ORIGINAL ARTICLE

Electrospun Microfiber Scaffolds with Anti-Inflammatory Tributanoylated N-Acetyl-D-Glucosamine Promote Cartilage Regeneration

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Tissue-engineering strategies offer promising tools for repairing cartilage damage; however, these strategies suffer from limitations under pathological conditions. As a model disease for these types of nonideal systems, the inflammatory environment in an osteoarthritic (OA) joint limits the efficacy of engineered therapeutics by disrupting joint homeostasis and reducing its capacity for regeneration. In this work, we investigated a sugar-based drug candidate, a tributanoylated N-acetyl-D-glucosamine analogue, called 3,4,6-*O*-Bu₃GlcNAc, that is known to reduce nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling in osteoarthritis. 3,4,6-*O*-Bu₃GlcNAc not only inhibited NFκB signaling but also exerted chondrogenic and anti-inflammatory effects on chondrocytes isolated from patients with osteoarthritis. 3,4,6-*O*-Bu₃GlcNAc also increased the expression of extracellular matrix proteins and induced cartilage tissue production in three-dimensional *in vitro* hydrogel culture systems. To translate these chondrogenic and anti-inflammatory properties to tissue regeneration in osteoarthritis, we implanted 3,4,6-*O*-Bu₃GlcNAc-loaded poly(lactic-co-glycolic acid) microfiber scaffolds into rats. The drug-laden scaffolds were biocompatible, and when seeded with human OA chondrocytes, similarly promoted cartilage tissue formation. 3,4,6-*O*-Bu₃GlcNAc combined with the appropriate structural environment could be a promising therapeutic approach for osteoarthritis.

Introduction

ARTICULAR CARTILAGE LACKS the innate ability to self-heal after the progressive cartilage damage due to its avascular nature and the low mitotic activity of the residing cells.¹ Cartilage tissue is homogeneous and has both a well-understood structure and function. It has therefore become a natural target of tissue engineering approaches.² Currently, natural and synthetic fiber scaffolds have seen implementation clinically as both cell-seeded and acellular structures in autologous repair operations.³ These efforts are working to establish scaffolds that can recapitulate the local extracellular matrix (ECM) environment, spatiotemporally direct cellular organization and differentiation, and integrate with host tissue.^{4–7} Scaffold-based tissue engineering has provided a promising option for regenerative medicine applications.⁸

Early uses for scaffold-based tissue engineering began with the implementation of a degradable polyester scaffold for cellular transplantation by Vacanti *et al.* in the late

1980s.⁹ These scaffolds provided structure to inform cellular function until natural metabolic processes can replace the degradable material with cell-derived components, ideally leading to integration with healthy host tissue. This is essential for chondrocytes and mesenchymal stem cells (MSCs) cultured *in vitro* before implantation, as a scaffold is necessary for ECM protein deposition.¹⁰ Different scaffold materials such as collagen type I, chitosan, hyaluronic acid, poly(lactic-co-glycolic acid) (PLGA), and many other synthetic polymers—and various combinations of these materials—have been tested with positive outcomes for the support of cartilage tissue growth/differentiation.^{11–13}

Typically, the canonical models for the study of these materials have been undertaken under nonpathological conditions with healthy cells and free from inflammatory factors. However, clinically articular cartilage is often needed in patients where the cells and local environment are less than ideal and include significant inflammatory components due to trauma, disease, or even surgical intervention, which can increase scaffold degradation, reduce

cellular proliferation, and reduce ECM deposition.¹⁴ Although some studies have introduced growth factors to combat the diseased environment, the short half-life and low stability of these materials make them ineffectual for long-term change in the slowly healing and tumultuous environment of the synovial joint.^{15,16} Currently, no scaffold and active signaling agent combination has been able to provide both long-term mechanical stability, for cellular proliferation and tissue integration with the host, and the chemical cues necessary to halt the malignant effects of pathological or traumatic inflammation due to surgery or disease.

Osteoarthritis is a leading cause of chronic disability throughout the world that is instigated by a local inflammatory reaction in the synovial joint.^{17,18} During the onset and progression of osteoarthritis, dominating catabolic regulators disturb the homeostatic balance, resulting in the cartilage tissue degradation.¹⁴ Of these mediators, proinflammatory cytokines and regulating factors, including matrix metalloproteinases (MMPs), interleukin-1 β (IL-1 β), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), are critical in the pathophysiology of osteoarthritis.^{19–22} In particular, NF- κ B activity modulates many degradation pathways, so small molecules that target NF- κ B activity have attracted considerable attention as a new class of therapeutics in the treatment of osteoarthritis.^{23,24} Furthermore, small molecules have various therapeutic advantages over growth factors because of their stability in the physiological environment, which can make them more compatible with hydrophobic polymer scaffolds than their growth factor counterparts.^{25,26}

Here we investigate electropun PLGA scaffold seeded with a sugar-based modulator of the NF- κ B pathway, tributanoyletated hexosamine analogue 3,4,6-*O*-Bu₃GlcNAc (GlcNAc-a, Fig. 1), which we have previously shown to have anti-inflammatory and further anabolic activity in bovine chondrocytes *in vitro*.²⁷ We have combined the chondrogenic potential of the scaffold with 3,4,6-*O*-Bu₃GlcNAc to provide a suitable three-dimensional (3D) microenvironment for tissue ingrowth and cartilage structural foundation while limiting local inflammation. We demonstrated biocompatibility of this drug-loaded scaffold *in vivo* in rodents and verified the anti-inflammatory and chondrogenic properties of the scaffold on primary human osteoarthritic (hOA) chondrocytes. Our tissue engineering and controlled OA drug release system use polymers and compounds safe for use in humans and promises a regenerative approach to a disease with limited options.

Results

Monolayer IL-1 β stimulated hOA chondrocytes

hOA chondrocytes isolated from osteoarthritis patients undergoing knee arthroplasty (NDRI) were used in this study to confirm the effects of 3,4,6-*O*-Bu₃GlcNAc previously observed in bovine cells in clinically relevant human cells. hOA chondrocytes were stimulated with 10 ng/mL of IL-1 β for 24 h of culture and compared with unstimulated cells. IL-1 β has been shown to be a key signaling molecule in the development of osteoarthritis and used for generating an *in vitro* OA disease model.^{28–30} After 24 h of monolayer culture, 100 μ M of 3,4,6-*O*-Bu₃GlcNAc was supplemented into the media of both stimulated and unstimulated cells. We observed negligible

FIG. 1. Anti-inflammatory effect of 3,4,6-*O*-Bu₃GlcNAc on hOA chondrocytes. **(A)** Chemical structure of 3,4,6-*O*-Bu₃GlcNAc (2-acetamido-2-deoxy-3,4,6-tri-*O*-butanoyl- α , β -D-glucopyranose, GlcNAc-a). **(B)** Expression levels of genes related to osteoarthritis and NF- κ B pathway. qRT-PCR results from monolayer hOA chondrocytes stimulated with IL-1 β (10 ng/mL). hOA chondrocytes were cultured in media supplemented with or without IL-1 β for 24 h and then further incubated with or without 3,4,6-*O*-Bu₃GlcNAc (100 μ M) in the presence of IL-1 β for another 24 h. Total RNA was collected at the 48 h time point and analyzed through qRT-PCR. Three individual samples were collected and averaged ($n = 3$, ** $p < 0.01$, *** $p < 0.001$). hOA, human osteoarthritic; IL-1 β , interleukin-1 β ; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

changes in cell viability at 100 μ M of 3,4,6-*O*-Bu₃GlcNAc in the presence of IL-1 β (10 ng/mL), which was confirmed by Alamar blue assay. Changes in cellular phenotype were measured using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Exposure to IL-1 β upregulated the expression levels of all genes transcriptionally regulated by NF- κ B, specifically, *NFKB1* and *IkB α* , and proinflammatory related genes, *IL-1 β* and *MMP13* (Fig. 1). IL-1 β -stimulated chondrocytes treated with 100 μ M of 3,4,6-*O*-Bu₃GlcNAc had significantly decreased expression levels of these proinflammatory genes (** $p < 0.01$, *** $p < 0.001$), confirming previous results from *in vitro* models.³¹

Anti-inflammatory effect of 3,4,6-*O*-Bu₃GlcNAc on hOA chondrocytes in 3D poly(ethylene glycol) hydrogel

We next evaluated the anti-inflammatory effect of 3,4,6-*O*-Bu₃GlcNAc in a 3D poly(ethylene glycol) (PEG)

hydrogel system that mimics the cartilage tissue environment and supports chondrogenesis.^{32–34} After encapsulation of the hOA chondrocytes in hydrogels, the constructs were treated for 21 days with 10 ng/mL of IL-1 β to maintain the OA phenotype. qRT-PCR was used to analyze expression of chondrogenic and inflammatory markers. Significant decreases in inflammatory genes, including *I κ B α* , *MMP13*, and *NFKB1* (Fig. 2A), and increases in *SOX9*, *AGGRECAN*, and *TYPE II COLLAGEN* expression were observed for hOA chondrocytes exposed to 3,4,6-*O*-Bu₃GlcNAc (Fig. 2B). The amount of sulfated glycosaminoglycans (sGAG) extracted from the hydrogel treated with 100 μ M of 3,4,6-*O*-Bu₃GlcNAc significantly increased, whereas DNA levels

did not change (Fig. 2C, D). Cartilage ECM accumulation was qualitatively assessed using Safranin-O staining and type II collagen immunohistochemistry (Fig. 2E, F). Together, the 3D environment with 3,4,6-*O*-Bu₃GlcNAc increased the cartilage phenotype of hOA cells.

Development of a cartilage regeneration system using electrospun microfibers

Electrospun microfibers allow the encapsulation of small molecules in a biodegradable fibrous scaffold for local drug delivery as well as structural support for regenerating tissues *in vivo*.^{35,36} PLGA can be electrospun and is both biodegradable and biocompatible, making it an ideal scaffold for drug delivery and cartilage tissue engineering.^{37–39} A total of 20% wt PLGA and 10% wt 3,4,6-*O*-Bu₃GlcNAc were electrospun with different flow rates and voltages to afford thick (diameter: 200 $\pm$ 20 μ m) and thin (diameter: 20 $\pm$ 2 μ m) fibers. The changes in the morphology of the fibers were analyzed by scanning electron microscopy (SEM) as shown in Figure 3A.

FIG. 2. 3,4,6-*O*-Bu₃GlcNAc in 3D hydrogels promotes cartilage formation by hOA chondrocytes. (A, B) Gene expression levels of the inflammatory (A) and chondrogenic (B) markers after 21 days of 3,4,6-*O*-Bu₃GlcNAc (GlcNAc-a) exposure to the hydrogels ($n = 3$) in the presence of IL-1 β (10 ng/mL). (C) DNA content, normalized to dry weight ($n = 3$). (D) sGAG, normalized to the DNA content ($n = 3$). In (A to D), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (E) Safranin-O and immunohistochemical type II collagen staining. 3D, three-dimensional; sGAG, sulfated glycosaminoglycans. Color images available online at www.liebertpub.com/tea

FIG. 3. Development of a tissue engineering and drug delivery scaffold using electrospun microfibers and 3,4,6-*O*-Bu₃GlcNAc. (A) Scanning electron microscopy images of the PLGA fibers. (B) Drug release of 3,4,6-*O*-Bu₃GlcNAc (GlcNAc-a) was achieved at a concentration of 20% (wt:wt) PLGA:DCM with linearity being maintained at different fiber diameters. Release from (1) thick and (2) thin fibers was compared with that from the (3) 3D poly(ethylene glycol) hydrogel system ($n = 3$). (C) Cytotoxicity of the thick and thin PLGA fibers was compared with that of tissue culture plate conditions. (D) Hematoxylin and eosin staining of subcutaneously implanted PLGA and PLGA-GlcNAc-a (3,4,6-*O*-Bu₃GlcNAc) microfibers at 1 and 4 week time points. Scale bars: 1 mm. PLGA, poly(lactic-co-glycolic acid). Color images available online at www.liebertpub.com/tea

Drug release profiles were characterized for both thick and thin fibers and compared with free drug release in the 3D PEG hydrogel system. Release profile of 3,4,6-*O*-Bu₃GlcNAc into aqueous media is independent of the thickness of the fiber (Fig. 3B). The fibers were tested for cytotoxicity by culturing chondrocytes for 6 days and comparing with tissue culture plate (TCP) conditions. No significant difference was observed between any of the test groups (Fig. 3C). Because the thick and thin fibers exhibited little difference in the 3,4,6-*O*-Bu₃GlcNAc release profiles ($R^2=0.97$ and $R^2=0.96$, respectively), the thick fibers were selected and used for all remaining experiments.

Cartilage regeneration in vivo using 3,4,6-*O*-Bu₃GlcNAc in polymer microfibers

A subcutaneous implantation of the PLGA fibers was performed in a rat model to assess the immunogenic response. PLGA fibers containing 3,4,6-*O*-Bu₃GlcNAc were compared with control fibers not loaded with the sugar analogue at both the 1 and 4 week time points. Typical fibrous encapsulation of the scaffold was observed at all time points (Fig. 3D), signifying a normal immune response to the implants.⁴⁰ The capsule containing 3,4,6-*O*-Bu₃GlcNAc demonstrated increased tissue ingrowth in all samples at both time points, whereas the sample containing PLGA had no tissue ingrowth, consistent with a previous report on rats.⁴¹

Anti-inflammatory effect of 3,4,6-*O*-Bu₃GlcNAc-loaded PLGA microfiber scaffolds

PLGA-GlcNAc-a and PLGA fibers were seeded with hOA chondrocytes, and ECM accumulation and expression of chondrogenic and inflammatory markers were compared. In the PLGA-GlcNAc-a scaffolds, we observed upregulated chondrocyte ECM markers (Fig. 4A), reductions of inflammatory markers *IL-1 β* , *IkB α* , *MMP13*, and *NFkB1* (Fig. 4B), and more sGAG within the matrix (Fig. 4C, D) compared with PLGA scaffolds alone. Cells seeded on the scaffold containing 3,4,6-*O*-Bu₃GlcNAc showed higher proteoglycan content, which was confirmed by Safranin-O and type II collagen staining (Fig. 4E, F).

Discussion

Current pharmacologic OA treatments focus on pain and joint function management, rather than a disease-modifying treatment; consequently there remains a considerable need to develop disease-modifying OA drugs (DMOADs) with an appropriate delivery strategy for encouraging the regrowth of damaged articular cartilage. In this work, we demonstrated the effectiveness of a sugar-based OA drug compound, 3,4,6-*O*-Bu₃GlcNAc, that exhibited chondroprotective effects on hOA chondrocytes in 3D scaffolds by modulating inflammatory response.

Chondrocytes located in articular cartilage play a key role in regulating homeostasis of the cartilage through modulating production of the ECM and enzymes to degrade aging ECM.⁴² During the onset of osteoarthritis, catabolic regulators become dominant and involved in the chronic inflammation in the joint. The inflammatory process secretes a number of inflammatory cytokines and mediators, which can generate a continuous positive feedback loop of the in-

FIG. 4. Anti-inflammatory effect of 3,4,6-*O*-Bu₃GlcNAc released from PLGA microfibers. hOA chondrocytes were cultured alongside fibers in the presence of IL-1 β (10 ng/mL) for 21 days. (A) Gene expression levels of the chondrogenic markers and (B) inflammation-related genes ($n=3$). (C) Biochemical analysis of DNA contents, normalized to dry weight and (D) sGAG, normalized to the DNA content ($n=3$, $*p<0.05$, $**p<0.01$). (E) Histological staining of the PLGA microfiber scaffolds with Safranin-O and immunohistochemical staining of type II collagen. Scale bars: 50 μ m. Color images available online at www.liebertpub.com/tea

flammatory process and further disturb the regeneration capability of the tissue. The transcription factor NF- κ B is one of the major inflammatory pathways in osteoarthritis.¹⁷ Inflammatory cytokines such as IL-1 β can initiate the mitogen-activated protein kinase signaling cascade resulting in activation of transcription factors, including NF- κ B. NF- κ B then promotes MMP13 expression among other inflammatory factors, which can reduce type II collagen deposition in the cartilage and synovial lining.⁴³ Several current DMOADs that focus on cytokine inhibition attempt to regulate individual cytokine expression such as TNF- α , iNOS, and IL-1 β . NF- κ B is activated when proinflammatory

cytokines such as IL-1 β stimulate toll-like receptors that promote the phosphorylation of I κ B that, in turn, releases NF κ B1 into the nucleus where it binds to DNA and promotes genes containing proinflammatory cytokines, such as IL-1 β .⁴⁴

In this study, hOA chondrocytes under pathophysiological conditions exhibited significant downregulation of gene expression level in the inflammatory targets involved in NF- κ B activities when treated with 3,4,6-*O*-Bu₃GlcNAc (Figs. 1 and 2). This anti-inflammatory effect of 3,4,6-*O*-Bu₃GlcNAc associated with inhibited NF- κ B activities was observed in our previous studies on cytokine-stimulated bovine chondrocytes.^{27,31} The consistent downregulation of the NF- κ B activity linked with inflammatory genes suggests that 3,4,6-*O*-Bu₃GlcNAc plays a role in the inhibition of this transcription factor. The cartilage tissue production associated with the inhibitory effect was investigated on 3D hydrogel *in vitro* culture. We confirmed increased proteoglycan accumulation along with upregulation of chondrogenic related genes (Fig. 2), indicating an anabolic effect of 3,4,6-*O*-Bu₃GlcNAc on the hOA chondrocytes. Further studies on the mechanism of action and target identification will be required to establish whether 3,4,6-*O*-Bu₃GlcNAc simply inhibits catabolic pathways or whether it also promotes anabolic activity directly.

Although intraarticular (IA) injection of drugs provides one option for drug delivery in a joint,¹⁷ the rapid clearance of the injected drugs through the draining lymphatic system of the synovial fluid limits therapeutic efficacy of the IA injection.¹⁸ Accordingly, incorporation of the drugs with a sustained release system will be necessary for clinical applications.⁴⁵ Electrospun fibers have been a well-established model system for sustained drug release and promoting tissue growth in *in vivo* applications.^{46,47} Our findings showed the biocompatibility of PLGA-microfibers (degradation half time: 3 weeks) with encapsulating 3,4,6-*O*-GlcNAc-a in a subcutaneous implantation *in vivo* model (Fig. 3).⁴⁸ In addition, we have demonstrated that sustained release of 3,4,6-*O*-Bu₃GlcNAc has the potential to modulate cellular behavior (Fig. 4). The promotion of anabolic activity by 3,4,6-*O*-Bu₃GlcNAc in conjunction with the scaffold gives it the potential as a possible implant for tissue reconstruction in the joint. Microfracture surgery, a first-line therapy to treat small cartilage lesions, stimulates progenitor cells in the bone marrow by creation of small holes in the cartilage defects, and scaffolds have been proposed for application within the defects to improve its efficacy.^{49,50} Although the microfracture technique recruits MSCs to the cartilage defects from subchondral bone, OA physiological condition may provide a suboptimal environment for the therapy because a large number of inflammatory cytokines present in an OA joint disrupt the regeneration capacity of the MSCs.^{51–53} By modulating NF- κ B activity, a 3,4,6-*O*-Bu₃GlcNAc-incorporated microfiber scaffold has the potential to reduce local inflammation from these procedures and protect local MSCs from inflammatory signaling—thus promoting the production of cartilaginous tissue.

In summary, we identified a sugar-based compound, 3,4,6-*O*-Bu₃GlcNAc, that has chondroprotective capacity by modulating inflammatory response associated with NF- κ B activity. Exposure of 3,4,6-*O*-Bu₃GlcNAc on hOA chondrocytes could improve cartilage tissue production and its

incorporation into PLGA microfibers showed sustained release delivery with biocompatibility for future clinical applications. Reproduction of these results in established translational models of osteoarthritis will be necessary to further evaluate the potential of this class of drugs.

Materials and Methods

Materials and cell culture

The sugar analogue compound, tributanoylated N-acetyl-D-glucosamine (3,4,6-*O*-Bu₃GlcNAc), was synthesized and characterized in the previous report.⁵⁴ Human articular cartilage samples explanted from OA patients (three patients) undergoing total knee arthroplasty were received from the National Disease Resource Institution (Philadelphia, PA) according to an IRB-approved protocol. The cartilage tissue was cut into 1 mm³ pieces, washed three times with phosphate-buffered saline (PBS) supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin (Pen/Strep), and digested on an orbital shaker for 16 h at 37°C with 0.17% (w/v) type II collagenase (Worthington Biochemical) in high-glucose Dulbecco's modified Eagle's medium (DMEM; In-vitrogen™, Life Technologies) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific). After the digestion, the filtrate was passed through a 70 μ m strainer and cells were rinsed three times with the DMEM supplemented with Pen/Strep and 10% FBS.

Cell culture in 3D PEG hydrogel and PLGA microfibers

Poly(ethylene glycol)-diacrylate (PEGDA, 100 mg; Sun-Bio) was dissolved in 1.0 mL sterile PBS. The photoinitiator Irgacure 2959 (BASF) was dissolved in 70% ethanol at a concentration of 10% and 5 μ L of the photoinitiator added to the 1.0 mL PEGDA solution. Cells (passage 1) were suspended in the PEGDA precursor solution at a density of 2×10^7 cells/mL and 100 μ L of the cell suspension transferred into sterile cylindrical molds. Polymer crosslinking was initiated through UV-A exposure (365 nm, 3.2 mW/cm²) for 5 min. Cell-laden hydrogels were transferred into 24-well plates and cultured in a 1.0 mL medium with or without 10 ng/mL of recombinant human IL-1 β . The medium consists of high-glucose DMEM, Pen/Strep, ITS premix (6.25 μ g/mL insulin, 6.25 μ g/mL transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL bovine serum albumin (BSA), 5.35 μ g/mL linoleic acid; BD Bioscience), 100 mM sodium pyruvate (Life Technologies), 40 mg/mL L-proline (Sigma-Aldrich), and 50 mM ascorbic acid-2-phosphate. After 3 days, the medium was changed to a medium containing 3,4,6-*O*-Bu₃GlcNAc with or without 10 ng/mL of IL-1 β . Similarly, for PLGA-fiber cell culture, 100 μ L of cell suspension (2×10^7 cells/mL) and 7 mg of PLGA microfibers were placed into sterile cylindrical molds. The molds were transferred into 24-well plates and cultured in a 1.0 mL of medium with or without 10 ng/mL of IL-1 β . The hydrogels and the PLGA fibers were cultured for an additional 21 days and the medium was changed three times per week ($n=3$ per group).

Biochemical analysis for GAG contents

The hydrogel or PLGA fiber constructs ($n=3$ per group) were lyophilized for 2 days and then digested overnight in

125 mg/mL papainase (Worthington Biochemical Corp.) for 16 h at 60°C. The sGAG content was determined by 1,9-dimethylmethylene blue (DMMB) dye assay and measuring the absorbance at 525 nm and using chondroitin sulfate as a standard.⁵⁵ The DNA content was determined by using Hoescht Dye 33342 DNA assay and calf thymus DNA as a standard.⁵⁶ The GAG content levels are expressed as micrograms of GAG per microgram of DNA for each construct.

Real-time PCR

Total mRNA was extracted from cells, hydrogels, and the PLGA fibers using Trizol reagent (Life Technologies) and cDNA was synthesized by using Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed using StepOnePlus Real Time PCR System (Applied Biosystems) with SYBR Green PCR Master Mix (Life Technologies). The relative expression of each target was calculated using the $\Delta\Delta CT$ method and β -actin and GAPDH were used as endogenous references. All expression levels of samples were normalized to controls. The PCR primers are listed as follows: β -actin (F: 5'-GCTCCTCCTGAGCG CAAGTAC-3' and R: 5'-GGACTCGTCATACTCCTGCT TGC-3'), GAPDH (F: 5'-TGAAGGTCTGGAGTCAACGGA TTTGGT-3' and R: 5'-CATGTGGGCCATGAGGTCCA CCAC-3'), Sox9 (F: 5'-GCATGAGCGAGGTGCACTC-3' and R: 5'-TCTCGCTTCAGGTCAGCCTTG-3'), type II collagen (F: 5'-CGCCGCTGTCCTTCGGTGTC-3' and R: 5'-AGGGCTCCGGCTTCCACACAT-3'), Aggrecan (F: 5'-TGGAACAGCCTATACCCAG-3' and R: 5'-CAGT TGCAGAAGGGCCTTCTGTAC-3'), MMP13 (F: 5'-TGGT CCAGGAGATGAAGACC-3' and R: 5'-TCCTCGGAGAC TGGTAATGG-3'), Ikb α (F: 5'-GCTGATGTCAATGCT CAGGA-3' and R: 5'-CCCCACACTTCAACAGGAGT-3'), IL-1 β (F: 5'-GGACAAGCTGAGGAAGATGC-3' and R: 5'-TCGTTATCCCATGTGTGCGAA-3'), NFkB1 (F: 5'-CTG GAAGCACGAATGACAGA-3' and R: 5'-CCTTCTGCTT GCAAATAGGC-3').

Histology and immunohistochemistry of hydrogel and PLGA fibers

The hydrogel and PLGA fiber constructs ($n=3$) were fixed in 4% paraformaldehyde overnight, dehydrated in increasing concentrations of ethanol, and embedded with paraffin. Five micrometer-thick sections were cut from the paraffin block and collected onto the glass slides. The sections were stained for proteoglycans with aqueous Safranin-O (0.1%) for 5 min, and then the specimens were mounted. For immunohistochemical staining, endogenous peroxidase of the sections was quenched using 2.5% (v/v) hydrogen peroxide in methanol and then incubated at 37°C with 0.25% (w/v) hyaluronidase for 1 h. AEC Broad Spectrum Histostain-SP Kit (Invitrogen) was used following the manufacturer's instructions. Primary antibodies for type II collagen (Abcam) using a 1:300 dilution factor in 4% BSA were dissolved in PBS.

Electrospun fiber preparation

The polymer solutions were loaded into a syringe and the flow rate was controlled using a syringe pump (NE-1000; New Era Pump Systems). A high voltage (Gamma High voltage Research) was applied to a needle as the solution

was being sprayed onto a stainless steel tray (32 cm L $\times$ 26 cm W $\times$ 6 cm H) that was placed at an angle of 35°C to the pump with a distance of 11 cm from the needle. The polymer solution compositions for the microfibers as well as electrospaying parameters were varied to produce fibers with different characteristics. The polymer solution of 20% wt PLGA and 10% wt GlcNAc-a dissolved in pure DCM was loaded in a 1 mL, BD syringe with a flow rate of 0.4 mL/hr and a voltage of 12 kV applied to the needle (22G 1 1/2 flattened tip). The polymer solution was electrospayed as a thick stream giving relatively thick microfibers of $200 \pm 20 \mu\text{m}$ in diameter. An identical solution was electrospayed using a 30 mL Norm-Ject syringe, a voltage of 11 kV, and a flow rate of 0.1 mL/hr to give microfibers of $20 \pm 2 \mu\text{m}$ in diameter. All microfibers were dried using lyophilization for 4 days. Fibers were then stored under inert gas at -20°C. The drug loading efficiency and drug loading content were 99.7% and 32.3%, respectively.

SEM characterization

The microparticles and microfibers were characterized using SEM. The samples were coated with 40 nm of Pd and the accelerating voltage was set to 5 kV. Representative images were chosen and diameter was measured using Adobe Photoshop CS5 software.

In vitro drug release study

PEGDA was dissolved in sterile PBS (10% [w/v]). The photoinitiator Irgacure 2959 and 10% GlcNAc-a were added to the PEGDA solution. The solution was then vortexed and 300 μL of the solution was set into the cylindrical cap of a 1.5 mL Eppendorf tube with a diameter of 10.9 mm. The caps were placed under UV light for ~5 min to crosslink (mesh size: 14 nm). The 3D hydrogel and the fibers were placed in a 24-multiwell TCP. The samples were incubated at 37°C and 5% CO₂ after 2 mL of PBS buffer was pipetted to each well. At specific time points, 1 mL was collected for high performance liquid chromatography (HPLC) analysis and the 2 mL buffer was refreshed. Three individual samples were tested to measure reproducibility and error.

High performance liquid chromatography

The collected analogue release samples were examined using a HPLC system composed of a pump, autosampler, analytical column (C18 column, 4.6 mm $\times$ 250 mm $\times$ 5 mm), and UV/VIS detector set at 230 nm. The mobile phase was 42:58 acetonitrile (with 10% water) and water with (0.1% trifluoroacetic acid). The flow rate was set at 1 mL/min and run time for each sample set to 10 min. The retention time for GlcNAc-a was 5.1 min. The standard calibration curve or plot of average area under curve versus drug concentration was linear with a regression coefficient of 0.99. The calibration curve was produced by plotting the results of four different standard solutions with concentrations of 500, 200, 40, and 8 $\mu\text{g/mL}$ that were repeatedly tested at the beginning, middle, and end of each HPLC run.

Cytotoxicity cell study

The electrospayed drug delivery vehicles were placed in inserts of the transwell and immersed in a well with primary

chondrocytes at a density of $\sim 2 \times 10^4$ cells/well. A positive control of primary chondrocytes was used to compare with the control fibers, to determine cytotoxicity of the fibers. A 24-transwell TCP was used and each condition was triplicated. Eighty-five microliters of Alamar Blue[®] (Life Technologies) was added to each sample and used to measure the cellular metabolism. The cell culture plate was incubated at 37°C and 5% CO₂. Three 110 μ L samples were drawn from each sample after 4 h and added to a 96-well culture plate (Falcon[™]). Cell viability was measured with the Synergy 2 Multi-Mode Reader. Percentage viability was calculated by dividing the fluorescence emission of cells exposed to 3,4,6-*O*-Bu₃GlcNAc by the fluorescence emission of untreated cells in media. (560 nm excitation, 590 nm emission).

Subcutaneous implantation of PLGA-GlcNAc-a fibers

To assess the inflammatory response of the electrospun fiber scaffolds, fiber scaffolds containing 10% GlcNAc-a were implanted into 6-week old male Sprague Dawley rats according to approved ACUC protocols (Johns Hopkins School of Medicine). Rats were anesthetized using 3% isoflurane and then shaved and sanitized with 70% ethanol and iodine before two midline incisions were made along the dorsum of the rat, and pockets were made for subcutaneous implantation using blunt dissection to the right and left of the incision. Sections (20 mg) of the 200 μ m microfiber scaffolds without cells ($n=3$) were implanted into each pocket and the incision was closed using 4-0 nylon fibers. Rats were then sacrificed after 1 and 4 weeks, and the dermal region containing the encapsulated fiber was surgically removed and fixed in 4% paraformaldehyde overnight to prepare for sucrose: optimum cutting temperature compound (OCT) infiltration. Samples were then processed with sucrose and OCT using standard methods before being immersed in pure OCT solution, frozen with liquid nitrogen, and finally stored at -80°C . Sections of the samples were then obtained using a Cryosectioner Leica CM3050 S rotary microtome at -80°C . Histological examination of the tissue was carried out using standard Harris hematoxylin and eosin Y staining.

Statistical analysis

Data are expressed as mean standard deviation and the statistical significance (p value) was determined by an unpaired t -test or one-way analysis of variance. The statistical significance was determined at $p < 0.05$.

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Disclosure Statement

No competing financial interests exist.

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