# A 'Clickable' Hydrogel for Annulus Fibrosus Repair After Intervertebral Disc Herniation

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**INTRODUCTION:** Aging, trauma, and overuse can lead to intervertebral disc (IVD) herniations, where the nucleus pulposus (NP) extrudes beyond the boundaries of the annulus fibrosus (AF). This disrupts the functional role of the IVD and can result in significant pain. Microdiscectomy, where the herniated NP tissue is surgically excised, is the gold standard of care, but does not restore AF mechanical integrity, and reherniations may occur through the unrepaired defect. Multifunctional biomaterials may overcome this limitation and achieve functional AF repair. We developed a norbornene-modified hyaluronic acid (NorHA) hydrogel that satisfies baseline AF repair criteria (biocompatible, injectable, mechanically compatible)[1] and is capable of spatiotemporal controlled delivery via click chemistry. Click chemistry is a class of reactions where two small molecules rapidly and strongly bind to one another and has recently been successfully leveraged for therapeutic delivery *in vivo*.[2] In this study, we evaluated the durability of click-functionalization, characterized hydrogel material properties, and demonstrated an *ex vivo* AF repair.

**METHODS:** Hydrogel Fabrication: NorHA synthesis and hydrogel fabrication were accomplished using established protocols.[3] Percent crosslinking was 50% (unless otherwise specified) and hydrogel weight percent varied between 3-5%. Click-functionalization was achieved via the addition of thiol-PEG-azide (TPA) to the hydrogel precursor solution. The level of TPA modification varied from ~3.3% (TPA1) to ~13.2% (TPA4). Upon UV exposure, hydrogel photocrosslinking and azide-modification occur simultaneously. Click Validation: Here, the click counterpart to azide is dibenzocyclooctyne (DBCO). 3 wt% NorHA and NorHA-TPA hydrogels, of varying degrees of azide-modification, were fabricated, allowed to swell overnight in PBS, and incubated in a 30 μM DBCO-modified Alexa Fluorophore (AF-488-DBCO) solution for 1 hour. Hydrogels were imaged via an Axiozoom microscope for up to 4 weeks and incubated in PBS on an orbital shaker at 4 C between imaging. Mechanical and Rheological Properties: 3 and 5 wt% NorHA and NorHA-TPA4 hydrogels were tested using a stress relaxation protocol (10% strain at 0.05%/s followed by stress relaxation for 10 min.) to determine the equilibrium modulus. To assess gelation kinetics, 5 wt% NorHA and NorHA-TPA4 precursor solutions were exposed to UV light for 180 seconds and storage modulus was assessed via rheology at 1% strain at 1 Hz. Curves were fitted with a one phase association exponential fit to determine Tau values (i.e., time to gelation). Cytocompatibility: Bovine AF cells (40,000 cells/cm$^2$) were seeded on sterile 3 wt% NorHA and NorHA-TPA4 hydrogels additionally modified with thiol-RGD peptide (1 mM). Cell viability and proliferation were monitored over 7 days via the AlamarBlue assay, and cells were imaged on day 3 to investigate cell adhesion. Gel Retention in AF defects: A cruciate injury to the AF was created in bovine caudal discs, and the defect was either left empty or filled with 5 wt% NorHA-TPA4 with a crosslinking density of 80%. The explant was cycled under physiologic loading (300 N) for 10,000 cycles using an Instron. Statistical Analysis: All statistics were conducted in GraphPad Prism with significance thresholds of *p<0.05 and **p<0.01. Outliers were removed from each dataset and significant differences were detected using the appropriate ANOVA test.

**RESULTS:** DBCO-modified fluorophores preferentially bound to NorHA-TPA hydrogels, and the degree of this attachment could be tailored based on degree of azide-modification (Fig. 1). Specifically, increasing azide-modification increased DBCO-fluorophore attachment and prolonged length of attachment. Hydrogel mechanics could be tailored by changing weight percent (Fig. 2A). While the addition of TPA did not impact bulk hydrogel mechanics, it slightly slowed gelation kinetics as indicated by larger Tau values for NorHA-TPA hydrogels; overall, gelation remained rapid (<30 s) (Fig. 2B). Additionally, AF cells readily adhered to the hydrogels by day 3 (Fig. 2C) and cell proliferation increased over time (Fig. 2D). Lastly, NorHA-TPA gel mediated repair of an annular defect and remained within the defect following 10,000 physiological compression cycles (Fig. 3).

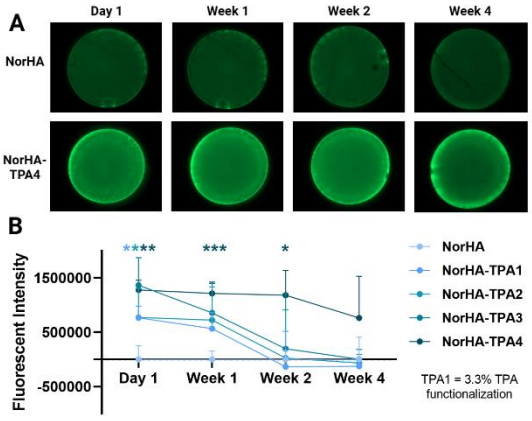


**Figure 1**: A) Hydrogels with varying levels of TPA incubated in AF-488-DBCO and washed for up to 4 weeks. B) Fluorescent intensity when varying TPA modification. n=4

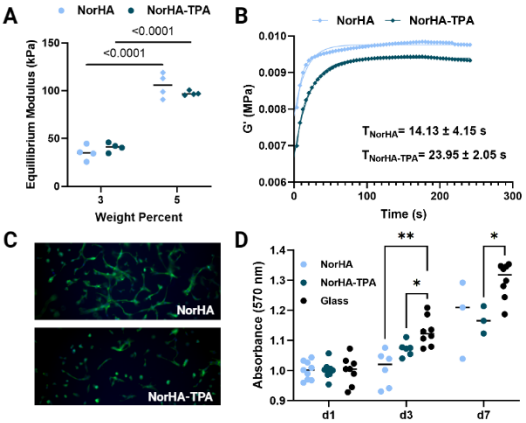


**Figure 2**: A) Equilibrium moduli for NorHA and NorHA-TPA4 hydrogels of varying weight percent. B) Gelation kinetics of hydrogels with respective Tau values. C) Day 3 images of AF cells seeded onto hydrogels. D) AlamarBlue data of cells seeded onto hydrogels for up to a week. n=≥3

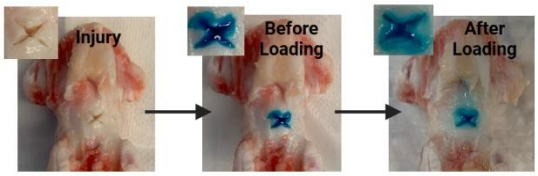


**Figure 3**: AF repair via NorHA-TPA (blue) before and after physiologic loading for 10,000 cycles. n=1

**DISCUSSION:** NorHA hydrogels are promising candidates for tissue repair due to their injectability, biocompatibility, and biodegradability, but do not inherently possess controlled delivery capacity. Here, we demonstrated that NorHA hydrogels can be functionalized to enable controlled delivery via secondary azide-alkyne click reactions. The addition of TPA enhanced attachment of DBCO-modified fluorophores and varying the degree of azide-modification enabled tailoring of the extent and duration of attachment. This suggests that DBCO-modified therapeutics or nanocarriers can be effectively tethered to the hydrogel, localizing the therapeutic with considerable control over its release profile. Importantly, TPA incorporation did not adversely impact hydrogel mechanics, mechanical tunability, or gelation kinetics. Cell adhesion and proliferation were also not affected by TPA addition, although there are observable differences in cell morphology, which may be due to increased hydrophobicity of the NorHA-TPA hydrogels. Excitingly, *ex vivo* testing demonstrated that NorHA-TPA remained within the annular defect over 10,000 cycles of physiologic loading. Future work will explore these hydrogels *in vivo* via subcutaneous implantation and disc injury models, in addition to evaluating DBCO-modified therapeutic and nanocarrier attachment to the hydrogel and therapeutic release profiles.

**SIGNIFICANCE:** Our 'clickable' hydrogel has the potential to repair the AF following herniation, transforming clinical practices and creating superior surgical alternatives and outcomes for patients. Broadly, this novel multifunctional biomaterial can be employed to repair multiple different tissue types and injuries, revolutionizing how we approach dense connective tissue repair.

**REFERENCES:** 1. Long, RG, *et. al*., J Biomech. Eng. (2016) 2. Peplow, M. Nature Biotech. (2023) 3. Gramlich, WM, *et. al*., Biomaterials (2013)

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