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In vitro and biomechanical screening of polyethylene glycol and poly(trimethylene carbonate) block copolymers for annulus fibrosus repair

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Abstract

Herniated intervertebral discs (IVDs) are a common cause of back and neck pain. There is an unmet clinical need to seal annulus fibrosus (AF) defects, as discectomy surgeries address acute pain but are complicated by reherniation and recurrent pain. Copolymers of polyethylene glycol with trimethylene carbonate (TMC) and hexamethylene diisocyanate (HDI) end-groups were formulated as AF sealants as the HDI form covalent bonds with native AF tissue. TMC adhesives were evaluated and optimized using the design criteria: stable size, strong adherence to AF tissue, high cytocompatibility, restoration of IVD biomechanics to intact levels following *in situ* repair, and low extrusion risk. TMC adhesives had high adhesion strength as assessed with a pushout test (150 kPa), and low degradation rates over 3 weeks *in vitro*. Both TMC adhesives had shear moduli (220 and 490 kPa) similar to, but somewhat higher than, AF tissue. The adhesive with three TMC moieties per branch (TMC3) was selected for additional *in situ* testing because it best matched AF shear properties. TMC3 restored torsional stiffness, torsional hysteresis area and axial range of motion to intact states. However, in a failure test of compressive deformation under fixed 5° flexion, some herniation risk was observed with failure strength of 5.9 MPa compared with 13.5 MPa for intact samples; TMC3 herniated under cyclic organ culture testing. These TMC adhesives performed well during *in vitro* and *in situ* testing, but additional optimization to enhance failure strength is required to further this material to advanced screening tests, such as long-term degradation. Copyright © 2016 John Wiley & Sons, Ltd.

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1. Introduction

Discectomy is the most effective surgical procedure to treat lower back pain relating to intervertebral disc (IVD) herniation (Asch *et al.*, 2002; Gray *et al.*, 2006; Weinstein *et al.*, 2008). This procedure consists of removing herniated and loose nucleus pulposus (NP) tissue through a small incision in the annulus fibrosus (AF). Defects in the AF due to herniation may be worsened during discectomy procedures, and this likely alters IVD biomechanical behaviours and could accelerate IVD degeneration. Incisions in the AF change the biomechanics of the IVD (Elliott *et al.*, 2008; Masuda *et al.*, 2005; Michalek and Iatridis, 2012), especially affecting torque range, disc height and neutral zone characteristics (Iatridis *et al.*, 2013). Discectomy procedures also have a risk of reherniation requiring revision surgery in up to 27% of the patients, depending on the size of the AF defect and

the amount of tissue removed (McGirt *et al.*, 2009; Watters and McGirt, 2009).

Currently, there are no widely used and clinically available AF sealants. The development of AF sealants is an active area of research with proposed solutions ranging from hydrogels to mechanical closures devices such as sutures and hardware (Guterl *et al.*, 2013). A full list of injectable AF repair solutions was reviewed (Long *et al.*, 2016), and all have some limitations associated with biological or mechanical incompatibility. Non-injectable AF repair solutions include sutures that do not restore intradiscal pressure (Ahlgren *et al.*, 2000) or that have been discontinued (Bailey *et al.*, 2013), and plugs that have a risk of extrusion (Bron *et al.*, 2010). The Barricaid (Intrinsic Therapeutics, Woburn, MA, USA) is a shield-like structure (Wilke *et al.*, 2013) that allowed retention of NP material and reduced risk of facet degeneration in a clinical trial (Trummer *et al.*, 2013), yet this device adds difficulty to the discectomy procedure, disrupts the endplate, and does not seal the AF defect. Considering available AF closure solutions, there remains a continued need for an effective and biologically compatible AF adhesive.

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An adhesive based on polyethylene glycol (PEG) with trimethylene carbonate (TMC) and hexamethylene diisocyanate (HDI) moieties is a good candidate for AF repair because it is injectable, and has material and adhesive properties that are amenable to AF repair. TMC adhesives bond with IVD tissue via nucleophilic attack of amine and hydroxyl groups (Six and Richter, 2000), and form cross-links via urethane, urea, allophanate and biuret linkages (Pocius, 2012). A formulation with two TMC–HDI moieties per PEG arm (TMC2) had an adhesive strength of 0.35 MPa, as assessed with a lap shear test (Bochyńska *et al.*, 2013). TMC2 had a tensile modulus of 21 MPa (Bochyńska *et al.*, 2013), which is in the range of the linear region of AF tissue (Acaroglu *et al.*, 1995; Green *et al.*, 1993; Guerin and Elliott, 2006; Long *et al.*, 2016; O'Connell *et al.*, 2009). PEG has been approved for therapeutic use alone and as conjugated to protein (FDA Center for Drug Evaluation and Research, 2016), so can be considered biocompatible.

This paper developed and evaluated PEG–TMC adhesives for AF repair. First, we screened two TMC formulations for AF repair and evaluated the effect of increasing the # of TMC–HDI moieties per arm, from two to three in TMC2 and TMC3 (Figure 1). This part had the following hypotheses: TMC2 and TMC3 have higher adhesion strength than non-adhered AF tissue, low degradation rates and similar shear moduli as native AF tissue. We also hypothesized that cells grown on TMC3 have higher proliferation than those grown on Dermabond. Next, we

tested whether TMC3, which performed better during screening tests, could perform well during *in situ* tests with biomechanics, failure test and organ culture measurements. We tested the hypotheses that TMC3 would effectively repair IVDs following discectomy in order to sufficiently restore *in situ* biomechanics and *in situ* failure strength to intact IVD levels, and withstand 4 days of cyclic loading without herniation in an organ culture model.

2. Materials and methods

2.1. Study design

This study progressed through a testing series of advancing complexity from *in vitro* screening to *in vitro* validation and *ex vivo* analysis (Figure 1). *In vitro* screening tests evaluated both TMC2 and TMC3 formulations with measurements of adhesion strength using a pushout test, shear modulus using a rheometer, and degradation rate by soaking the adhesive in phosphate-buffered saline (PBS) and measuring dry weight. Based on these results, TMC3 was selected for further screening. *In vitro* validation included a cytocompatibility test, assessed by growing cells directly on TMC3 and Dermabond, and measuring DNA concentration. *In situ* validations were also applied to TMC3, and included biomechanical tests, failure tests and organ culture tests to assess cyclic loading.

Figure 1. Polyethylene glycol (PEG) with hexamethylene diisocyanate (HDI) reactions with native tissue proteins; study design progresses from screening to validation. (A) Trimethylene carbonate (TMC)2 and TMC3 are block copolymers of PEG and HDI with $n = 2$ and 3, and molecular weights of about 1038 g mol^{-1} and 1186 g mol^{-1} , respectively. (B) The isocyanate groups in the HDI end-groups form urethane, urea, allophanate and biuret linkages when reacted with alcohols and amines in intervertebral disc (IVD) tissue. Reactions from Pocius (2012). (C) The study design progresses from simpler screening tests to increasingly complex tests, including *in vitro*, *in situ* and *ex vivo* validation

2.2. Synthesis

Trimethylene carbonate adhesives were synthesized in a two-step reaction as described and validated previously (Bochyńska *et al.*, 2013). Briefly, ring-opening polymerization was performed on TMC initiated by PEG with Sn(Oct)₂ as a catalyst. The molar ratios, 4:1 and 6:1 for TMC2 and TMC3, resulted in two and three TMC moieties on each end of the PEG molecule, respectively (Figure 1). Oligomers were added dropwise to a volume of HDI under a flow of nitrogen to ensure an excess of HDI and an inert environment. Excess HDI was removed by precipitation in dry diethyl ether and the PEG400-(TMC₃-HDI)₂ was dried in a vacuum.

The chemicals were purchased from the following vendors: TMC from ForYou (China); HDI from Merck Schuchardt (Germany); diethyl ether from Biosolve (the Netherlands); PBS from B Braun Melsungen AG (Germany); and PEG ($M_w = 400 \text{ g mol}^{-1}$), acetic anhydride, stannous octoate (tin 2-ethylhexanoate, SnOct₂) and chloroform-d (CDCl₃) from Sigma Aldrich (the Netherlands). PEG was dried at 120 °C under vacuum, and diethyl ether was dried over molecular sieves prior to use.

2.3. Adhesion strength

Trimethylene carbonate2 ($n = 9$) and TMC3 ($n = 12$) block copolymers were evaluated using a pushout test described previously (Guterl *et al.*, 2014; Maher *et al.*, 2010). Briefly, PEG-TMC formulations were applied to the centre of cylindrical AF specimen (3 mm × ϕ 8 mm with concentric ϕ 3 mm hole) and cured for 12 h at 4 °C before testing. An indenter (ϕ 2.8 mm) applied displacement at 0.01 mm s^{-1} until the adhesive plug underwent a simulated 'herniation'. Adhesion strength was calculated by dividing the failure force by area of contact between adhesive and AF tissue. Failure force was defined by the maximum force before abrupt reduction of force, indicating failure. Adhesion strength for AF press-fit control was obtained from prior tests (Guterl *et al.*, 2014), and is the failure force of passively placed AF tissue.

2.4. Degradation

Trimethylene carbonate2 and TMC3 ($n = 4$ per adhesive per time point) adhesives were prepared and formed into cylindrical plugs by injecting the adhesive into a custom-made Teflon mold (ϕ 5 mm, 1.5 mm thick; Guterl *et al.*, 2014). Each plug was soaked in PBS for 1, 7, 14 or 21 days. At each timepoint, glue plugs from both groups were weighed (W_w), dried in vacuum and weighed again (W_d). Swelling ratio (W_w/W_d) was calculated from wet weight (W_w) and dry weight (W_d).

2.5. Shear modulus

Trimethylene carbonate2 and TMC3 ($n = 6$ per group) adhesives were prepared and formed into cylindrical

plugs by injecting the adhesive into a custom-made Teflon mold (3 mm × ϕ 5 mm). AF tissue plugs were prepared by dissecting AF tissue from frozen bovine caudal IVDs and punched with ϕ 5 mm biopsy punch. Each gel cured for 4 h at 37 °C submerged in water. Each gel was tested in a parallel plate rheometer (AR2000ex, TA Instruments, New Castle, DE, USA) with a frequency sweep (0.05–10 Hz) at 1% strain under an axial normal force of 2 N (0.1 MPa). Shear modulus is the complex modulus at 1 Hz and 1% strain.

2.6. Cytocompatibility

Bovine caudal AF cells ($n = 3$) were seeded in triplicate in each condition in 48-well plates at $24\,000 \text{ cells well}^{-1}$; the wells were either coated with TMC3 or Dermabond, or left uncoated (No Adhesive; i.e. tissue culture plastic: polystyrene). TMC3 was selected for further screening because TMC3 and TMC2 had similar adhesion strengths and degradation profiles, and TMC3 had similar shear modulus to AF tissue. Cells were cultured for 1, 3 or 7 days at 37 °C, 5% CO₂ and ambient oxygen. Cells were grown in high glucose Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 1% Pen/Strep and 0.2% ascorbic acid. Cells were then lysed, and the double-stranded DNA content was quantified using fluorescent dye (QuantiFluor® dsDNA System, Promega, Madison, WI, USA) and a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA, USA). Dermabond and cell culture plate plastic (tissue culture polystyrene) were used as limiting case controls. Dermabond has known cytotoxicity, and is used as a control here as it is a strong adhesive and it has also been tested *in vivo* in the disc (Kang *et al.*, 2017). Conversely, cell culture plastic is the control for the limiting case of fastest cell growth expected for these cells.

2.7. Biomechanics

Six skeletally mature bovine tails were obtained from a local abattoir (Green Village Packing, Green Village, NJ, USA), and 12 motion segments (vertebra-IVD-vertebra) were dissected from two caudal levels (cc 2–3 and cc 3–4). Each motion segment per tail was distributed to one of two groups ($n = 6$ per group): Sham or TMC3 (repaired). Bovine coccygeal IVDs were chosen because the size is comparable to human lumbar IVD (Illien-Jünger *et al.*, 2013). After dissection, motion segments were wrapped in saline-soaked tissue and frozen until testing. The night before testing, samples were thawed overnight in PBS (Fisher BioReagents, Fischer Scientific, Pittsburgh, PA, USA), and potted in polymethylmethacrylate (Henry Schein, Melville, NY, USA).

All motion segments were evaluated in a repeated-measures test that included three tests: Intact; Discectomy; and in the Experimental condition: either TMC3 (repaired) or Sham (not repaired; Figure 2). The

Figure 2. *In situ* biomechanics testing study design and sample results. (A) Each specimen was tested under axial force and torsion conditions three times as a repeated-measured test: Intact; Discectomy; and Experimental. The experimental condition involved trimethylene carbonate (TMC)3 repair (TMC3) or non-treated injured control (Sham). (B) The loading protocol consisted of a 1 min ramp, 10 min compression, 20 cycles of tension and compression (0.25 to -0.50 MPa), and 20 cycles of torsion ($\pm 4^\circ$). The second to last (C) force-displacement and (D) torque-rotation traces were analysed; the representative discectomy trace (red) was different than the intact (green)

first test, in the Intact condition, occurred after overnight swelling equilibration in PBS. The second test, in the Discectomy condition, occurred after overnight swelling equilibration in PBS and discectomy. The discectomy consisted of a 5 mm biopsy punch defect through the posterolateral AF and removal of approximately 25% of the NP (145 ± 17 mg). The third test, in the Experimental condition, occurred after group-specific treatment (Figure 2). The TMC3 group was repaired with TMC3 by retrograde injection, left at room temperature for 30 min to cure, submerged in PBS overnight and subjected to the final mechanical test. The Sham group was submerged in PBS overnight and subjected to the final mechanical test.

The mechanical test consisted of multistep cyclic loading in axial compression, tension and torsion. First, there was a 1-min ramp to -50 N preload and a 10-min hold at this load. The preload was followed by 20 cycles of axial loading to forces equivalent to 0.25 MPa (tension) and 0.50 MPa (compression) nominal axial stresses at 0.1 Hz. The axial loading was followed by 20 torsion

cycles from $\pm 4^\circ$ at 0.1 Hz (Figure 2). The displacement of the torsion cycles is approximately the maximum physiological rotational range of motion (ROM) of human lumbar motion segments (Li *et al.*, 2009; Pearcy and Tibrewal, 1984; Xia *et al.*, 2010). Specimens were tested with an MTS Servohydraulic system (Bionix 858, MTS, Eden Prairie, MN, USA).

Herniation was monitored with a video camera throughout the loading procedures. Force, displacement, torque and rotation were recorded at 100 Hz. The biomechanical variables were calculated using the second to last cycle using a custom MATLAB code (Mathworks, Natick, MA, USA). The compressive, tensile and torsional stiffness were calculated as the top 20% of the axial load or rotation of the force-displacement or torque-rotation curves. The axial ROM and torque range were calculated as the total displacement or torque developed. All parameters except hysteresis area were normalized by dividing by the corresponding value from the first (Intact) test. The hysteresis area was calculated as the area between the loading and unloading force-displacement or torque-

rotation curves. The neutral zone stiffness was calculated as the slope of 10 data points (0.1 s) in the centre of the force–displacement of torque–rotation curves.

2.8. Failure strength

Vertebra–IVD–vertebra segments from nine tails (levels cc2–3, cc3–4 and cc4–5) were dissected as previously described, except that the vertebral bodies were sectioned approximately 4–5 mm above endplates with a diamond blade saw (IsoMet 1000 Precision Cutter, Buehler, Lake Bluff, IL, USA). The motion segments were distributed to either Intact, Discectomy or TMC3 groups ($n = 9$ per group). Intact samples underwent no treatment. The Discectomy and TMC3 samples underwent a posterolateral 4 mm biopsy punch defect and removal of 150–170 mg of nuclear and annular material using a rongeur. The TMC3 samples were repaired as described above. All samples were soaked in PBS overnight before testing. The diameter and disc height of each sample were measured three times at different locations and averaged. Each sample was subjected to monotonically increasing compressive axial displacement (2 mm min^{-1}) under a fixed 5° bending with the posterolateral injured or repaired face at the outside of the bend (i.e. the defect face had a larger disc height than the opposite side) as described previously (Vergroesen *et al.*, 2015a). Force and subsidence were measured through the test and extrusion was monitored by video. Failure strength and subsidence until failure were identified by inspecting the force trace and corresponding video to determine the point at which NP tissue breached the boundary of the IVD. Extrusion of the TMC3 glue was also monitored. The mechanism of failure was identified as follows: endplate failure occurred with a single maximum force in a monotonically increasing force trace. Nucleus herniation failure occurred with incremental micro-failures and a subsequent increase in the force trace.

2.9. Organ culture

Bovine IVDs with endplates were randomly distributed among three groups: Intact; Discectomy; and TMC3 repair ($n = 3$ per group), and prepared as described previously (Gantenbein *et al.*, 2006; Illien-Jünger *et al.*, 2010; Walter *et al.*, 2014). For Discectomy and TMC3 IVDs, the discectomy was initiated on the posterolateral side of the IVD with a 4 mm biopsy punch to the centre of the IVD, followed by tissue removal with a rongeur. IVDs were then cultured for 4 days with applied loading that simulated the diurnal cycle and two bouts of rigorous loading starting on Day 2. Loading consisted of diurnal loading (8 h: 0.2 MPa /16 h: 0.3 MPa) with two 5-h bouts of ‘exercise’ ($0.3 \pm 0.2 \text{ MPa}$ @ 0.1 Hz) during the daytime cycle, similar to that described (Walter *et al.*, 2014). Over the 4-day culture period, 10 800 cycles of loading were applied. Immediately after culture, discs were fixed in zinc formalin (Z-Fix®, Anatech LTD, Battle Creek MI,

USA) for at least 48 h, embedded in methyl-methacrylate and sectioned as previously described (Laudier *et al.*, 2007; Walter *et al.*, 2015). Sections were then stained with Picrosirius Red/Alcian Blue (Gruber *et al.*, 2009), and imaged with an upright light microscope (AxioImager Z1, Zeiss).

2.10. Statistics

A one-way non-parametric ANOVA (Friedman’s) with correction for multiple comparisons (Dunn’s) was used to assess differences between treatments for adhesion, shear modulus, failure strength and subsidence. A one-way non-parametric ANOVA with multiple comparisons test was also used to test differences between means of the Intact condition, Discectomy condition and the final condition (either TMC3 or Sham) for biomechanics. A two-way non-parametric ANOVA with correction for multiple comparisons was used to detect differences between treatments and timepoints for cytocompatibility and degradation. Significance was assessed at $\alpha = 0.05$ level. All statistics were calculated with Prism version 6.04 for Windows (GraphPad Software, La Jolla, CA, USA). All data are presented mean $\pm$ standard deviation, and all error bars are standard deviation.

3. Results

3.1. Adhesion strength

For the pushout test, TMC2 and TMC3 had significantly higher adhesion strength than the AF pressfit control (Figure 3).

3.2. Degradation

The swelling ratio (W_w/W_d), wet weight over dry weight, remained unchanged for TMC3 and TMC2 for 3 weeks (Figure 3).

3.3. Shear modulus

The shear modulus of TMC2 was greater than AF tissue (Figure 3). Because the shear modulus of TMC3 was not significantly greater than AF tissue, TMC3 was prioritized for further analysis.

3.4. Cytocompatibility

DNA concentration significantly increased from Day 1 to 7 for the Plastic (i.e. no adhesive) group, while no changes in DNA content were observed for Dermabond- or TMC3-treated cells. DNA concentration was higher for cells grown on plastic ($3759.8 \pm 2797.0 \text{ ng mL}^{-1}$) compared with Dermabond ($17.1 \pm 6.6 \text{ ng mL}^{-1}$) and TMC3 ($1026.3 \pm 955.3 \text{ ng mL}^{-1}$) at Day 7 ($P < 0.05$), and

Figure 3. Polyethylene glycol (PEG)-based adhesives have high adhesion strength, low degradation rate and high shear modulus. (A) Both formulations of PEG-based adhesives, trimethylene carbonate (TMC)2 and TMC3, had higher adhesion strength than the pressfit annulus fibrosus (AF) control. (B) The swelling ratio (W_w/W_d) of both formulations remained constant for 3 weeks. (C) The shear modulus of TMC2 was significantly greater than AF tissue (bar $P \leq 0.05$, letters indicate distinct statistical groups)

Figure 4. Annulus fibrosus (AF) cells grow less rapidly on trimethylene carbonate (TMC)3 and Dermabond than on tissue culture polystyrene (plastic). Cells grown on plastic had higher DNA concentration than cells grown on Dermabond and TMC3 at Day 7. Cells grown on TMC3 had a trend of higher DNA concentration than cells grown on Dermabond and tended to increase with time. Cells grown on plastic had higher DNA concentration at Day 7 than at Days 3 and 1 ($P \leq 0.001$ from Day 3 and Day 1, bar $P \leq 0.05$, $^*P \leq 0.10$)

DNA concentration trended higher for cells grown on TMC3 compared with Dermabond ($P = 0.09$; Figure 4).

3.5. Biomechanics

Discectomy resulted in increased axial ROM, decreased torsion hysteresis area, torsion stiffness and torque range (Figure 5) compared with the intact condition for both groups: Sham and TMC3 repaired. This change was present in the third test for the Sham group for only axial ROM, torsion hysteresis area and torsional stiffness, indicating the effect of the injury on these parameters was constant through the 2 days and both tests for the Sham group. Of these parameters, the TMC3 group had similar values to those of the Intact condition for axial ROM,

Figure 5. Trimethylene carbonate (TMC)3 partially restored *in situ* biomechanical behaviours to intact levels. Following discectomy. (A) Discectomy increased axial range of motion (ROM) and TMC3 repair restored to Intact condition. (B) Discectomy reduced torsion hysteresis area and TMC3 restored to Intact condition. (C) Discectomy reduced torsional stiffness and TMC3 repair restored to Intact condition. (D) Discectomy reduced torque range (bar $P \leq 0.05$, $^*P \leq 0.01$). All parameters except torsional hysteresis were normalized to Intact condition

torsion hysteresis area and torsional stiffness, indicating restoration to intact values. There was no change between Intact and Discectomy conditions for axial hysteresis, compressive stiffness and tensile stiffness (not shown).

None of the six successfully repaired specimens herniated.

3.6. Failure strength

The Injured and TMC3-repaired samples had lower failure strengths (4.5 ± 2.7 MPa and 5.9 ± 4.2 MPa, respectively) and lower subsidence to failure (2.09 ± 0.67 and 2.29 ± 0.69 mm, respectively), defined by the extrusion of NP material, than did the intact samples (13.5 ± 4.2 MPa and 3.30 ± 0.54 mm; Figure 6). TMC3 repair extruded at lower nominal axial stress (force/area) and subsidence than the extrusion of nuclear tissue (0.80 ± 0.63 MPa and 0.945 ± 0.402 mm, respectively). In both the injured and TMC3 groups, 9/9 samples failed by herniation compared with 1/9 in the intact group, in which the mechanism of failure was endplate fracture.

3.7. Organ culture

In organ culture, two of three TMC3 repairs herniated after 4 days of culture. In Picrosirius red/Alcian blue-stained histological sections, the TMC3-repaired group had a rectangular shaped defect where the TMC3 adhesive was injected, whereas the discectomy group had a triangular shaped defect due to NP swelling and additional tissue deformations. Upon high-magnification inspection, there was evidence that the TMC3 plug dislodged some AF tissue upon herniation (Figure 7B), and the discectomy sample

had herniated NP material. At the AF defect surface, the TMC3-repaired sample had less disrupted AF structure than the discectomy group, which had a more disorganized AF structure at the defect edge (Figure 7).

4. Discussion

Trimethylene carbonate² and TMC3 PEG–TMC compositions were evaluated to assess their capacity to serve as AF adhesives following discectomy. TMC2 and TMC3 had similarly high adhesion strengths and low degradation rates, but TMC2 had a shear modulus substantially higher than native AF tissue. Because TMC2 had a material property mismatch, TMC3 was prioritized for further *in vitro* and *in situ* validation tests with assessments of cytocompatibility, *in situ* biomechanics, *in situ* failure and organ culture. TMC3 allowed cell proliferation, suggesting good cytocompatibility, partially restored motion segment biomechanics to intact levels, and was easy to inject into the IVD. However, TMC3 had a high risk of herniation during the *in situ* failure test and cyclic organ culture. Further optimization is therefore required and may consist of chemical modifications to change the TMC material properties to be more similar to the AF, and increasing adhesion strength by reducing hydrophobicity and increasing wetting behaviours.

Trimethylene carbonate² and TMC3 had high adhesion strengths (150 kPa) as measured by pushout testing, likely due to the formation of covalent bonds with the native IVD tissue. Comparing with other proposed AF sealants proposed in the literature, TMC2 and TMC3 both had higher adhesive strength than fibrin (72.2 ± 29 kPa), fibrin

Figure 6. Trimethylene carbonate (TMC)3 repaired samples failed at similar loads to non-repaired Discectomy samples. (A) One time loss of force in the force–displacement trace of a Discectomy sample indicated nucleus herniation. The solid line is the ultimate load, which is divided by cross-sectional area to get strength, and the dashed line is the subsidence to failure. (B) Gradual reduction in force of an Intact sample indicated endplate failure. (C) The TMC3 and Discectomy samples had similar failure strengths and subsidences that were lower than those of Intact samples ($P \leq 0.05$). Failure was confirmed with video and dissection

Figure 7. Trimethylene carbonate (TMC)3 better maintained intervertebral disc (IVD) structure, although expulsion occurred after cyclic loading. Picrosirius red/Alcian blue-stained histological sections clearly demonstrated differences in (A) the overall structure of the IVD for intact, discectomy and TMC3 groups (bar 0.2 cm). (B) The TMC3 repair had a rectangular shaped defect where the TMC3 'plug' was injected and expulsion of the TMC3 plug following cyclic loading dislodged some annulus fibrosus (AF) tissue (arrow). In contrast, the discectomy sample had a triangular shaped defect and more herniated NP material (bar 500 μ m). (C) At AF defect surface, TMC3 maintained a smoother AF structure at the interface (arrow), while the discectomy group had a more disorganized AF structure at defect edge (arrow). Failure of the TMC3 repair predominantly occurred between AF tissue and TMC3, although some TMC3 remained adhered to the AF tissue (arrow; bar 200 μ m)

cross-linked with genipin (67.5 ± 31 kPa; Guterl *et al.*, 2014), poly(*N*-isopropylacrylamide)–poly(ethylene glycol)/poly(ethylene imine) with glutaraldehyde cross-linker (1.4–2.5 kPa; Vernengo *et al.*, 2010), poly(*N*-isopropylacrylamide) + chondroitin sulphate $\pm$ aldehyde modified chondroitin sulphate (1.1–1.8 kPa; Wiltsey *et al.*, 2015) and aligned nanofibrous poly- ϵ -caprolactone scaffold (55–125 kPa; Nerurkar *et al.*, 2009), but not Dermabond (700 kPa; Bochyńska *et al.*, 2016). The electrophilic isocyanate groups are vulnerable to nucleophilic attack from amine and hydroxyl groups in IVD tissue, yielding urethanes and ureas. Amine and hydroxyl groups are found in sulphated glucosaminoglycans present in the IVD (Melrose and Roughley, 2014). The urea and urethane groups can further react with excess isocyanate to form allophanate and biuret linkages, also resulting in cross-linkages and adherence (Pocius, 2012). Both TMC2 and TMC3 were stable through time and were not prone to hydrolytic cleavage in PBS after 3 weeks, as compared with fibrin, which is known to degrade rapidly (Guterl *et al.*, 2014).

Cyanoacrylates have toxic byproducts resulting in seroma formation, tissue necrosis and chronic foreign body giant cell reaction when placed beneath the skin (Mobley *et al.*, 2002), and shorter polymers degrade faster, resulting in higher toxicity. Dermabond therefore has a reduced cytocompatibility, which presumably allowed it to be approved only for topical use in the USA (Administration, 1998; Medhekar and Melkerson, 2010). We hypothesized that TMC3 would have increased cytocompatibility compared with Dermabond (2-octyl cyanoacrylate, $M_w = 209.20$ g mol $^{-1}$) as PEG–TMC adhesives have higher molecular weights (TMC2 $M_w \approx 1038$ g mol $^{-1}$, TMC3 $M_w \approx 1186$ g mol $^{-1}$; Figure 1) and

form large interbranching networks, thus degrading more slowly and enabling transport and release of potential cytotoxic degradation products. Additionally, PEG-based adhesives such as Duraseal and Coseal are currently in clinical use (Mehdizadeh and Yang, 2012). We found that cells grew substantially more rapidly on TMC3 than on Dermabond. However, decreased cytocompatibility for both groups was identified since we found that at Day 7, Dermabond and TMC3 had lower DNA concentrations than cells grown on plastic. The toxicity of Dermabond is likely due to cyanoacrylate degradation into cyanoacetate and formaldehyde via the inverse Knoevenagel reaction, but could be due to other metabolic pathways (Hubbard *et al.*, 2014). The reduction of DNA content of cells grown on TMC3 may be due to the buildup of carbonic acid resulting from the release of carbon dioxide after reaction with water (Six and Richter, 2000), or may be due to decreased adhesion. Because the DNA concentration of cells grown on TMC3 was similar to that of cells grown on plastic and trended to be higher than those on Dermabond, we considered this acceptable.

Discectomy resulted in significantly increased axial ROM, and significantly reduced torsion hysteresis, torsion stiffness and torque range. These results are consistent with previous findings that torsion mechanics are sensitive to AF injury, including loss of stiffness and increased ROM (Iatridis *et al.*, 2013). Defects larger than 40% of the disc height are known to induce repeatable and measurable effects (Elliott *et al.*, 2008), and we used a 5 mm injury ($\sim 50\%$ disc height) for biomechanics to induce large effects. TMC3 resulted in recovery of torsional stiffness, hysteresis and axial ROM to the intact condition, but not in the torque range. This restoration of three biomechanical parameters shows promise.

Failure testing and organ culture testing demonstrated a risk of herniation. Specifically, failure strength was similar for TMC3 and discectomy groups, and some herniation of TMC3 was observed during the physiological cyclic loading in organ culture testing. The failure strength of intact (13.5 ± 4.2 MPa) and injured bovine IVDs (4.5 ± 2.7 MPa) was similar to the failure strength of intact (12.5 ± 4.4 MPa) and injured (punctured with 2.4 mm hypodermic needle; 6.5 ± 3.6 MPa) lumbar IVDs from skeletally mature Dutch milk goats (Vergroesen *et al.*, 2015b). A 4-mm-diameter defect was used in our failure strength and organ culture studies as a large repeatable defect approximately 40% of the disc height. The failure strength of TMC3 (5.9 ± 4.2 MPa) was similar to that of TMC1 (9.8 ± 6.1 MPa; Vergroesen *et al.*, 2015b). Interestingly, TMC3 failed at lower stresses than TMC1, and we believe it is likely that TMC3 did not wet the surface of the AF as it has greater hydrophobicity. Water content in both TMC2 and TMC3 was low, indicating some hydrophobicity from the TMC groups, possibly influencing the interaction with the AF, which is about 75% water (Antoniou *et al.*, 1996). However, it is possible that the shear modulus of TMC3 was too high and this AF–TMC3 material mismatch resulted in stress concentrations that were responsible for the herniation risk. Consequently, new formulations of TMC must be developed that can result in better wetting characteristics, perhaps by increasing the hydrophilic PEG to hydrophobic TMC ratio while also providing biochemical modifications allowing better matching to native AF tissue material properties.

Trimethylene carbonate³ herniated in two out of three samples during organ culture experiments. A useful future direction elucidated by this work is the importance of applying a physiologically relevant failure test early in the screening process to assess *in vivo* extrusion risk. Adhesion strength and failure strength test used loading rates that have been published to allow better comparison with the literature, but herniations are expected to occur at

higher rates and results may be rate dependent. Extrusion is a risk that has sidelined many annular repair strategies (Bron *et al.*, 2010), and it is important to address this risk early in the development for annular repair strategies. Another important parameter to assess in future tests is long-term degradation and cytocompatibility, as the effect of degradation byproducts of these adhesives is unknown and may impact *in vivo* performance.

5. Conclusions

Polyethylene glycol–TMC-based adhesives were screened and validated as AF sealants using a robust testing paradigm. TMC3 exhibited high adhesive strength, slow degradation, high cytocompatibility and good *in situ* biomechanical performance, but extruded at high stresses and under cyclic loading. Further optimization of TMC is necessary to promote better tissue integration and prevent herniation, possibly with formulations that increase wetting behaviours and allow better matching of AF compressive and shear material properties.

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