of the isocyanate (2261 cm^{-1}) and the disappearance of the hydroxyl (3489 cm^{-1}) bands. When the adhesive loses its reactivity, which can be attested by the disappearance of the 2260 cm^{-1} band, new peaks appear around 3328 cm^{-1} (N–H stretching), 1242 cm^{-1} and 1581 cm^{-1} referring to the urethane group. The band at 1242 cm^{-1} (C–O stretching from carbonate group) is also found on the PCD spectrum [31-33].

3.2. PU-based adhesive as an annulus fibrosus sealant

3.2.1. Dynamical mechanical response

The mechanical viability of the materials was evaluated through the compressive modulus, by simulating slightly higher efforts than typical physiological loading [34]. Fig. 1 shows the storage modulus of the PUs synthesized from different polyols as a function of frequency. Frequencies at 1−10 Hz are usually observed in common daily activities [35]. All samples showed a linear increase of storage modulus with frequency and also a pseudo-solid like behavior in these experimental conditions. This behavior concerning storage curves has already been reported for an *ex vivo* sheep model at 0.1–10 Hz frequency range [36].

The average compressive modulus of the U500-1, U1000-1, and U2000-1 samples was 12.4 ± 3.6 MPa, 5.0 ± 2.4 MPa, and 3.1 ± 2.3 MPa, respectively. These values are superior to similar tests performed on native IVD tissue [34]. The compressive moduli of U2000-1 sample seemed to be the closest of the IVD outer region (0.22−0.54 MPa) [37, 38], and thus the most adequate for restoration. So, only this sample proved viable for future analyses.

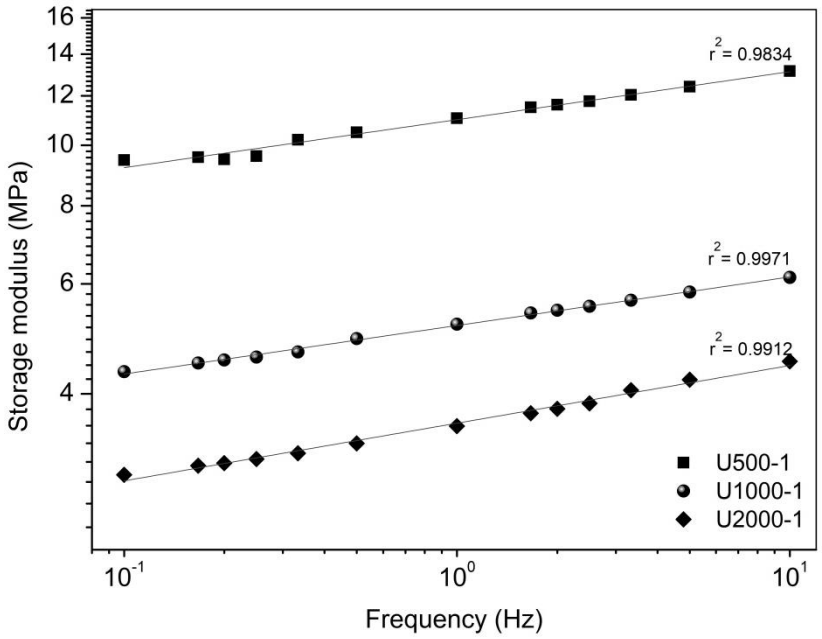

Fig. 1. Storage modulus for U500-1, U1000-1 and U2000-1 solid samples

3.2.2. Reactivity with gelatin film

An AF repair sealant must firmly adhere to the native tissue to resist stresses generated from IVD intradiscal pressures during physiologic loading [39]. This adhesiveness is an essential requirement for clinical translation [12]. The tensile strength results were not dependent on the NCO/OH molar ratio, and consequently on the amount of free –NCO groups. As a result, no clear difference could be discerned between the formulations tested. A tensile strength magnitude of 21.31 ± 3.06 MPa was reached in the rupture of gelatin films by fracture, not by detachment. Here gelatin was used to simulate the living tissue [25, 40]. In all tests, the glued area in the specimens remained intact, which indicates the excellent adhesive capacity of the material. For a sealant to be effective in restoring the AF, it must have a tensile strength of about 4.0–8.0 MPa, and an adhesion force of 177 kPa [41, 42].

Free –NCO contents higher than NCO/OH = 1.2 did not contribute to adhesiveness because gelatin itself has limited reactivity. Gelatin contains approximately 31−35 free amine groups and 77−118 carboxylic acids per 1000 amino acids depending on the pre-treatment received during its production [43, 44]. Besides increasing toxicity, high concentrations of free –NCO groups also significantly affect the viscoelastic behavior of the material. Low concentrations of free –NCO produce high viscosity prepolymers, which difficult the gel penetration in the incision to be sealed. Prepolymers produced with high –NCO contents, however, have low viscosity and flow through AF adjacent tissues. The U2000-2 proved to be

the most appropriate sample when considered the parameters injectability and adhesiveness. This sample presented 8−9% of free –NCO groups according to titration analysis.

To advance tissue adhesive technology, understanding the physicochemical interactions at the collagen/biomaterial interface is indispensable. From ATR-FTIR spectroscopic evaluation, the probable bonding mechanism between the polymer and gelatin functional groups can be monitored [45, 46]. It is assumed that the free isocyanate groups of the adhesive will react with the $–NH_2$, –OH, –COOH or –NHCO− groups presented in sulfated glucosaminoglycans IVD tissue, yielding urethanes, ureas, and amides functional substances, as well as biuret and allophanates secondary products [25, 47]. Fig. 2 shows the spectra of gelatin, U2000-2 adhesive, and the gelatins surface in which the glued PU was solvent extracted. The spectroscopic results corroborated previous evaluations of the geltin/PU bonding chemistry [43, 48, 49]. Fig. 2 attests the reaction of the carboxylic group of gelatin by the narrowing of the broad 3700−2500 cm^{-1} region (related to the carboxylic group asymmetric stretching) in the spectrum of the extracted gelatin film [50, 51]. Bands at 3326, 1620, 1570, 1242, 732−790, 620 cm^{-1} corresponding to urea and amide groups, namely N–H stretching vibration, amide I, amide II, amide III, amide IV, and amide V, respectively, were detected [52]. The C=O groups of the urethane bonds appear at about 1730 cm^{-1} and 1740 cm^{-1} in the adhesive and extracted gelatin film spectra, respectively [53]. Since there is no peak at 2260 cm^{-1} it may be said that all free –NCO groups from the adhesive have reacted [54]. Therefore, these results prove the presence of urea and amide functional groups on the gelatin/adhesive glued interface [49]. It is suggested that only a strong covalent bond between gelatin and adhesive could explain the films breakage rather than its detachment when subjected to traction.

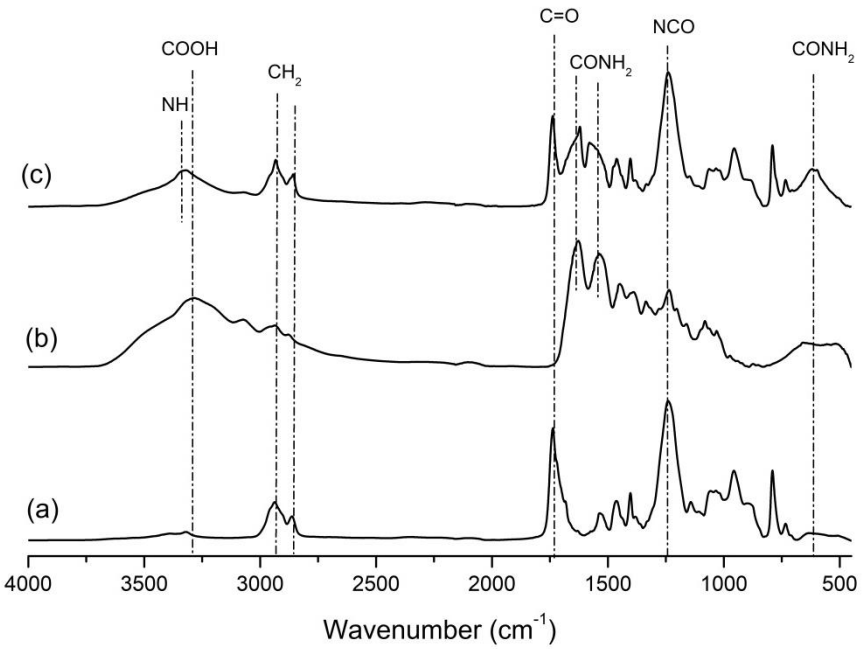

Fig. 2. ATR-FTIR spectra of: (a) U2000-1, (b) gelatin and (c) extracted gelatin film

3.2.3. Behavior when in contact with water

Figuring out how biomaterial behaves in an aqueous environment is essential to predict their stability under physiological conditions. The affinity of biological molecules for an implanted scaffold is profoundly affected by the nature of the biomaterial surface [55]. Increased adhesive wettability also improves implant tissue integration. The Owens-Wendt theory determines the polar and dispersive contributions to the surface free energy of a solid using the known polar and dispersive components of the probe liquids and their contact angles with the solid [56]. The higher the surface free energy value, the higher the adhesiveness and bioactivity of a biomaterial [25]. The adhesive and the gelatin film presented total free surface energies about 61.8 and 46.2 mN/m, respectively (Table 2), which is in agreement with the literature values [25, 32]. The surface tension of blood assessed in a group of 150 healthy people (72 men and 78 women aged from 20 to 65 years) by the drop-weight method at a temperature of 22 ºC was 63.8 mN/m [57]. As these substrates present near-surface energy values, it can be inferred that the adhesive will present good spreadability when in contact with the biological tissue [25]. The polar component of the PU-based adhesive is significantly higher than its dispersive element, which explains the weak cohesion forces in this polymer and why the adhesion forces are preferred [25].

Table 2. Contact angles (θ, ºC) and surface free energy (γ_S) of the solid surfaces, including dispersive (γ_S^D) and polar (γ_S^P) contributions

Solid surfaces	**Contact Angle (θ, ºC)**				**Surface free energy (mN/m)**			**R^2**
	Water	Glycerin	Dimethyl sulfoxide	Hexadecane	γ_S	γ_S^D	γ_S^P	
U2000-1	74.8 ± 0.8	88.6 ± 0.8	44.6 ± 0.8	13.9 ± 0.8	61.8	10.5	51.3	0.9872
Gelatin film	87.1 ± 0.8	80.7 ± 0.8	51.3 ± 0.8	17.2 ± 0.8	46.2	6.4	39.9	0.9935

An excessive volume increase of the material when in contact with the physiological environment may damage the surrounding tissues [25]. The adhesive presented a low swelling ratio of approximately 1.03 ± 0.06%, which suggests that there will not be a significant volume increase capable of preventing its use. However, the adhesive will continue to present some hydrophilicity, which ensures its biocompatibility through interactions between the hydrated network and tissue proteins [25].

Analyzing the stability of –NCO groups under humidity conditions is of paramount importance when considering the ability of urethanes to favor the adhesion with living tissues [25]. When exposed to a saturated water atmosphere, the free isocyanate end groups of the pre-polymer react with moisture, leading to the formation of an unstable carbamic acid that decomposes to carbon dioxide and an aminic ending polymer [47]. Further reactions with additional –NCO groups results in urea substances and their secondary products [58]. Although this moisture reaction phenomenon will necessarily occur in the living tissues, it is essential to avoid it while the adhesive is only being manipulated or stored. The stability of the –NCO groups was monitored by ATR-FTIR technique, through the evolution of the peak at 2260 cm^{-1} relative to the free isocyanate. After 24 h under moisture atmosphere, almost 10 mol% of the –NCO groups reacted. On the seventh day, this percentage increased to 51 mol%. The conversion rate of the –NCO group decelerates over time. Initially, few monomers are polymerized, and the viscosity of the reactional medium is rather low, which allows reactants to flow and mix quickly. As the polymerization progresses, the medium becomes more viscous and the reaction rate decreases [59]. The total reaction of the isocyanate groups with water occurred after 18 days. When in contact with the living tissues, however, it is expected that *in situ* polymerization of the adhesive occurs much faster since the isocyanate reactivity with amines ($–NH_2$) is 1000 times faster than its reactivity with water and a primary hydroxyl group. Moreover, the reaction between –NCO and amino groups is

thermodynamically favorable to the ambient temperature and does not need to be catalyzed [50, 60].

3.2.4. Preparation conditions for clinical use

Here we discuss two parameters involved in the preparation of adhesives: (i) time between intended use and application, which was simulated by mathematical modeling of the DSC measurements, and (ii) viscosity, which is directly related to the injectability of the prepolymer. Concerning the time of preparation, it is essential to consider the type of clinical procedure to which the adhesive's application is linked. Microdiscectomy, because it is an elective procedure, allows for planned and previous preparation of the adhesive. The polymerization kinetic mechanism indicated in the DSC results determined the adhesive preparation time. Fig. 3 shows the dependence of the $E_{\alpha(T)}$ *vs.* $\alpha_{(T)}$ for U2000-1 material. $E_{\alpha(T)}$ values were determined using the FWO, FR and KAS isoconversional model-free methods, and presented a practically constant value ~49 kJmol. These results are in agreement with the literature for polymerizations reactions of PU-based prepolymers [61]. The constant behavior of $E_{\alpha(T)}$ *vs.* α (T) curves suggests that polymerization is limited by a single step process [62]. Also, the $E_{\alpha(T)}$ values provided by the FWO, FR, and KAS methods were quite close, proving the accuracy of the models. The $E_{\alpha(T)}$ values found for the U500-1 and U1000-1 remained between 55−60 kJ/mol.

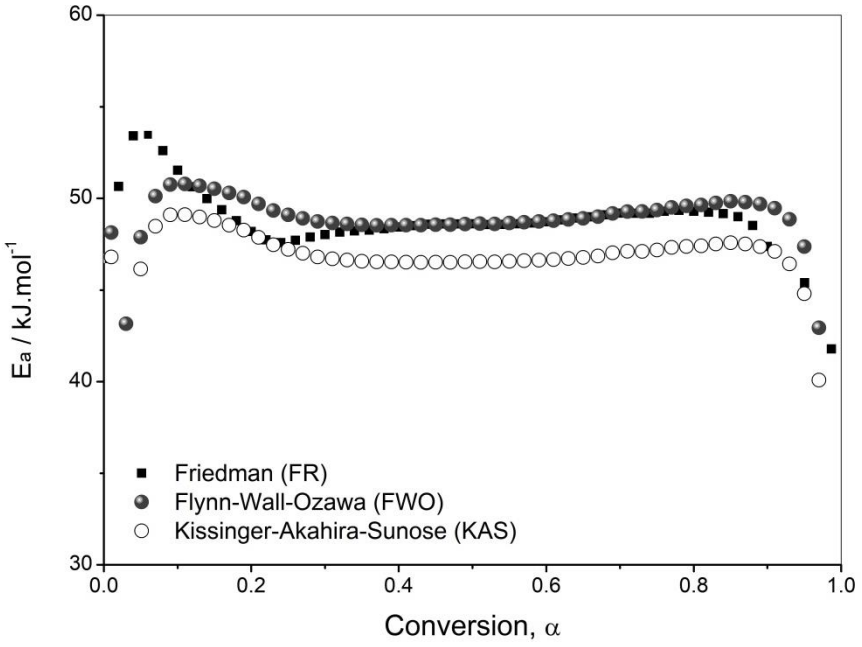

Fig. 3. Dependence of the $E_{\alpha(T)}$ vs. α(T) for U2000-1 sample

Then the nonisothermal kinetic parameters were investigated through multivariate non-linear regression (see Table S1) using the DSC data obtained at different heating rates. The accuracy of the 18 kinetics models adopted for determining the kinetic parameters was validated using F statistical test. Considering the correlation coefficient (r) and *F-test* values given in Table 3, it could be concluded that the model proposed by Prout-Tompkins (B_{na} mechanism) was the most appropriate for describing the polymerization mechanism. This model presented *r* and *F-test* values close to 1, and an activation energy (Ea) values nearest to those found in the FWO, FR, and KAS models.

Fig. 4 shows a comparison between results obtained experimentally by DSC and those simulated mathematically by the B_{na} model. An almost perfect overlapping between experimental and theoretical data was observed, suggesting that B_{na} model can satisfactorily describe the polymerization kinetics of the adhesive. It is not surprising that this model has been the most appropriate since it is known in the literature for describing autocatalyzed (sigmoidal shape) reactions [61]. The polymerization of urethane materials is based on an equilibrium reaction of isocyanate and alcohol chemical groups that has an autocatalytic behavior [61]. The autocatalysis phenomenon occurs when the products catalyze the reaction [63].

The non-isothermal results obtained from B_{na} model were then used in simulations to predict an isothermal polymerization behavior. Fig. 5a shows the effect of temperature on the reactional conversion and, consequently, on the preparation time of the adhesives. It can be observed that U2000-1 sample needs ~600 min at 60 ºC to reach a full conversion. In this condition, the material displays a viscosity of 461.3 Pa·s (liquid-like behavior) (Fig. 5b) and can be injected using a needle-free syringe of 12.34 mm inner diameter [64]. Sample U2000-2 was expected to be less viscous than sample U2000-1; however, this behavior was not observed. This sample may have suffered dimerization [47] and also reacted with the humidity in the rheometer. At 37 ºC, the U2000-1 material polymerizes slowly, and a 461.3 Pa·s viscosity is only achieved within 1097 min of the reaction (results not shown).

Table 3. Nonisothermal kinetic parameters obtained from nonlinear regression method

f(α)	***Ea (kJ.mol^{-1})***	***log A (s^{-})^{1}***	***Correlation coefficient (r)***	***F-$_{test}$***
B_{na} (*n = 1.2042; a = 0.3485*)	49.39	4.20	0.9867	1.00
C_{nB} (*n =1.5372; log K_{cat} = 0.6678*)	50.21	3.78	0.9830	1.35
F_n (*n = 1.0715*)	64.89	5.97	0.9759	2.24
F_2	90.11	9.33	0.9442	3.60
A_n (*n = 1.2915*)	48.39	3.84	0.9805	1.49
F_1	62.60	5.67	0.9762	2.19
C_{1B} (*log K_{cat} = 6.83E-3*)	51.70	4.14	0.9768	2.05
D_3	132.76	13.43	0.8202	10.76
D_{3F}	131.04	13.05	0.8053	11.23
D_{1F}	112.47	10.88	0.7982	12.79
R_3	51.80	3.74	0.9596	3.44
A_2	33.50	1.90	0.8853	4.72
R_2	46.80	3.24	0.9316	4.95
D_4	124.27	12.29	0.7675	14.13
D_2	98.74	9.73	0.5243	26.55
A_3	23.89	0.65	0.6470	13.53
D_1	103.85	10.45	0.5591	25.76
B_1	-1.40	0.00	-0.5799	37.44

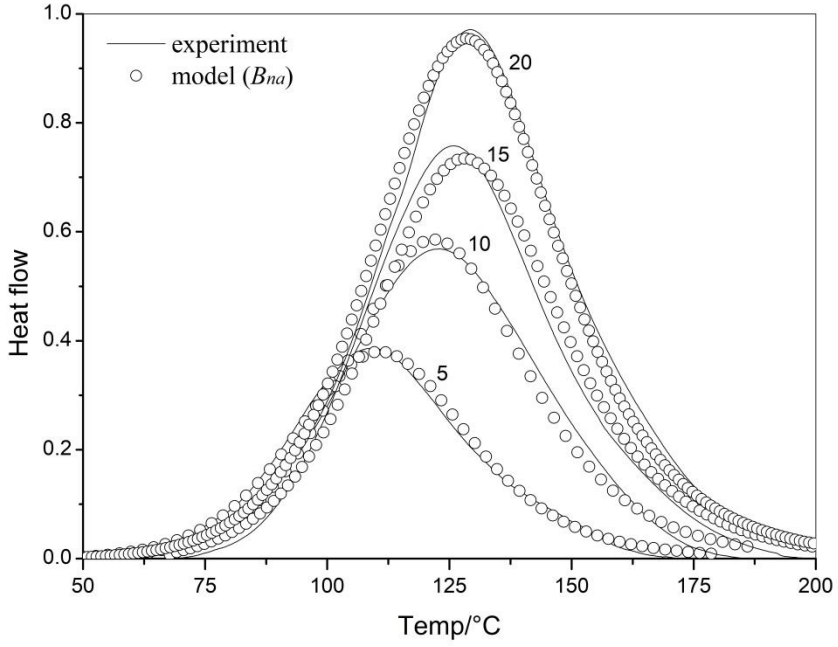

Fig. 4. Model prediction of U2000-1 polymerization reaction using Prout-Tompkins (B_{na}) n-th order approach. The different heating rates (in °C/min) employed at DSC experiments were indicated in each curve.

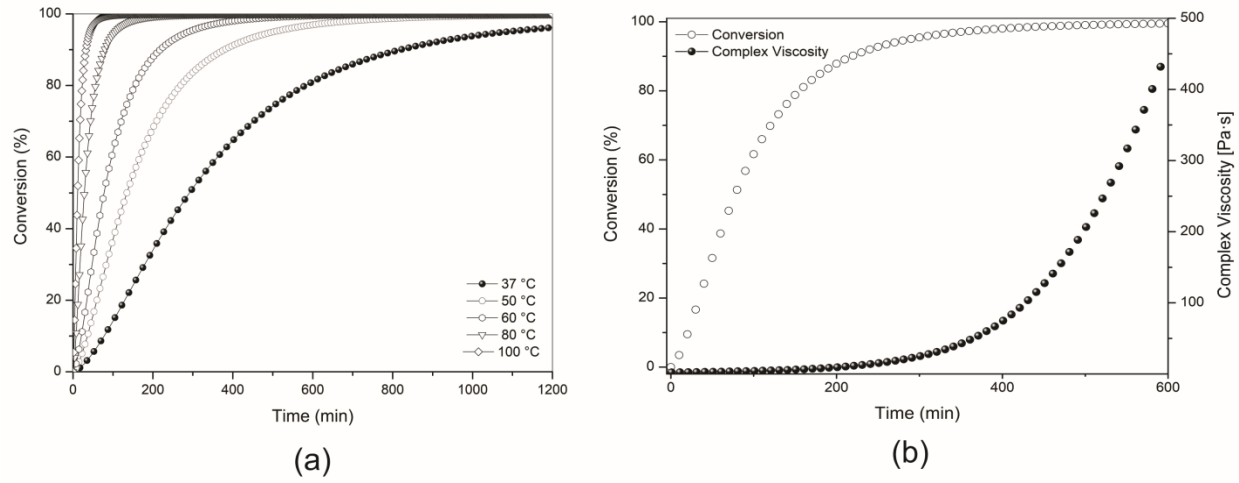

Fig. 5. (a) U2000-1 reaction simulation at different temperatures and (b) U2000-1 rheological profile as a function of conversion

3.2.5. In vitro cytocompatibility

The cytotoxic effects of the adhesives were evaluated by MTT assay using NIH/3T3 and VERO cell lines. The U2000-1 (non-reactive sample) and U2000-2 (adhesive) materials were tested and compared to the influence of free –NCO groups on cell response. The solid U2000-1 did not display any cytotoxicity in fibroblast NIH/3T3 and VERO lineages in up to 72 h, as shown in Fig. 6a. When the sample containing reactive isocyanate end groups was added to the medium, significant cytotoxicity was observed for VERO cells, being this effect less pronounced for NIH/3T3 cells (Fig. 6b). Interestingly, this inflammatory response coincides with the time required by the prepolymer to react, as revealed by the kinetic analysis. During polymerization, the pH of the biological environment drops due to the formation of carbamic acid, as well as urethane and urea fragments with terminal acid groups, negatively impacting cell viability [48, 65, 66]. The CO_2 generated during the *in situ* reaction might also harm cell survivability. Guo *et al*. (2015) [67] cytoprotected cells during polymerization by encapsulating them in alginate beads to provide a barrier to CO_2 diffusion. Cells survived the polymerization at > 70% viability, and rapid dissolution of alginate beads after the scaffold cured created interconnected macropores that facilitated cell adhesion to the biomaterial *in vitro*. Moreover, any residual monomer released from the underpolymerized material is incorporated into the lipid bilayer of the cell membrane, causing its solubilization and death [66, 68, 69]. Nevertheless, enhancement of cell viability after 48 h was denoted for both VERO and NIH/3T3 lineages, a phenomenon already reported earlier in the literature [54, 70, 71]. Although the non-polymerized adhesive initially moderately affects cell viability,

once the *in situ* polymerization occurs, this toxicity tends to disappear. As no direct relationship between the excess of isocyanate and adhesiveness was experimentally observed, the use of lower levels of this monomer will be preferable to ensure cell survival and growth [43, 54].

The cell adhesion affinity by the U2000-2 was investigated in 24, 48 and 72 h post-seeding NIH/3T3 fibroblasts deposited onto the material surfaces (Fig. 7). In the first 24 h of culture, the fibroblast showed a rounded morphology (d_i= 1.04 ± 0.1 µm) and some adherent contact points with the adhesive surface [72]. Favorable cell adhesion and growth pattern toward the adhesive were observed for 48 and 72 h, with extensive releasing of extracellular matrix (ECM) [71]. This biological mechanism is linked to the chemical nature and surface properties of the polyurethane substrate, which confirms the surface free energy results [71]. The high superficial energy from the polar chemical groups on the PU-based adhesive surface favors fibroblast affinity and attachment [73-75]. Moreover, the polyurethane surface supports cell spreading due to the presence of its hard segments, which are distributed throughout the soft domains. The high elasticity of the PU microstructure can tolerate the tension forces imposed by the cells [76].

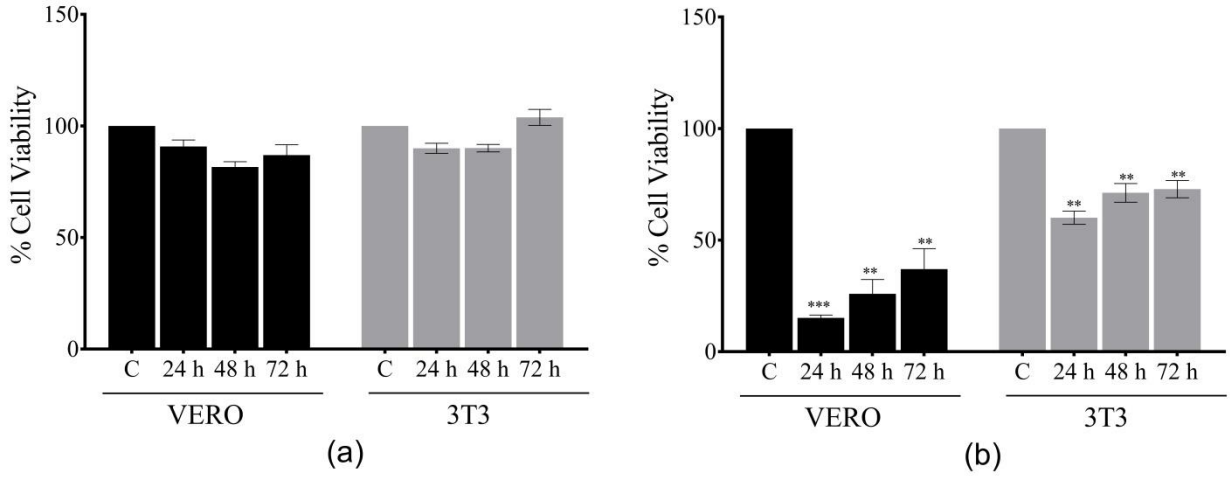

Fig. 6. Effect of incubation with (a) U2000-1 and (b) U2000-2 on cell viability of NIH/3T3 fibroblast and VERO cells after 24, 48 and 72 h. Each column represents the mean ± SEM. *p<0.05 versus control (C).

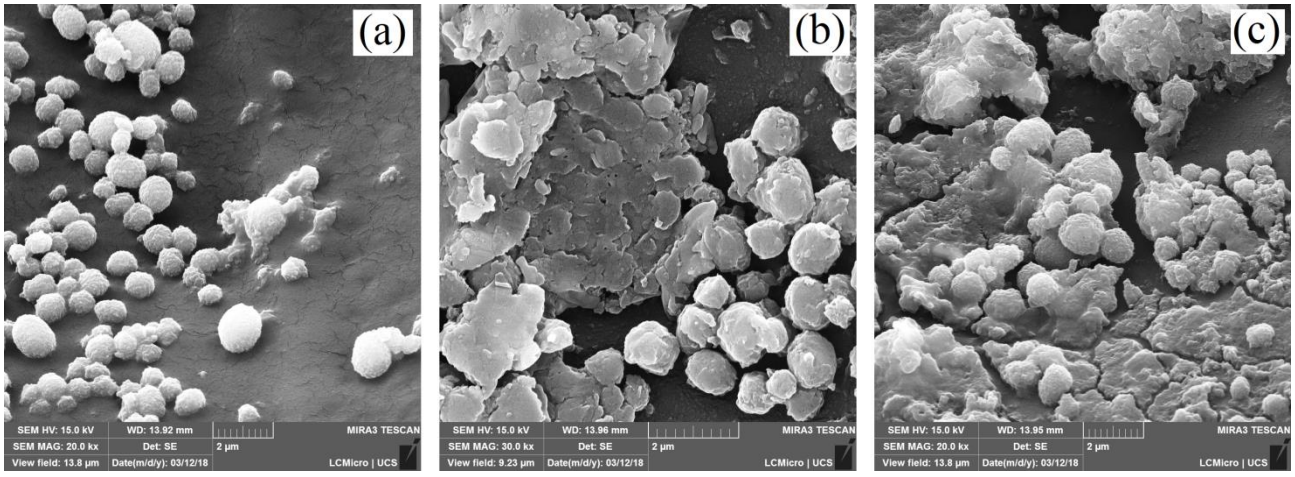

Fig. 7. Fibroblasts NIH/3T3 adhesion in U2000-2 at (a) 24, (b) 48 and (c) 72 h (FEG-SEM magnification 20.0 kx)

4. Conclusion

A PU-based tissue adhesive for annulus fibrosus repair has been evaluated through physicochemical, mechanical, kinetic and rheological, and cytotoxic testing. The outstanding features of U2000 prepolymer result from its dynamic compress behavior, excellent adhesiveness to gelatin, minimum swelling, and injectability characteristic. This adhesive bounded gelatin covalently without the use of a catalyst or initiator. The U2000-2 formulation presented a shelf life stability of 18 days and required 10 h of preparation time at 60 ºC before use. This material also showed a moderate cytotoxic effect for NIH/3T3 fibroblasts in the first 24 h, which disappeared after polymerization. Favorable cell adhesion and proliferation toward the adhesive were observed after 48 h of culture. These findings provide evidence of the U2000 suitability. Additional long term studies are being planned to decrease the storage module, and experiments in animals with a degenerative precondition should be conducted.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors acknowledge financial support from the Brazilian Agency *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)* through a scholarship to Lucas Dall Agnol and a post-doctoral fellowship to Fernanda Dias. The authors also acknowledge FINEP (01.13.0359.00) and Laboratório Central de Microscopia Prof. Israel Baumvol for FEG-SEM

analysis. We would like to thank Dr. Jaderson Costa da Costa (Brain Institute of Rio Grande do Sul -BraIns) for the assistance in cytotoxicity experiments. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico- CNPq, Brazil (308241/2015-0).

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SUPPLEMENTARY FILES

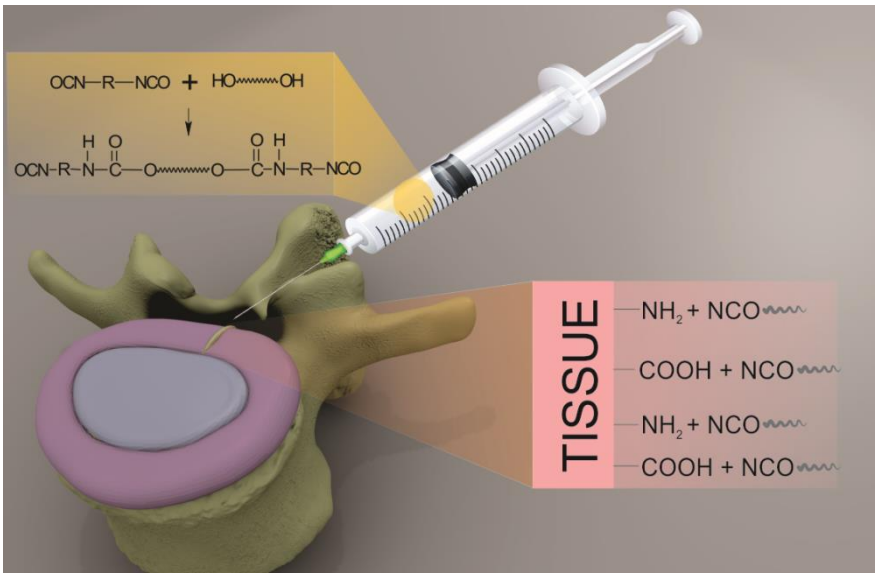

Fig. S1: Schematic illustration of the adherence mechanism between tissue and PU-based adhesive

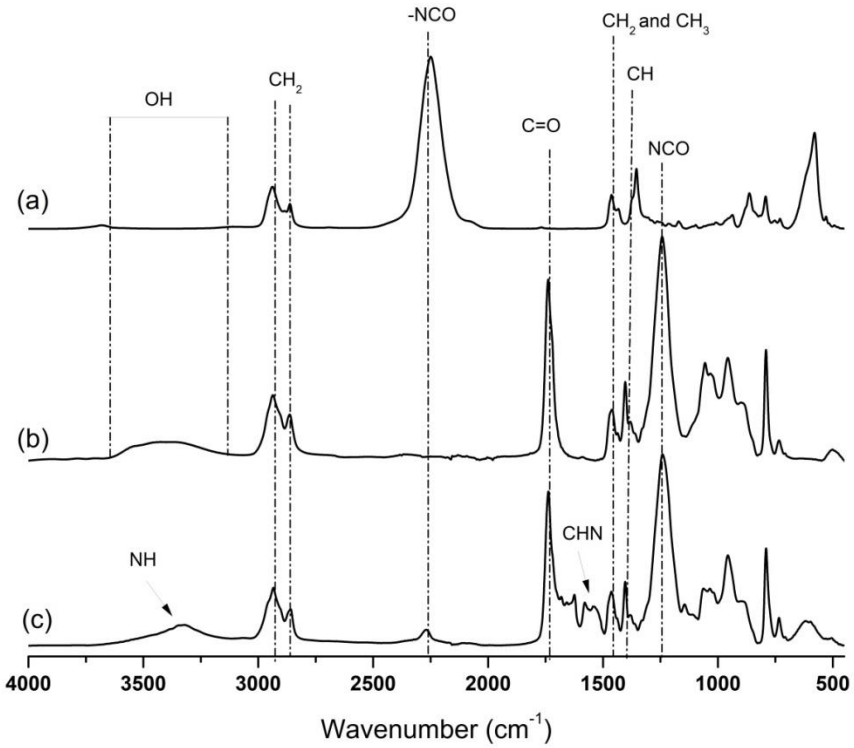

Fig. S2: ATR-FTIR spectra of: (a) HDI, (b) PCD monomers and (c) urethane-based prepolymer (U2000-2)

Table S1. Reaction model types and corresponding reaction equations $de/dt = -A \exp(E/RT) f(e, p)$ of Netzsch Thermokinetics software

Code	***f(e, p)***	**Reaction type**
F_1	e	First-order reaction
F_2	e^2	Second-order reaction
F_n	e^n	n^{th}-order reaction
R_2	$2e^{1/2}$	Two-dimensional phase boundary reaction
R_3	$3e^{2/3}$	Three-dimensional phase boundary reaction
D_1	$0.5/(1 - e)$	One-dimensional diffusion
D_2	$-1/\ln(e)$	Two-dimensional diffusion
D_3	$1.5e^{1/3}/(e^{-1/3} - 1)$	Three-dimensional diffusion (Jander's type)
D_4	$1.5/(e^{-1/3} - 1)$	Three-dimensional diffusion (Ginstling–Brounstein type)
B_1	ep	Simple Prout–Tompkins equation
B_{na}	$e^n p^a$	Expanded Prout–Tompkins equation (na)
$C_{1\text{-}X}$	$e(1 + K_{cat}X)$	First-order reaction with autocatalysis through the reactants, X $X = a$ product in the complex model, frequently $X = p$
$C_{1\text{-}X}$	$e^n(1 + K_{cat}X)$	n^{th}-order reaction with autocatalysis through the reactants, X
A_2	$2e(-\ln(e))^{1/2}$	Two-dimensional nucleation
A_3	$3e(-\ln(e)^{2/3}$	Three-dimensional nucleation
A_n	$ne(-\ln(e))^{(n-1)/n}$	n-dimensional nucleation/nucleus growth according to Avrami–Erofeev

Where A is the pre-exponential factor; E is the activation energy; R is the gas constant; T is the temperature; α is the conversion degree; e is the starting concentration of the reactant ($e = 1 - \alpha$), and p is the concentration of the final product ($p = \alpha$).

5 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Dos resultados discutidos neste trabalho, pode-se concluir que os fatores de maior influência no desenvolvimento de um biomaterial para o reparo do AF são: similaridade mecânica e reatividade com o tecido nativo, citocompatibilidade, tempo de preparo e facilidade de manuseio. Tais fatores foram exaustivamente abordados na revisão sistemática "*Polyurethane as a strategy for annulus fibrosus repair and regeneration: a systematic review",* submetida no periódico Regenerative Medicine em abril de 2018. Dos dezesseis estudos incluídos nesta revisão, apenas dois avaliaram a aplicação *in vivo* do biomaterial à base de PU. A maior parte dos trabalhos experimentais foi conduzida *in vitro*, sem a preocupação de se considerar a arquitetura e o microambiente nativo do disco.

Apesar de exibirem propriedades mecânicas capazes de suportar e estimular a proliferação celular, estes implantes ainda encontram limitações quanto à aderência ao tecido do disco e possibilidade de migração. Neste caso, uma solução viável seria o uso de um implante injetável que permitisse o selamento imediato do defeito e, ao mesmo tempo, a formação de um tecido funcional. Apenas dois trabalhos na literatura reportam o uso de colas à base de PU no reparo do anel fibroso. Além disso, todos os dispositivos de selamento do AF produzidos até o momento baseiam-se em materiais degradáveis (~6 semanas). Entretanto, a escolha por um selante longevo (de baixa degradabilidade) parece ser a opção mais adequada no caso do AF, região de baixo potencial regenerativo. Desta forma, a proposta do presente trabalho consistiu em desenvolver um bioadesivo poliuretânico injetável e polimerizável *in situ* para atuar de forma eficiente no selamento de incisões do AF.

Os bioadesivos produzidos foram caracterizados quanto à sua estrutura química, tempo de preparo, comportamento viscoelástico, resistência adesiva PU-colágeno (gelatina), características superficiais (molhabilidade e inchamento) e viabilidade/adesão celular. O pré-polímero U2000 foi o material que apresentou as propriedades mais interessantes e, quanto ao seu desempenho, é possível destacar: (i) elevada resistência à compressão dinâmica; (ii) excelente adesividade à gelatina (interação por ligações covalentes sem a necessidade de catalisador ou iniciador); (iii) inchamento mínimo (<1% de alteração volumétrica); (iv) injetabilidade

comprovada reologicamente; (v) vida útil de 18 dias e 10 hs de preparo a 60 ºC, quando formulado com uma razão molar NCO/OH de 2 e (vi) proliferação e adesão celular favorecidas a partir de 48 hs de contato com fibroblastos NIH/3T3.

Apesar de promissor, o material U2000 ainda necessita de otimização em sua formulação, bem como de testes adicionais referentes aos seus efeitos citotóxicos no local de implantação. O uso de um extensor de cadeia ou monômero trifuncional na pré-formulação deste adesivo, por exemplo, contribuiria para a obtenção de módulos elásticos mais próximos ao do disco e para a redução do tempo total de polimerização. Um método eficiente para a esterilização deste biomaterial também necessita ser desenvolvido. Além disso, ensaios futuros devem investigar a resistência adesiva, de compressão e de falha do material por meio de um ensaio biomecânico com modelo *ex vivo*, com a finalidade de prever o comportamento do implante ao longo do tempo. A cultura deste material com células isoladas de DIV's de humanos também forneceria informações a respeito da sua biocompatibilidade *in vivo*. Estes adesivos ainda podem ser combinados com fármacos, fatores de crescimento ou terapias celulares para atuarem na regeneração de defeitos maiores no DIV.