Structural and Chemical Modification to Improve Adhesive and Material Properties of Fibrin-Genipin for Repair of Annulus Fibrosus Defects in Intervertebral Disks

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Annulus fibrosus (AF) defects from intervertebral disk (IVD) herniation and degeneration are commonly associated with back pain. Genipin-crosslinked fibrin hydrogel (FibGen) is an injectable, space-filling AF sealant that was optimized to match AF shear properties and partially restored IVD biomechanics. This study aimed to enhance mechanical behaviors of FibGen to more closely match AF compressive, tensile, and shear properties by adjusting genipin crosslink density and by creating a composite formulation by adding Poly(D,L-lactide-co-glycolide) (PDLGA). This study also evaluated effects of thrombin concentration and injection technique on gelation kinetics and adhesive strength. Increasing FibGen genipin concentration from 1 to 36 mg/mL significantly increased adhesive strength (~5 to 35 kPa), shear moduli (~10 to 110 kPa), and compressive moduli (~25 to 150 kPa) with concentration-dependent effects, and spanning native AF properties. Adding PDLGA to FibGen altered the material microstructure on electron microscopy and nearly tripled adhesive strength, but did not increase tensile moduli, which remained nearly 5× below native AF, and had a small increase in shear moduli and significantly decreased compressive moduli. Increased thrombin concentration decreased gelation rate to <5 min and injection methods providing a structural FibGen cap increased pushout strength by ~40%. We conclude that FibGen is highly modifiable with tunable mechanical properties that can be formulated to be compatible with human AF compressive and shear properties and gelation kinetics and injection techniques compatible with clinical discectomy procedures. However, further innovations, perhaps with more efficient fiber reinforcement, will be required to enable FibGen to match AF tensile properties. [DOI: 10.1115/1.4036623]

Keywords: annulus fibrosus repair, intervertebral disk herniation, tissue engineering, adhesive biomaterial, genipin

1 Introduction

Lower back pain is the leading cause of global disability [1] and affects up to 17% of the world population [2]. Low back pain and spinal pathologies also cause a substantial economic burden with direct costs of \$253 billion, plus indirect costs such as lost work days [3]. The causes of low back pain are complex and multifactorial, although commonly IVD degeneration and herniation are direct causes of pain [4,5]. In herniated IVDs, the nucleus pulposus penetrates through the AF causing compression of nerve roots [6,7]. The current gold standard for treatment of symptomatic IVD herniation after failed conservative care is lumbar microdiscectomy. Unrepaired AF defects exhibit accelerated IVD degeneration [8] and the most common complications are reherniation, recurrent pain, and repeated discectomy or fusion at the operated level [9–15].

Previous AF repair strategies have not successfully restored function, or prevented IVD degeneration. Enhanced suturing techniques such as Xclose, Inclose, and others have been removed from the market, for lack of efficacy or complicated techniques that are difficult to apply clinically [16–19]. Barbed polyethylene plugs for AF closure resulted in device expulsion and significant endplate damage in large animal studies [20]. Another strategy using a titanium bone anchor implanted into the adjacent vertebrae of the disk did not herniate after 100,000 load cycles; however, it requires strong bone quality and 12 months after implantation, showed no statistically significant difference [21,22].

An ideal AF repair biomaterial would be injectable in a minimally invasive manner, seal AF defects, match native AF properties, restore IVD biomechanics, and promote regeneration. Repairs must be able to gel rapidly to be compatible with current discectomy procedures and almost immediately withstand high intradiscal pressures and complex loading conditions without risk of herniation, which remains a challenge for effective clinical translation [23–25]. Fibrin used in IVD repair had encouraging

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Table 1 Study design

Aim	Groups	Test	Dependent variable
Effects of crosslinking	Press-fit AF control fibrin Fibrin + 1 mg/mL genipin (FG1) Fibrin + 2 mg/mL genipin (FG2) Fibrin + 4 mg/mL genipin (FG4) Fibrin + 6 mg/mL genipin (FG6) Fibrin + 36 mg/mL genipin (FG36)	Ninhydrin assay Shear test Unconfined compression test Pushout test Structure evaluation	Percent crosslinking $ G^* $ E_C Adhesive strength SEM and TEM
Effects of microstructure	FG6 FG6-PDLGA small particle (FG6-S2 and FG6-S20) FG6-PDLGA Large particle (FG6-L2 and FG6-L20)	Pushout test Rheology frequency sweep Unconfined compression Tensile test Structure evaluation	Adhesive strength $ G^* $ E_C E_T SEM and TEM
Clinical Optimization	FG6 FG6-Cap FG6-1× Thrombin concentration FG6-4× Thrombin concentration	Pushout test Gelation kinetics test	Adhesive strength Gelation time

results in large animals and early clinical trials [23,26,27]. However, clinical trials were discontinued because Fibrin did not outperform saline-injected controls [27–29]. We postulate that failure was due to rapid degradation of fibrin as well as lower mechanical stiffness values than native AF, which can be improved with the addition of a crosslinker [30,31].

Genipin is a natural crosslinker with low cytotoxicity that has been used as a food dye and in research for tissue engineering of articular cartilage and IVD [32–35]. Fibrin crosslinked with genipin created FibGen, capable of being tuned to match AF shear modulus and which exhibited substantially slower degradation rates than fibrin [31,36]. When FibGen was injected into injured IVDs, it restored IVD height and compressive stiffness of injured IVDs after 14,400 cycles of compressive loading in a large defect bovine IVD organ culture model [37]. However, tensile and torsional stiffness of injured motion segments were not restored with FibGen [38], likely providing an opportunity for further optimization of FibGen material behaviors.

The purpose of this three-part study was to modify FibGen formulations to determine if modifications of crosslinking density, microstructure, formulation, and injection technique could create a hydrogel with greater adhesive strength, with material properties that more closely match AF shear, compressive and tensile material properties, and is compatible with discectomy procedures. Aim 1 modified crosslink density by adjusting genipin concentration. Aim 2 adjusted microstructure by creating a composite mixture of FibGen with Poly (D,L-lactide-co-glycolide) (PDLGA). Aim 3 investigated ways to enhance clinical translation of FibGen by reducing gelation time and evaluating injection techniques to be more amenable for discectomy procedures. PDLGA was used because it is biocompatible and useable in many forms [39–44], and since it has a higher tensile modulus and failure strength [45] than native AF, PDLGA may function to enhance those properties of FibGen when used in a composite formulation.

2 Methods

2.1 FibGen Formulations. This study evaluated effects of crosslinking, microstructure, and clinical optimization conditions (Table 1).

2.1.1 FibGen Gel Preparation. A dual barrel syringe with mixing tip (4:1 syringe, Pacific Dental, Walnut, CA) was used to thoroughly mix following components of the gel: Fibrinogen (dissolved in phosphate-buffered saline) was pipetted into the large barrel and a solution of thrombin, genipin, and PDGLA (all dissolved in dimethylsulfoxide (DMSO)) was pipetted into the small barrel of the syringe. After mixing and injection into molds (gel size dependent on test, explained further below), gels solidified rapidly and then further cured in a humidified atmosphere for 4 h

at 37 °C to final mechanical properties. Fibrin and all FibGen gels had a final concentration of 140 mg/mL fibrinogen (Sigma-Aldrich, St. Louis, MO) and 28 U/mL of thrombin (Sigma-Aldrich). For crosslinking experiments, the final concentration of genipin (Wake, Richmond, VA) ranged from 0 mg/mL to 36 mg/mL (Table 1). The groups of Fibrin, FG6 and FG36, were chosen for further characterization of adhesive strength and by electron microscopy to examine the relationship between structure and function over a wide range of concentrations.

For clinical optimization experiments, the injection surface area of the gel was increased by adding a structural “cap” (1 mm thick and 8 mm in diameter) with a custom-designed mold. Then, pushout tests were performed to compare the samples with and without the cap (Table 1) to a press-fit control. The force was applied to an “inner” cap to simulate an excess of FibGen near the nucleus pulposus region interior to the AF. To accelerate the gelation time, the thrombin concentration was modified by increasing thrombin from 28 U/mL (1×) to 112 U/mL (4×).

2.1.2 PDLGA Fiber Generation. PDLGA polymer (molecular weight 222.6×10^3 g/mol), at a ratio of 85:15 lactic to glycolic acid, was dried under vacuum and extruded to a 0.2 mm diameter fiber (Brabender, Plasticorder PL-2100, Duisberg, Germany). Fibers were then subjected to hot-drawing to straighten the polymer chain (heater zone 6 cm, total length of 21 cm, temperature 125–155 °C). PDLGA fibers were snap frozen in liquid nitrogen and homogenized for either 4 min or 10 s (SamplePrep GenoG-rinder, SPEX Metuchen, NJ) to create small (S) and large (L) PDLGA fibers, which were added at two concentrations (2 and 20 mg/mL), to the FG6 prior to gelation resulting in four groups (FG6-L2, FG6-L20, FG6-S2, and FG6-S20).

2.2 Mechanical Tests. Complex shear modulus ($|G^*|$, 0.5 Hz; $n = 4$ –6/group) of gels (5 mm diameter and 3 mm height) was measured using a frequency sweep (0.05–5 Hz at 1% strain) on a parallel-plate rheometer (AR2000ex, TA Instruments, New Castle, DE). Gelation time ($n = 5$ –6/group) was measured using a time sweep and multiple frequency (0.5, 2, 3.5, and 5 Hz) test for 25 min duration at 37 °C. The time at which the tangent loss converged at all frequencies tested is the time of liquid to solid conversion and is referred to in this paper as gelation time [46].

Young’s tensile modulus (E_T ; $n = 6$ /group) was measured using “dog-bone” Type V specimens according to ASTM Standard D638-02a [47]. Specimens were placed into tensile-testing grips and stretched at 0.2% strain/s (Instron 8872, Norwood, MA). Young’s compressive modulus (E_C , $n = 6$ –8/group) of gels (5 mm diameter, 5 mm height) was measured using unconfined compression at a 0.2% strain/s rate (Electro Force 3200 Bose, Eden

Prairie, MN). A single strain rate was used for tensile and compressive loading to represent relatively slow loading conditions.

Adhesive strength ($n=6-8/\text{group}$) was determined by pushout test (Electro Force Bose) using radially oriented bovine AF tissue plugs (3 mm thick, 8 mm diameter) using a custom chamber similar to that described [48]. A 3 mm diameter defect was created in the center of the AF plug using a 3 mm biopsy punch. The punched defect of the annular ring was filled with FibGen (3 mm diameter, 3 mm height) or removed AF was reinserted as press-fit control. Adhesive strength was calculated from the load at failure divided by the area of contact between the gel and the AF tissue. The 3-mm thick specimens spanned most of the bovine AF

(bovine AF is thinner than human AF) and the flat, parallel samples (cut with cryostat) controlled the surface contact area and volume of FibGen added to each test sample simplifying comparisons across studies.

2.3 Structural and Chemical Characterizations

2.3.1 Structural Characterization. To determine the gel structure, samples were analyzed by scanning electron microscopy (SEM) or transmission electron microscopy (TEM). Samples were fixed in 3% glutaraldehyde and followed by 1% osmium tetroxide, dehydrated in ethyl alcohol and dried and gold sputtered for SEM

Fig. 1 Genipin crosslinking increased adhesive strength, compressive and shear moduli, but with a threshold effect. (a) Greater genipin concentration increased crosslinking as measured by significantly reduced free amines. Increased crosslinking increased (b) complex shear modulus ($|G^*|$), (c) Young's compressive modulus (E_c), and (d) adhesive strength. (e) SEM and TEM of selected formulations show that increased crosslinking resulted in structures with larger fibers that were more densely connected. Data displayed as mean $\pm$ standard deviation. Values of x in FG x correspond to the genipin concentration in mg/mL. * = $p < 0.05$ compared to FG6, † = $p < 0.05$ compared to Fibrin, bar = $p < 0.05$.

Fig. 2 FibGen with PDLGA partially increased adhesive strength but did not increase all moduli. Small (S) and large (L) PDLGA was mixed in with FG6 at 2 mg/mL and 20 mg/mL. FibGen-PDLGA materials were characterized for (a) adhesive strength; (b) $|G^*|$ at 0.5 Hz frequency; (c) E_C ; and (d) Young's tensile modulus (E_T). Data displayed as mean $\pm$ standard deviation, "*" indicates significant difference from FG6, $p < 0.05$.

(5 kV, S4300 SEM Hitachi High Technologies) or embedded in Embed 812 for TEM (H760 TEM Hitachi High Technologies, Northridge, CA).

2.3.2 Chemical Characterization. Relative crosslinking was assessed using a ninhydrin assay ($n = 4-5$ gels per formulation). FibGen was prepared, weighed, frozen in liquid nitrogen, and lyophilized overnight followed by boiling in ninhydrin solution (30 min, 80 °C). Free amine groups were calculated by correlating optical density to a glycine standard (Spectramax M5, Molecular Devices, Sunnyvale, CA) [49,50]. ^1H nuclear magnetic resonance (NMR) spectrum was obtained for PDLGA dissolved in CDCl_3 (Sigma-Aldrich) at 400 MHz, with a ^1H 5 mm probe (BrukerAvanceDrx400 spectrometer, LabX, Midland, ON).

2.4 Statistical Methods. Statistical analyses were performed using Student's t -test or one-way Analysis of Variance with subsequent Tukey's (comparing all groups) or Dunnett's (comparing to FG6 as control) posthoc tests. All data were analyzed using Graphpad Prism 5 with $p < 0.05$ considered significant.

3 Results

3.1 Effects of Crosslinking. Increased genipin concentration significantly increased crosslinking density (Fig. 1(a)), significantly increased $|G^*|$ and E_C in a dose-dependent manner (Figs. 1(b) and 1(c)). All concentrations of genipin had significantly higher E_C than fibrin. Fibrin, FG6 and FG36 adhesive strength and electron microscopy were investigated based on increased E_C and $|G^*|$. FG6 and FG36 had increased adhesive strength, surface roughness, and highly integrated structure compared to Fibrin (Figs. 1(d) and 1(e)).

3.2 Effects of PDLGA. FG6-PDLGA mixtures affected adhesive and biomechanical behaviors regardless of concentration or particle size. FG6-L2 and FG6-S2 had significantly increased adhesive strength and FG6-L2 and FG6-S20 had significantly increased $|G^*|$ compared to FG6 alone (Figs. 2(a) and 2(b)). For all PDLGA groups, E_C was decreased (Fig. 2(c)) while no differences were observed for E_T (Fig. 2(d)). PDLGA groups had

elongated and denser whisker-type structures that were randomly oriented within the gel compared to FG6. FG6 large particulate groups L2 and L20 had a roughened surface area with larger bundles than the original FG6 group as visualized on SEM. SEM imaging demonstrated that FG6-S2 and FG6-S20 groups had finer structures compared to the other PDLGA groups (Fig. 3). TEM imaging revealed large bundles and thick or long fibrillar whiskers for FG6-L2 and FG6-L20 groups, while FG6-S2 and FG6-S20 groups appeared to have a higher density fine and short highly branched fibrils.

PDLGA ^1H NMR confirmed chemical structure and monomer ratio (see Supplemental Fig. 1(a), which is available under the "Supplemental Materials" tab for this paper on the ASME Digital Collection). The original particulates (see Supplemental Fig. 1(b), which is available under the "Supplemental Materials" tab for this paper on the ASME Digital Collection) added to the syringe were not detected under electron microscopy. Surface area, aspect ratio, and length were significantly smaller for S-PDLGA compared to L-PDLGA structures, yet the aspect ratio remained below five so that both structures were considered particulates (see Supplemental Fig. 1(c), which is available under the "Supplemental Materials" tab for this paper on the ASME Digital Collection).

3.3 Clinical Optimization. Adding a structural cap to FG6 (Fig. 4(a)) significantly increased the adhesive strength of the standard formulation ($p < 0.05$, Fig. 4(b)). Increasing the concentration of thrombin four times decreased the gelation time significantly from 12 to 4 min ($p < 0.05$, Fig. 4(c)).

4 Discussion

This three-part study modified FibGen crosslinking, microstructure, formulation, and injection technique to provide an injectable, adhesive hydrogel that could be used to seal AF defects during discectomy procedures, and better matched AF material properties of tensile modulus (0.3–30 MPa, depending on orientation, region, and strain level), shear modulus (≥ 100 kPa), and compressive modulus (~ 200 kPa) [25]. Of these mechanical parameters, our priority was to increase tensile modulus and adhesive strength. FibGen formulations were modified with increasing genipin

Fig. 3. PDLGA FibGen formulations modify hydrogel microstructure. The addition of PDLGA altered FibGen microstructure to increase the fibril density and to create larger structural features. PDLGA had randomly oriented, elongated, and denser whisker-type structures compared to FG6. FG6-L2 and FG6-L20 had a roughened surface area with larger bundles than the original FG6 group as visualized on SEM. FG6-S2 and FG6-S20 groups had finer structures compared to the other PDLGA groups. TEM imaging revealed large bundles and thick or long fibrillar whiskers for FG6-L2 and FG6-L20 groups, while FG6-S2 and FG6-S20 groups appeared to have a higher density of fine and short highly branched fibrils.

crosslink density to obtain FibGen that matched AF compressive and shear properties and increased adhesive strength until a crosslinking threshold was reached. Adding PDLGA particulates also increased adhesive strength and shear modulus, yet decreased compressive modulus. Tensile modulus was not enhanced with PDLGA with values that matched AF radial tensile moduli (~ 300 kPa) but did not approach AF circumferential tensile moduli (~ 30 MPa). Increased thrombin concentration decreased gelation time to be less than 5 min, and injecting in a way that added a structural cap increased adhesive strength. AF dynamic tensile moduli are insensitive to strain rate [51], so viscoelastic effects are not expected to change the finding that FibGen can match radial but not achieve circumferential AF tensile moduli values. Overall, FibGen was highly modifiable by adjusting genipin, thrombin, and PDLGA particulate concentrations to tune FibGen to match AF compressive and shear properties, and to accelerate gelation time to support its effective use as an AF sealant compatible with constraints of discectomy procedures. However, further innovations, perhaps with more efficient fiber reinforcement, are required to enable FibGen to match circumferential AF tensile properties.

Fig. 4 Optimization of formulation and injection technique to enhance clinical compatibility. (a) Schematic illustrating push-out testing samples that had a structural cap to increase hydrogel contact area. The structural cap increased (b) adhesive strength. (c) Gelation time was decreased with formulations containing higher concentrations of thrombin. Data displayed as mean $\pm$ standard deviation, bar indicates significant difference between groups, $p < 0.05$.

Crosslinking is often used to increase the modulus of biomaterials [35,52–54]. Genipin binds with primary amine groups in the presence of oxygen in a ring-opening mechanism and can form both intra and interfibril crosslinks and polymer chains [55,56]. Free amines were reduced with increasing genipin concentration, as quantified with the ninhydrin assay, and the increased crosslinking was visible on SEM and TEM. Increased crosslinking agrees with genipin's use as a crosslinker as previously reported [34,36,57] and generally increased shear and compressive moduli in a dose-dependent manner. This was consistent with electron microscopy that showed FG6 and FG36 had larger fiber structures that were more densely packed than fibrin alone. However, adhesive strength and compressive moduli were not significantly different between FG6 and FG36 despite a $6\times$ increase in genipin concentration, suggesting a functional threshold effect even though free amines continued to decrease. FG6 was chosen for continued evaluation because this was the highest effective concentration and high genipin concentrations are known to have cytotoxic effects [36,58,59]. We believe this threshold suggested that maximum stiffness that crosslinking can add to this fibrin-based material was achieved, and since it was still lower than native AF, we sought alternative strategies of stiffening and added PDLGA.

FibGen with PDLGA was intended to create a composite material that would increase tensile and compressive properties due to the larger moduli of PDLGA (2 GPa) [45]. The addition of PDLGA increased adhesive strength and shear modulus, but compressive modulus was reduced and tensile modulus was unchanged. Furthermore, PDLGA mixtures had lack of strong size or concentration effects suggesting reactions may have been occurring at nearly saturated concentrations. The reduced compressive modulus was surprising since it did not follow a rule of mixtures relationship and may be associated with the PDLGA and FibGen materials reacting chemically to copolymerize. Copolymerization could occur between hydroxyl groups on PDLGA and the opened genipin ring, and might be facilitated because PDLGA solubilizes in DMSO [60,61], which is a necessary solvent for genipin. Further, the original PDLGA particulates (>100 μm size) were not observed within gels using SEM or TEM. Electron microscopy also demonstrated that PDLGA greatly altered FibGen microstructure with larger structural features for PDLGA. We speculate that the larger structural features of the PDLGA observed on EM may have enabled enhanced interdigitation of FibGen with AF tissue to increase the measured adhesive strength. However, compressive modulus was reduced suggesting that strength of the newly formed fibrillar network was diminished. The presumed interaction between FibGen and PDLGA suggested

that additional genipin concentrations could have been investigated to optimize genipin concentration. However, the reduced compressive modulus of the PDLGA groups provided little justification for future investigations with PDLGA in this particulate form, suggesting that alternate materials and form factors with greater fiber reinforcement (i.e., smaller diameter and larger aspect ratio) may be more beneficial.

FibGen is an injectable hydrogel; the capacity for it to solidify in situ allows maximal tissue integration and adherence, filling of irregularly shaped defects, and allows potential translation with minimally invasive procedures. Increased thrombin concentration reduced gelation time from 12 min, consistent with previous findings [31], to less than 4.5 min. This experiment was a proof of concept that FibGen could gel in less time than that required to close the skin after surgery, although the use of only two thrombin concentrations does not allow conclusions regarding linearity of this effect. These findings are clinically significant because adoption of new technologies occurs when 1) techniques are easy to learn, 2) are modifications of existing procedures (as opposed to an entirely new procedure) and 3) do not greatly increase operation time [62,63].

Injection procedures that filled AF defects with an excess of FibGen to create a structural cap increased adhesion strength. The pushout test is a repeatable experiment intended to simulate herniation, but adhesive strength values obtained do not relate to physiological stresses or intradiscal pressures. While the adhesive strength values are somewhat low, the FG6 formulation had no or little herniation risk under extreme torsion and 14,000 cycles of physiological compression loading so that substantial improvements in adhesive strength are expected to reduce herniation risk further [37,38]. During discectomy procedures, loose nucleus pulposus tissue is removed, and the increased adhesion strength of this inner structural cap within the nucleus pulposus space suggested increased protection against nucleus pulposus herniation. Pilot studies showed a similar increase in adhesive strength for an outer cap that would represent extra FibGen on the outside of the IVD. We postulate that the addition of excess FibGen both inside of and outside of the AF defect would best decrease herniation risk. However, excess FibGen outside a herniation must be no more than a thin layer because of the increased proximity to neural structures, and further study is required to assess effects of FibGen on neural structures and cells.

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References

- [1] Hoy, D., March, L., Brooks, P., Blyth, F., Woolf, A., Bain, C., Williams, G., Smith, E., Vos, T., Barendregt, J., Murray, C., Burstein, R., and Buchbinder, R., 2014, "The Global Burden of Low Back Pain: Estimates From the Global Burden of Disease 2010 Study," *Ann. Rheum. Dis.*, **73**(6), pp. 968–974.
- [2] Vos, T., Flaxman, A. D., Naghavi, M., Lozano, R., Michaud, C., Ezzati, M., Shibuya, K., Salomon, J. A., Abdalla, S., Aboyans, V., Abraham, J., Ackerman, I., Aggarwal, R., Ahn, S. Y., Ali, M. K., AlMazroa, M. A., Alvarado, M., Anderson, H. R., Anderson, L. M., Andrews, K. G., Atkinson, C., Baddour, L. M., Bahalim, A. N., Barker-Collo, S., Barrero, L. H., Bartels, D. H., Basáñez, M.-G., Baxter, A., Bell, M. L., Benjamin, E. J., Bennett, D., Bernabé, E., Bhalla, K., Bhandari, B., Bikbov, B., Abdulhak, A. B., Birbeck, G., Black, J. A., Blencowe, H., Blore, J. D., Blyth, F., Bolliger, I., Bonaventure, A., Boufous, S., Bourne, R., Boussinesq, M., Braithwaite, T., Brayne, C., Bridgett, L., Brooker, S., Brooks, P., Brugh, T. S., Bryan-Hancock, C., Bucello, C., Buchbinder, R., Buckle, G., Budke, C. M., Burch, M., Burney, P., Burstein, R., Calabria, B., Campbell, B., Canter, C. E., Carabin, H., Carapetis, J., Carmona, L., Cella, C., Charlson, F., Chen, H., Tai-An Cheng, A., Chou, D., Chugh, S. S., Coffeng, L. E., Colan, S. D., Colquhoun, S., Colson, K. E., Condon, J., Connor, M. D., Cooper, L. T., Corriere, M., Cortinovis, M., de Vaccaro, K. C., Couser,

- W., Cowie, B. C., Criqui, M. H., Cross, M., Dabhadkar, K. C., Dahiya, M., Dahodwala, N., Damsere-Derry, J., Danaei, G., Davis, A., De Leo, D., Degenhardt, L., Dellavalle, R., Delossantos, A., Denenberg, J., Derrett, S., Des Jarlais, D. C., Dharmaratne, S. D., Dherani, M., Diaz-Torne, C., Dolk, H., Dorsey, E. R., Driscoll, T., Duber, H., Ebel, B., Edmond, K., Elbaz, A., Ali, S. E., Erskine, H., Erwin, P. J., Espindola, P., Ewoigbokhan, S. E., Farzadfar, F., Feigin, V., Felson, D. T., Ferrari, A., Ferri, C. P., Fèvre, E. M., Finucane, M. M., Flaxman, S., Flood, L., Foreman, K., Forouzanfar, M. H., Fowkes, F. G. R., Franklin, R., Fransen, M., Freeman, M. K., Gabbe, B. J., Gabriel, S. E., Gakidou, E., Ganatra, H. A., Garcia, B., Gaspari, F., Gillum, R. F., Gmel, G., Gosselin, R., Grainger, R., Groeger, J., Guillemin, F., Gunnell, D., Gupta, R., Haagsma, J., Hagan, H., Halasa, Y. A., Hall, W., Haring, D., Haro, J. M., Harrison, J. E., Havmoeller, R., Hay, R. J., Higashi, H., Hill, C., Hoen, B., Hoffman, H., Hotez, P. J., Hoy, D., Huang, J. J., Ibeanusi, S. E., Jacobsen, K. H., James, S. L., Jarvis, D., Jasrasaria, R., Jayaraman, S., Johns, N., Jonas, J. B., Karthikeyan, G., Kassebaum, N., Kawakami, N., Keren, A., Khoo, J.-P., King, C. H., Knowlton, L. M., Kobusingye, O., Koranteng, A., Krishnamurthi, R., Lalloo, R., Laslett, L. L., Lathlean, T., Leasher, J. L., Lee, Y. Y., Leigh, J., Lim, S. S., Limb, E., Lin, J. K., Lipnick, M., Lipshultz, S. E., Liu, W., Loane, M., Ohno, S. L., Lyons, R., Ma, J., Mabweijano, J., MacIntyre, M. F., Malekzadeh, R., Mallinger, L., Manivannan, S., Marcenes, W., March, L., Margolis, D. J., Marks, G. B., Marks, R., Matsumori, A., Matzopoulos, R., Mayosi, B. M., McAnulty, J. H., McDermott, M. M., McGill, N., McGrath, J., Medina-Mora, M. E., Meltzer, M., Memish, Z. A., Mensah, G. A., Merriman, T. R., Meyer, A.-C., Miglioli, V., Miller, M., Miller, T. R., Mitchell, P. B., Mocumbi, A. O., Moffitt, T. E., Mokdad, A. A., Monasta, L., Montico, M., Moradi-Lakeh, M., Moran, A., Morawska, L., Mori, R., Murdoch, M. E., Mwaniki, M. K., Naidoo, K., Nair, M. N., Naldi, L., Venkat Narayan, K. M., Nelson, P. K., Nelson, R. G., Nevitt, M. C., Newton, C. R., Nolte, S., Norman, P., Norman, R., O'Donnell, M., O'Hanlon, S., Olives, C., Omer, S. B., Ortblad, K., Osborne, R., Ozgediz, D., Page, A., Pahari, B., Durai Pandian, J., Panozo Rivero, A., Patten, S. B., Pearce, N., Padilla, R. P., Perez-Ruiz, F., Perico, N., Pesudovs, K., Phillips, D., Phillips, M. R., Pierce, K., Pion, S., Polanczyk, G. V., Polinder, S., Arden Pope, C., III, Popova, S., Porrini, E., Pourmalek, F., Prince, M., Pullan, R. L., Ramaiah, K. D., Ranganathan, D., Razavi, H., Regan, M., Rehm, J. T., Rein, D. B., Remuzzi, G., Richardson, K., Rivara, F. P., Roberts, T., Robinson, C., De León, F. R., Ronfani, L., Room, R., Rosenfeld, L. C., Rushton, L., Sacco, R. L., Saha, S., Sampson, U., Sanchez-Riera, L., Sanman, E., Schwebel, D. C., Scott, J. G., Segui-Gomez, M., Shahraz, S., Shepard, D. S., Shin, H., Shivakoti, R., Silberberg, D., Singh, D., Singh, G. M., Singh, J. A., Singleton, J., Sleet, D. A., Sliwa, K., Smith, E., Smith, J. A., Stapelberg, N. J. C., Steer, A., Steiner, T., Stolk, W. A., Jacob Stovner, L., Sudfeld, C., Syed, S., Tamburlini, G., Tavakkoli, M., Taylor, H. R., Taylor, J. A., Taylor, W., Thomas, B., Thomson, W. M., Thurston, G. D., Teyjeh, I. M., Tonelli, M., Towbin, J. A., Truelsen, T., Tsilimbaris, M. K., Ubeda, C., Undurraga, E. A., van der Werf, M. J., van Os, J., Vavilala, M. S., Venketasubramanian, N., Wang, M., Wang, W., Watt, K., Weatherall, D. J., Weinstock, M. A., Weintraub, R., Weisskopf, M. G., Weissman, M. M., White, R. A., Whiteford, H., Wiersma, S. T., Wilkinson, J. D., Williams, H. C., Williams, S. R. M., Witt, E., Wolfe, F., Woolf, A. D., Wulf, S., Yeh, P.-H., Zaidi, A. K. M., Zheng, Z.-J., Zonies, D., Lopez, A. D., and Murray, C. J. L., 2012, "Years Lived With Disability (YLDs) for 1160 Sequelae of 289 Diseases and Injuries 1990-2010: A Systematic Analysis for the Global Burden of Disease Study 2010," *Lancet*, **380**(9859), pp. 2163–2196.
- [3] Watkins-Castillo, S., and Andersson, G., 2014, "United States Bone and Joint Initiative: The Burden of Musculoskeletal Diseases in the United States (BMUS)," BMUS, Rosemont, IL, accessed May 8, 2017, <http://www.boneandjointburden.org>
- [4] Adams, M. A., and Dolan, P., 2012, "Intervertebral Disc Degeneration: Evidence for Two Distinct Phenotypes," *J. Anat.*, **221**(6), pp. 497–506.
- [5] Li, Z., Liu, H., Yang, H., Wang, J., Wang, H., Zhang, K., Ding, W., and Zheng, Z., 2014, "Both Expression of Cytokines and Posterior Annulus Fibrosis Rupture Are Essential for Pain Behavior Changes Induced by Degenerative Intervertebral Disc: An Experimental Study in Rats," *J. Orthop. Res.*, **32**(2), pp. 262–272.
- [6] Sharifi, S., Bulstra, S. K., Grijpma, D. W., and Kuijer, R., 2015, "Treatment of the Degenerated Intervertebral Disc: Closure, Repair and Regeneration of the Annulus Fibrosus," *J. Tissue Eng. Regen. Med.*, **9**(10), pp. 1120–1132.
- [7] Jensen, M., Brant-Zawadzki, M., Obuchowski, N., Modic, M., Malkasian, D., and Ross, J., 1994, "Magnetic Resonance Imaging of the Lumbar Spine in People Without Back Pain," *N. Engl. J. Med.*, **331**(2), pp. 69–73.
- [8] Iatridis, J. C., Nicoll, S. B., Michalek, A. J., Walter, B. A., and Gupta, M. S., 2013, "Role of Biomechanics in Intervertebral Disc Degeneration and Regenerative Therapies: What Needs Repairing in the Disc and What Are Promising Biomaterials for Its Repair?," *Spine J.*, **13**(3), pp. 243–262.
- [9] Lee, C. K., 1988, "Accelerated Degeneration of the Segment Adjacent to a Lumbar Fusion," *Spine*, **13**(3), pp. 375–377.
- [10] McGirt, M. J., Garcés Ambrossi, G. L., Dato, G., Sciubba, D. M., Witham, T. F., Wolinsky, J. P., Gokaslan, Z. L., and Bydon, A., 2009, "Recurrent Disc Herniation and Long-Term Back Pain After Primary Lumbar Discectomy: Review of Outcomes Reported for Limited Versus Aggressive Disc Removal," *Neurosurgery*, **64**(2), pp. 338–344.
- [11] Lebow, R. L., Adogwa, O., Parker, S. L., Sharma, A., Cheng, J., and McGirt, M. J., 2011, "Asymptomatic Same-Site Recurrent Disc Herniation After Lumbar Discectomy: Results of a Prospective Longitudinal Study With Two-Year Serial Imaging," *Spine*, **36**(25), pp. 2147–2151.
- [12] Carragee, E. J., Han, M. Y., Suen, P. W., and Kim, D., 2003, "Clinical Outcomes After Lumbar Discectomy for Sciatica: The Effects of Fragment Type and Anular Competence," *J. Bone Joint Surg. Am.*, **85-A**(1), pp. 102–108.

- [13] Carragee, E. J., Spinnickie, A. O., Alamin, T. F., and Paragioudakis, S., 2006, "A Prospective Controlled Study of Limited Versus Subtotal Posterior Discectomy: Short-Term Outcomes in Patients With Herniated Lumbar Intervertebral Discs and Large Posterior Annular Defect," *Spine*, **31**(6), pp. 653–657.
- [14] Daneyemez, M., Sali, A., Kahraman, S., Beduk, A., and Seber, N., 1999, "Outcome Analyses in 1072 Surgically Treated Lumbar Disc Herniations," *Minimally Invasive Neurosurg.*, **42**(2), pp. 63–68.
- [15] Henriksen, L., Schmidt, K., Eskesen, V., and Jantzen, E., 1996, "A Controlled Study of Microsurgical Versus Standard Lumbar Discectomy," *Br. J. Neurosurg.*, **10**(3), pp. 289–293.
- [16] Bailey, A., Araghi, A., Blumenthal, S., and Huffmon, G. V., 2013, "Prospective, Multicenter, Randomized, Controlled Study of Annular Repair in Lumbar Discectomy: Two-Year Follow-Up," *Spine*, **38**(14), pp. 1161–1169.
- [17] Ahlgren, B. D., Lui, W., Herkowitz, H. N., Panjabi, M. M., and Guiboux, J. P., 2000, "Effect of Annular Repair on the Healing Strength of the Intervertebral Disc: A Sheep Model," *Spine*, **25**(17), pp. 2165–2170.
- [18] Chiang, Y. F., Chiang, C. J., Yang, C. H., Zhong, Z. C., Chen, C. S., Cheng, C. K., and Tsuang, Y. H., 2012, "Retaining Intradiscal Pressure After Annulotomy by Different Annular Suture Techniques, and Their Biomechanical Evaluations," *Clin. Biomech.*, **27**(3), pp. 241–248.
- [19] Chiang, C.-J., Cheng, C.-K., Sun, J.-S., Liao, C.-J., Wang, Y.-H., and Tsuang, Y.-H., 2011, "The Effect of a New Annular Repair After Discectomy in Intervertebral Disc Degeneration: An Experimental Study Using a Porcine Spine Model," *Spine*, **36**(10), pp. 761–769.
- [20] Bron, J. L., Van Der Veen, A. J., Helder, M. N., Van Royen, B. J., and Smit, T. H., 2010, "Biomechanical and In Vivo Evaluation of Experimental Closure Devices of the Annulus Fibrosus Designed for a Goat Nucleus Replacement Model," *Eur. Spine J.*, **19**(8), pp. 1347–1355.
- [21] Wilke, H.-J., Widmann, L., Graf, N., and Heuer, F., 2011, "Can Herniation Be Prevented? Establishment of a Herniation Model and Experiments With an Annulus Reconstruction Implant," *Spine J.*, **11**(10), pp. S148–S149.
- [22] Trummer, M., Eustacchio, S., Barth, M., Klassen, P. D., and Stein, S., 2013, "Protecting Facet Joints Post-Lumbar Discectomy: Barricaid Annular Closure Device Reduces Risk of Facet Degeneration," *Clin. Neurol. Neurosurg.*, **115**(8), pp. 1440–1445.
- [23] Buser, Z., Kuelling, F., Liu, J., Liebenberg, E., Thorne, K. J., Coughlin, D., and Lotz, J. C., 2011, "Biological and Biomechanical Effects of Fibrin Injection Into Porcine Intervertebral Discs," *Spine*, **36**(18), pp. E1201–E1209.
- [24] Claus, A., Hides, J., Moseley, G. L., and Hodges, P., 2008, "Sitting Versus Standing: Does the Intradiscal Pressure Cause Disc Degeneration or Low Back Pain?," *J. Electromyogr. Kinesiol.*, **18**(4), pp. 550–558.
- [25] Long, R., Torre, O., Hom, W., Assael, D., and Iatridis, J., 2016, "Design Requirements for Annulus Fibrosus Repair: Review of Forces, Displacements and Material Properties of the Intervertebral Disc and a Summary of Candidate Hydrogels for Repair," *ASME J. Biomech. Eng.*, **138**(2), p. 021007.
- [26] Buser, Z., Liu, J., Thorne, K. J., Coughlin, D., and Lotz, J. C., 2014, "Inflammatory Response of Intervertebral Disc Cells Is Reduced by Fibrin Sealant Scaffold In Vitro," *J. Tissue Eng. Regener. Med.*, **8**(1), pp. 77–84.
- [27] Yin, W., Pauza, K., Olan, W. J., Doerzbacher, J. F., and Thorne, K. J., 2014, "Intradiscal Injection of Fibrin Sealant for the Treatment of Symptomatic Lumbar Internal Disc Disruption: Results of a Prospective Multicenter Pilot Study With 24-Month Follow-Up," *Pain Med.*, **15**(1), pp. 16–31.
- [28] Sabins, G., and Wheeler, J., 2013, "Spinal Restoration, Inc. Announces Disappointing Phase III Study Results for the Biostat® System," Business Wire, Inc., San Francisco, CA, accessed May 8, 2017, <http://www.businesswire.com/news/home/20130718005215/en/spinal-restoration-announces-disappointing-phase-iii-study>
- [29] Spinal Restoration, Inc., 2014, "Clinical Trial Identifier: NCT01011816," Spinal Restoration, Inc., Austin, TX, accessed May 8, 2017, <https://clinicaltrials.gov/ct2/show/study/NCT01011816>
- [30] Likhitanichkul, M., Kim, Y., Torre, O. M., See, E., Kazezian, Z., Pandit, A., Hecht, A. C., and Iatridis, J. C., 2015, "Fibrin-Genipin Annulus Fibrosus Sealant as a Delivery System for Anti-TNF α Drug," *Spine J.*, **15**(9), pp. 2045–2054.
- [31] Guterl, C. C., Torre, O. M., Purmessur, D., Dave, K., Likhitanichkul, M., Hecht, A. C., Nicoll, S. B., and Iatridis, J. C., 2014, "Characterization of Mechanics and Cytocompatibility of Fibrin-Genipin Annulus Fibrosus Sealant With the Addition of Cell Adhesion Molecules," *Tissue Eng. Part A*, **20**(17–18), pp. 2536–2545.
- [32] Bigi, A., Cojazzi, G., Panzavolta, S., Roveri, N., and Rubini, K., 2002, "Stabilization of Gelatin Films by Crosslinking With Genipin," *Biomaterials*, **23**(24), pp. 4827–4832.
- [33] Touyama, R., Inoue, K., Takeda, Y., Yatsuzuka, M., Ikumoto, T., Moritome, N., Shingu, T., Yokoi, T., and Inouye, H., 1994, "Studies on the Blue Pigments Produced From Genipin and Methylamine II on the Formation Mechanisms of Brownish-Red Intermediated Leading to the Blue Pigment Formation," *Chem. Pharm. Bull.*, **42**(8), pp. 1571–1578.
- [34] Dare, E. V., Griffith, M., Poitras, P., Kaupp, J. A., Waldman, S. D., Carlsson, D. J., Dervin, G., Mayoux, C., and Hincke, M. T., 2009, "Genipin Cross-Linked Fibrin Hydrogels for In Vitro Human Articular Cartilage Tissue-Engineered Regeneration," *Cell Tissue Organs*, **190**(6), pp. 313–325.
- [35] Mwale, F., Iordanova, M., Demers, C. N., Steffen, T., Roughley, P., and Antoniou, J., 2005, "Biological Evaluation of Chitosan Salts Cross-Linked to Genipin as a Cell Scaffold for Disk Tissue Engineering," *Tissue Eng.*, **11**(1–2), pp. 130–140.
- [36] Schek, R. M., Michalek, A. J., and Iatridis, J. C., 2011, "Genipin-Crosslinked Fibrin Hydrogels as a Potential Adhesive to Augment Intervertebral Disc Annulus Repair," *Eur. Cells Mater.*, **21**, pp. 373–383.
- [37] Likhitanichkul, M., Dreischarf, M., Illien-Junger, S., Walter, B. A., Nukaga, T., Long, R. G., Sakai, D., Hecht, A. C., and Iatridis, J. C., 2014, "Fibrin-Genipin Adhesive Hydrogel for Annulus Fibrosus Repair: Performance Evaluation With Large Animal Organ Culture, In Situ Biomechanics, and In Vivo Degradation Tests," *Eur. Cells Mater.*, **28**, pp. 25–38.
- [38] Long, R. G., Bürki, A., Zysset, P., Eglin, D., Grijpma, D. W., Blanquer, S. B. G., Hecht, A. C., and Iatridis, J. C., 2016, "Mechanical Restoration and Failure Analyses of a Hydrogel and Scaffold Composite Strategy for Annulus Fibrosus Repair," *Acta Biomater.*, **30**, pp. 116–125.
- [39] Mundargi, R. C., Babu, V. R., Rangaswamy, V., Patel, P., and Aminabhavi, T. M., 2008, "Nano/Micro Technologies for Delivering Macromolecular Therapeutics Using Poly(D,L-Lactide-Co-Glycolide) and Its Derivatives," *J. Controlled Release*, **125**(3), pp. 193–209.
- [40] Ulery, B. D., Nair, L. S., and Laurencin, C. T., 2011, "Biomedical Applications of Biodegradable Polymers," *J. Polym. Sci. B Polym. Phys.*, **49**(12), pp. 832–864.
- [41] Yu, S. C., Park, S. N., and Suh, H. K., 2005, "Adipose Tissue Engineering Using Mesenchymal Stem Cells Attached to Injectable PLGA Spheres," *Biomaterials*, **26**(29), pp. 5855–5863.
- [42] Rezwani, K., Chen, Q. Z., Blaker, J. J., and Boccaccini, A. R., 2006, "Biodegradable and Bioactive Porous Polymer/Inorganic Composite Scaffolds for Bone Tissue Engineering," *Biomaterials*, **27**(18), pp. 3413–3431.
- [43] Sahoo, S., Toh, S. L., and Goh, J. C. H., 2010, "A BFGF-Releasing Silk/PLGA-Based Biohybrid Scaffold for Ligament/Tendon Tissue Engineering Using Mesenchymal Progenitor Cells," *Biomaterials*, **31**(11), pp. 2990–2998.
- [44] Baker, S. C., Rohman, G., Southgate, J., and Cameron, N. R., 2009, "The Relationship Between the Mechanical Properties and Cell Behaviour on PLGA and PCL Scaffolds for Bladder Tissue Engineering," *Biomaterials*, **30**(7), pp. 1321–1328.
- [45] Gentile, P., Chiono, V., Carmagnola, I., and Hatton, P. V., 2014, "An Overview of Poly(Lactic-co-Glycolic) Acid (PLGA)-Based Biomaterials for Bone Tissue Engineering," *Int. J. Mol. Sci.*, **15**(3), pp. 3640–3659.
- [46] Jiao, Y., Gyawali, D., Stark, J. M., Akcora, P., Nair, P., Tran, R. T., and Yang, J., 2012, "A Rheological Study of Biodegradable Injectable PEGMC/HA Composite Scaffolds," *Soft Matter*, **8**(5), pp. 1499–1507.
- [47] ASTM, 2014, "Standard Test Method for Tensile Properties of Plastics," ASTM International, West Conshohocken, PA, Standard No. **ASTM D638-02A**.
- [48] Maher, S. A., Mauck, R. L., Rackwitz, L., and Tuan, R. S., 2010, "A Nanofibrous Cell-Seeded Hydrogel Promotes Integration in a Cartilage Gap Model," *J. Tissue Eng. Regener. Med.*, **4**(1), pp. 25–29.
- [49] Yuan, Y., Chesnutt, B. M., Utturkar, G., Haggard, W. O., Yang, Y., Ong, J. L., and Bumgardner, J. D., 2007, "The Effect of Cross-Linking of Chitosan Microspheres With Genipin on Protein Release," *Carbohydr. Polym.*, **68**(3), pp. 561–567.
- [50] Liang, H.-C., Chang, W.-H., Lin, K.-J., and Sung, H.-W., 2003, "Genipin-Crosslinked Gelatin Microspheres as a Drug Carrier for Intramuscular Administration: In Vitro and In Vivo Studies," *J. Biomed. Mater. Res. A*, **65A**(2), pp. 271–282.
- [51] Sen, S., Jacobs, N. T., Boxberger, J. I., and Elliott, D. M., 2012, "Human Annulus Fibrosus Dynamic Tensile Modulus Increases With Degeneration," *Mech. Mater.*, **44**, pp. 93–98.
- [52] Wang, Y., Ameer, G. A., Sheppard, B. J., and Langer, R., 2002, "A Tough Biodegradable Elastomer," *Nat. Biotechnol.*, **20**(6), pp. 602–606.
- [53] Kosaraju, S. L., Puvanenthiran, A., and Lillford, P., 2010, "Naturally Cross-linked Gelatin Gels With Modified Material Properties," *Food Res. Int.*, **43**(10), pp. 2385–2389.
- [54] Chik, T. K., Ma, X. Y., Choy, T. H., Li, Y. Y., Diao, H. J., Teng, W. K., Han, S. J., Cheung, K. M. C., and Chan, B. P., 2013, "Photochemically Crosslinked Collagen Annulus Plug: A Potential Solution Solving the Leakage Problem of Cell-Based Therapies for Disc Degeneration," *Acta Biomater.*, **9**(9), pp. 8128–8139.
- [55] Mi, F. L., Shyu, S. S., and Peng, C. K., 2005, "Characterization of Ring-Opening Polymerization of Genipin and pH-Dependent Cross-Linking Reactions Between Chitosan and Genipin," *J. Polym. Sci. Part A Polym. Chem.*, **43**(10), pp. 1985–2000.
- [56] Butler, M. F., Ng, Y. F., and Pudney, P. D. A., 2003, "Mechanism and Kinetics of the Crosslinking Reaction Between Biopolymers Containing Primary Amine Groups and Genipin," *J. Polym. Sci. Part A Polym. Chem.*, **41**(24), pp. 3941–3953.
- [57] Gamboa-Martínez, T. C., Luque-Guillén, V., González-García, C., Gómez Ribelles, J. L., and Gallego-Ferrer, G., 2015, "Crosslinked Fibrin Gels for Tissue Engineering: Two Approaches to Improve Their Properties," *J. Biomed. Mater. Res. A*, **103**(2), pp. 614–621.
- [58] Sung, H. W., Huang, R. N., Huang, L. L., and Tsai, C. C., 1999, "In Vitro Evaluation of Cytotoxicity of a Naturally Occurring Cross-Linking Reagent for Biological Tissue Fixation," *J. Biomater. Sci. Polym. Ed.*, **10**(1), pp. 63–78.
- [59] Tsai, C. C., Huang, R. N., Sung, H. W., and Liang, H. C., 2000, "In Vitro Evaluation of the Genotoxicity of a Naturally Occurring Crosslinking Agent (Genipin) for Biologic Tissue Fixation," *J. Biomed. Mater. Res.*, **52**(1), pp. 58–65.
- [60] Hung, L.-H., Teh, S.-Y., Jesterb, J., and Lee, A. P., 2010, "PLGA Micro/Nanosphere Synthesis by Droplet Microfluidic Solvent Evaporation and Extraction Approaches," *R. Soc. Chem.*, **10**, pp. 1820–1825.
- [61] Jeon, H. J., Jeong, Y. I., Jang, M. K., Park, Y. H., and Nah, J. W., 2000, "Effect of Solvent on the Preparation of Surfactant-Free Poly(DL-Lactide-Co-Glycolide) Nanoparticles and Norfloxacin Release Characteristics," *Int. J. Pharm.*, **207**(1–2), pp. 99–108.
- [62] Wilson, C. B., 2006, "Adoption of New Surgical Technology," *BMJ*, **332**(7533), pp. 112–114.
- [63] Ahn, H., Bhandari, M., and Schemitsch, E. H., 2009, "An Evidence-Based Approach to the Adoption of New Technology," *J. Bone Joint Surg. Am.*, **91**(Suppl. 3), pp. 95–98.