Localized delivery of ibuprofen via a bilayer delivery system (BiLDS) for supraspinatus tendon healing in a rat model

Brittany L. Taylor1,2 | Dong Hwa Kim1,2 | Julianne Huegel1,2 | Harina A. Raja1,2 | Sophie J. Burkholder2 | Stephanie N. Weiss1,2 | Courtney A. Nuss1,2 | Louis J. Soslowsky1,2 | Robert L. Mauck1,2 | Andrew F. Kuntz1,2 | Joseph Bernstein1,2

1Translational Musculoskeletal Research Center, Corporal Michael J. Crescenz VA Medical Center, Philadelphia, Pennsylvania
2McKay Orthopaedic Research Laboratory, Department of Orthopaedic Surgery, University of Pennsylvania, Philadelphia, Pennsylvania

Correspondence
Joseph Bernstein, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6081.
Email: Orthodoc@uphs.upenn.edu

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Abstract
The high prevalence of tendon retear following rotator cuff repair motivates the development of new therapeutics to promote improved tendon healing. Controlled delivery of non-steroidal anti-inflammatory drugs to the repair site via an implanted scaffold is a promising option for modulating inflammation in the healing environment. Furthermore, biodegradable nanofibrous delivery systems offer an optimized architecture and surface area for cellular attachment, proliferation, and infiltration while releasing soluble factors to promote tendon regeneration. To this end, we developed a bilayer delivery system (BiLDS) for localized and controlled release of ibuprofen (IBP) to temporally mitigate inflammation and enhance tendon remodeling following surgical repair by promoting organized tissue formation. In vitro evaluation confirmed the delayed and sustained release of IBP from Labrafil-modified poly(lactic-co-glycolic) acid microspheres within sintered poly(ε-caprolactone) electrospun scaffolds. Biocompatibility of the BiLDS was demonstrated with primary Achilles tendon cells in vitro. Implantation of the IBP-releasing BiLDS at the repair site in a rat rotator cuff injury and repair model led to decreased expression of proinflammatory cytokine, tumor necrotic factor-α, and increased anti-inflammatory cytokine, transforming growth factor-β1. The BiLDS remained intact for mechanical reinforcement and recovered the tendon structural properties by 8 weeks. These results suggest the therapeutic potential of a novel biocompatible nanofibrous BiLDS for localized and tailored delivery of ibuprofen to mitigate tendon inflammation and improve repair outcomes. Future studies are required to define the mechanical implications of an optimized BiLDS in a rat model beyond 8 weeks or in a larger animal model.

KEYWORDS
biomaterials, tendon, tissue engineering

Statement of Clinical Significance: This study investigates a biocompatible nanofibrous bilayer delivery system (BiLDS) for localized and controlled delivery of ibuprofen to mitigate inflammation following rotator cuff repair. Further evaluation is necessary to elucidate the prolonged beneficial effects of the system in a larger animal model.
1 | INTRODUCTION

Musculoskeletal injuries and conditions affect over 28 million people in the United States annually. Rotator cuff tendon tears due to overuse or degeneration are common shoulder injuries and result in pain, instability, or decreased shoulder function. Surgical repair to restore function is a common procedure and non-steroidal inflammatory drugs (NSAIDs) may be prescribed during the postsurgical period to mitigate pain and inflammation. Unfortunately, many studies have shown the detrimental effects of traditional and cyclooxygenase-2 specific (COX-2) NSAIDs on tendon healing, which is likely a contributor to the high postoperative retear rate. The mechanistic relationship between NSAIDs and tendon healing remains unclear but it is hypothesized that the inhibition of COX decreases prostaglandin E2 (PGE2) levels which hinders tendon-to-bone healing. Several studies utilizing the rat rotator cuff injury model demonstrated that early systemic delivery of NSAIDs impaired healing during the proliferative phase while delayed delivery improved remodeling by decreasing inflammation.

We observed this time-dependent effect in a recent study where early oral administration of NSAIDs adversely affected rat rotator cuff tendon healing, but these effects were offset by delayed oral administration.

Delivery systems can be utilized to release NSAIDs, such as ibuprofen (IBP), in vivo in a temporally controlled manner to modulate the tendon healing environment. Previously, we evaluated a biodegradable scaffold with IBP incorporated throughout the nanofibrous network for localized delivery. Unfortunately, this system resulted in rapid IBP release both in vitro in physiological conditions and subcutaneously in a rat model. Therefore, we identified a need for a delivery system with tunable properties to deliver IBP in a sustained manner without adversely affecting the tendon healing environment. Furthermore, a local delivery system would require a lower dose of IBP than systemic administration and minimize the negative side effects of NSAIDs on the gastrointestinal and cardiovascular systems.

To this end, we developed a bilayer delivery system (BiLDS), which dually offers a threedimensional surface for cellular attachment and infiltration, and localized delayed release of IBP. BiLDS is a biodegradable scaffold system that incorporates poly (lactic-co-glycolic) acid (PLGA) microspheres sandwiched between two sintered poly(ε-caprolactone) (PCL) nanofibrous scaffolds (Figure 1A). Previous work assessed the use of this novel system both in vitro and implanted subcutaneously to deliver basic fibroblast growth factor (bFGF), but the in vivo effects and release kinetics of IBP from this system on tendon healing are unknown.

Therefore, the purpose of this study was to (a) evaluate the release profile of IBP from microspheres alone and within the BiLDS, (b) elucidate the effect of IBP on primary tendon cells in vitro, and (c) investigate the biological and mechanical effects of the sustained release of IBP from BiLDS implanted at the repair site in our well-established rat supraspinatus injury and repair model. We hypothesized that IBP would release at a faster rate in physiological-like conditions compared to the release in standard buffer solution and that the in vitro delivery of IBP would not have an adverse effect on cellular viability. Furthermore, we hypothesized that the release of IBP from the BiLDS in vivo would improve tendon healing by decreasing the expression of proinflammatory cytokines, thus improving tendon remodeling and mechanical properties.

2 | METHODS

2.1 | Microspheres and BiLDS fabrication

PLGA (75:25 PLGA; molecular weight [MW] = 70 kDa; 0.15 g/mL) microspheres were created using an oil-in-water emulsion technique with an external phase of 1% poly(vinyl alcohol) (PVA). Labrafil M 1944 CS oil was added to the PLGA, PVA, dichloromethane mixture in 30, 300, or 600 µL amounts and stirred overnight to create the L30, L300, and L600 PLGA microspheres. The L0 microspheres did not contain any Labrafil. Labrafil is a polyethylene glycol (PEG) derivative and surfactant used to increase the hydrophilicity of polymeric surfaces for drug delivery. A total of 30 mg/mL of IBP (MW = 206.28 g/mol) was also added to the mixture for the IBP-loaded microspheres and stirred for 4 hours at room temperature.

![Figure 1](https://example.com/figure1.png)

**FIGURE 1** (A) Schematic of the bilayer delivery system (BiLDS) and (B) Study design for the rat supraspinatus detachment and repair in vivo study. The groups were supraspinatus tendon injury and repair: without BiLDS (No_BiLDS), with empty BiLDS (BiLDS), and with ibuprofen-releasing BiLDS (BiLDS_IBP). The animals were sacrificed at 1 (n = 6/group), 4 (n = 12/group), and 8 (n = 12/group) weeks post-surgery. BiLDS, bilayer delivery system [Color figure can be viewed at wileyonlinelibrary.com]
Based on the sustained linear release of IBP from the L300 microspheres in vitro, BiLDS were created by entrapping 10 mg of the L300 microspheres in phosphate buffer saline (PBS), with and without IBP (BiLDS_IBP and BiLDS, respectively), between two heat sintered 6 × 8 mm electrospun PCL scaffolds to create approximately 100 µm thick BiLDS. The microspheres and BiLDS were then imaged with an environmental scanning electron microscope (ESEM).Abbreviations for the groups are outlined in Table 1.

### 2.2 | In vitro release and cell studies

Two in vitro release studies were conducted. In the first release study, 20 mg of free IBP-loaded microspheres with varying amounts of Labrafil were submerged in 5 mL of normal rat serum or 1X PBS and incubated at 37°C. The quantity of total IBP released (µg/mL) was measured over 14 days with enzyme-linked immunosorbent assay (ELISA) and UV spectrophotometer (λ = 223 nm). In the second release study, BiLDS and BiLDS_IBP were incubated on a shaker at 37°C in 5 mL serum for up to 14 days. In one group, the total 5 mL of serum was collected at 0.5, 3, 7, 14 days (BiLDS and BiLDS_IBP) and in the “continuous” groups (BiLDS_Cont and BiLDS_IBP_Cont) 2 mL of serum was collected at the same timepoints and replaced with fresh serum to assess the effect of serum exchange on the release of IBP from the BiLDS over time.

Primary tendon cells were isolated from Achilles tendons excised from adult male Sprague-Dawley rats (400–450 g). The tendon was incubated on tissue culture polystyrene (TCP) in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS) and 2X penicillin-streptomycin (P/S) to allow the cells to migrate out of the tissue. The tendon cells were then cultured in the supplemented media for 1 week. Biocompatibility of the BiLDS was tested both directly and indirectly. For the direct study, the cells (passage 1) were seeded at 5 × 10^3 cells per construct on 6 × 8 mm BiLDS without microspheres (BiLDS), BiLDS with empty microspheres (BiLDS_MS), and BiLDS with IBP-loaded microspheres (BiLDS_IBP). The cell-seeded constructs were maintained in DMEM with 1% FBS up to 10 days. For the indirect study, identical BiLDS, BiLDS_MS, and BiLDS_IBP constructs were submerged in culture media for 1 week and the conditioned media from these samples was then added to cells cultured on TCP at 5 × 10^3 cells per well up to 10 days. The control group was cells cultured in normal culture media. MTT assay was employed to quantify cell metabolic activity.

### 2.3 | Rotator cuff surgical procedure

This study was approved by the Philadelphia Veteran Affairs Medical Center Animal Component of Research Protocol Committee. Ninety adult male Sprague-Dawley rats (400–450 g) underwent bilateral supraspinatus detachment with immediate repair. Animals were randomly divided into three groups: repair without BiLDS (No_BiLDS), repair with 3 × 5 mm BiLDS which did not contain microspheres or IBP (BiLDS), and repair with 3 × 5 mm BiLDS_IBP

### Table 1 | Microspheres and BiLDS groups

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td><strong>In vitro release studies</strong></td>
<td></td>
</tr>
<tr>
<td>L0</td>
<td>PLGA microspheres with 0 µL Labrafil and 30 mg/mL IBP</td>
</tr>
<tr>
<td>L30</td>
<td>PLGA microspheres with 30 µL Labrafil and 30 mg/mL IBP</td>
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<tr>
<td>L300</td>
<td>PLGA microspheres with 300 µL Labrafil and 30 mg/mL IBP</td>
</tr>
<tr>
<td>L600</td>
<td>PLGA microspheres with 600 µL Labrafil and 30 mg/mL IBP</td>
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<tr>
<td>BiLDS</td>
<td>BiLDS construct without microspheres or IBP in static serum conditions</td>
</tr>
<tr>
<td>BiLDS_Cont</td>
<td>BiLDS construct without microspheres or IBP in serum exchange conditions</td>
</tr>
<tr>
<td>BiLDS_IBP</td>
<td>BiLDS construct with L300 IBP-loaded microspheres in static serum conditions</td>
</tr>
<tr>
<td>BiLDS_IBP_Cont</td>
<td>BiLDS construct with L300 IBP-loaded microspheres in serum exchange conditions</td>
</tr>
<tr>
<td><strong>In vitro cell studies (direct and indirect)</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Cells seeded on tissue culture polystyrene (TCP) or in normal culture media</td>
</tr>
<tr>
<td>BiLDS</td>
<td>Cells seeded on empty BiLDS constructs (no microspheres or IBP) or cultured in BiLDS-conditioned media</td>
</tr>
<tr>
<td>BiLDS_MS</td>
<td>BiLDS constructs with empty microspheres (no IBP) or cultured in media conditioned with BiLDS_MS constructs</td>
</tr>
<tr>
<td>BiLDS_IBP</td>
<td>BiLDS constructs with L300 IBP-loaded microspheres or cultured in media conditioned with BiLDS_IBP constructs</td>
</tr>
<tr>
<td><strong>In vivo rotator cuff study</strong></td>
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<tr>
<td>No_BiLDS</td>
<td>Repaired tendon without BiLDS</td>
</tr>
<tr>
<td>BiLDS</td>
<td>Repaired tendon with empty BiLDS constructs (no microspheres or IBP)</td>
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<tr>
<td>BiLDS_IBP</td>
<td>Repaired tendon with L300 IBP-loaded microspheres</td>
</tr>
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Abbreviations: BiLDS, bilayer delivery system; IBP, ibuprofen; PLGA, poly(lactic-co-glycolic) acid.
containing 170 µg of IBP-loaded L300 PLGA microspheres in 1 µL of PBS (BiLDS_IBP) (n = 30/group). The L300 IBP-loaded microspheres with 30 mg/mL of IBP and BiLDS constructs were created as described above. Based on previous in vitro release and in vivo systemic IBP delivery studies, we predicted the BiLDS_IBP constructs would deliver approximately 270 µg of cumulative IBP to the injury site over an 8-week period.\(^\text{12}\)

Animals were anesthetized via inhalation of 5% isoflurane and maintained at 2% to 3% throughout surgery. Supraspinatus detachment was performed as previously described using sterile technique,\(^\text{13,14}\) and the BiLDS were overlaid on the repair site and attached by suturing the BiLDS proximally to the tendon and distally to the 0.5 mm bone tunnel drilled through the greater tuberosity. Rats were allowed ad lib cage activity and administered buprenorphine every 12 hours for 3 days postoperation. Rats were euthanized at 1, 4, and 8 weeks post-surgery and all of the supraspinatus tendons were utilized for biological or mechanical assays (Figure 1B). The right supraspinatus tendons were immediately grossly dissected and processed for histological assessment (n = 6 tendons per group per all timepoints), or ELISA (n = 6 tendons per group per 4- and 8-week timepoints). The left supraspinatus tendons from the 1-week animals were immediately placed in liquid nitrogen upon harvest and stored in −80°C for quantitative real-time polymerase chain reaction (qRT-PCR) (n = 6 tendons per group per 1-week timepoint). Blood serum was also collected from all animals. Rats with the left intact shoulders from the 4- and 8-week timepoints (n = 12 tendons per group per timepoint) were stored in −20°C prior to thawing for quasi-static mechanical testing.

### 2.4 Histology and immunohistochemistry

The grossly dissected muscle-tendon-bone complexes (n = 6/group/timepoint) were fixed in paraformaldehyde, decalcified, processed in paraffin, and bisected along the sagittal plane. Five micrometers thick sections were stained with hematoxylin and eosin (H&E) to evaluate tendon cellularity and nuclear shape between groups and over time. Blind semi-quantitative grading was performed on a scale of 1 (more spindle shape or low cellularity) to 3 (more rounded shape or high cellularity). Immunohistochemistry was also performed using antibodies to assess cellularity and nuclear shape between groups and over time.

Histology and immunohistochemistry were performed on 4- and 8-week timepoint specimens. Stain specificity was maintained according to the instructions provided by the manufacturers.

### 2.5 Gene and protein expression

Total RNA from pulverized 1-week specimens was precipitated using centrifugation phase separation and Trizol (Thermo Fisher Scientific) (n = 6/group). qRT-PCR was run in quadruplicate using TaqMan assays (Applied Biosystems by Life Technologies) and on the BioMark HS System (Fluidigm, San Francisco). The reverse transcription reaction was carried out with High Capacity cDNA synthesis kit with RNase inhibitor (Applied Biosystems by Life Technologies) in 20 µL reactions containing 1.0 µg RNA. All of the primers were purchased from Thermo Fisher Scientific as selected markers for gene expression in inflammation, tendon remodeling, and tendon repair: tumor necrotic factor-α (TNF-α), interleukins-1b (IL-1b), -6 (IL-6), and -10 (IL-10), PGE2, CD68, and CD163, transforming growth factors-1 (TGF-β1) and -3 (TGF-β3), bFGF1, vascular endothelial growth factor B, collagen types-1 (COL I), -3 (COL III), and -4 (COL IV), MMP2, MMP3, MMP9, and MMP13, tenascin-C (TNC), tenomodulin (Tmn), and aggrecan (ACAN). The TaqMan assay ID information is shown in Table 2. The cycle threshold values were calculated with Fluidigm Real-Time PCR Analysis Software and normalized to the internal control housekeeping (glyceraldehyde 3-phosphate dehydrogenase, Rn01775763_g1). The fold change was calculated by normalizing the treatment groups to the control group (No_BiLDS).

ELISAs for TNF-α (RAB0480; Sigma-Aldrich, St Louis), IL-6 (RAB3012; Sigma-Aldrich), and TGF-β1 (ab119668; Abcam, Cambridge) were performed on protein isolates from the excised tendons. Competitive ELISA for IBP (Neogen, Lexington) was also performed on the blood serum of all animals at 4 and 8 weeks. ELISA analysis was performed according to the instructions provided by the manufacturers.

### 2.6 Biomechanical testing

Left supraspinatus tendons and humeri were finely dissected from animals sacrificed at 4 and 8 weeks (n = 12/group/timepoint). Stain lines were placed on the tendon at the insertion, and 2, 4, and 8 mm proximal to the insertion for optical strain tracking. Cross-sectional area was measured using a custom laser device. The distal humerus was then potted with polymethylmethacrylate in a custom fixture with the inferior border of the humeral head resting on the lip of the pot. The tendon was sandwiched and adhered between two sandpaper squares and a custom gripping fixture. Tensile testing was performed on an Instron 5543 as follows: preload to 0.1N, preconditioning between 0.1 and 0.5N for 10 cycles, followed by ramp to 5% strain at 5% per second and stress relaxation for 10 minutes. The tendons were then ramped to failure at 0.3% strain per second.
Stress relaxation, modulus, stiffness, maximum load, and maximum stress were computed.

2.7 | Statistical analysis

Two-way analysis of variance (ANOVA) and normality tests were performed on all datasets. One-way ANOVAs with Tukey's post hoc test or Kruskall-Wallis test and Welch’s t tests or Mann-Whitney U tests were performed, as appropriate, with Bonferroni Correction. Comparisons were made between groups at each timepoint, and over time for cytokine expression measured quantitatively. Sample size for the animal study was determined based on power analysis. Significance was set at *P < .05 (*). Data is presented as mean ± standard deviation or median ± interquartile range when specified in figure legend.

3 | RESULTS

3.1 | In vitro release and cell studies

The microspheres in each group were qualitatively consistent in morphology and similar to the representative image of L300 microspheres (Figure 2A), with diameters ranging between 5 and 50 µm. ESEM images of the BiLDS confirmed the pocket of microspheres between the two sintered 6 × 8 mm nanofibrous scaffolds (Figure 2B,C). The IBP released more rapidly from the L0 to L30 microspheres in serum than in PBS, and in comparison to L300 and L600 microspheres (Figure 3A). The L0 microspheres released 74% more IBP than the L600 microspheres through day 14 (Figure 3A). At day 14, 165 µg/mL of total IBP was released from the BiLDS in serum, which was about 70% less than the total amount of IBP released from free L300 microspheres in serum; which indicates a slower and more controlled release of IBP from the microspheres pocketed in the BiLDS than free microspheres (Figure 3A,B). The BiLDS with IBP in the continuous group (BiLDS_IBP_Cont) released significantly more total IBP between days 7 and 14 in comparison to the BiLDS with IBP in static serum conditions (BiLDS_IBP) (Figure 3B).

In direct culture, metabolic activity of the tendon cells seeded on TCP was significantly greater than the cells seeded on the BiLDS, BiLDS_MS, and BiLDS_IBP constructs at day 7 (Figure 3C). At day 1 of the indirect culture, the cells cultured in media from the control, BiLDS_MS, and BiLDS_IBP groups had increased metabolic activity in comparison to the BiLDS alone group (Figure 3D). By day 3, the cellular metabolic activity of the cells in the BiLDS and BiLDS_MS groups were greater than the control group. Metabolic activity of the BiLDS groups was significantly greater than the control group at day 7 (Figure 3D).

### TABLE 2 Taqman assays used for qRT-PCR

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Abbreviations: CD, clusters of differentiation; COL, collagen; bFGF, basic fibroblast growth factor; IL, interleukin; MMP, matrix metalloproteinase; TNC, tenascin-C; TNF, tumor necrotic factor.
3.2 | Histologic analysis and immunohistochemistry

Semi-quantitative grading from images from the insertion was made between groups at each timepoint. Representative images of samples stained for H&E, MMP13, CD68, and CD183 are shown in Figure 4A-D. H&E images of representative samples used as a key for histological semi-quantitative grading of cellularity, nuclear shape, and expression of MMP13, CD68, and CD163 are included in the Supporting Information (Figure S1). There were no statistically significant differences between groups at any timepoint in cellularity (Figure 4E), nuclear shape (Figure 4F), and expression of MMP13 (Figure 4G), CD68 (Figure 4H), and CD163 (Figure 4I). Percent positive areas of CD45+, types I and III collagen, and TNF-α+ cells in the insertion were computed using a Matlab program. Representative images are shown in Figure 5A-C. The percent positive area of type I collagen in the insertion of the BiLDS_IBP treated tendons significantly decreased over time, from 1 to 8 weeks and from 4 to 8 weeks (Figure 5D). The percent positive area of type III collagen in the insertion of the BiLDS_IBP treated tendons was significantly greater than the No_BiLDS untreated and BiLDS treated tendons at 1 week. The BiLDS alone treated tendons (BiLDS) had increased type III collagen over time (Figure 5E). CD45, a leucocyte surface marker, significantly decreased in the BiLDS_IBP tendons over time (Figure 5F). There were no significant differences in the ratio of type I collagen to type III collagen over time or between groups at any timepoint (data not shown).

3.3 | Cytokine and gene expression analysis

Cytokine expression of TNF-α significantly decreased over time in the BiLDS and BiLDS_IBP treated tendons (Figure 5G). Cytokine expression of TNF-α significantly decreased over time in the BiLDS and BiLDS_IBP treated tendons (Figure 5G).
expression of TGF-β1 was greater in the BiLDS_IBP treated tendons than the No_BiLDS untreated and BiLDS treated tendon at 8 weeks (Figure 5H). Cytokine expression of IL-6 significantly decreased over time in the tendons treated with BiLDS (Figure 5I). qPCR demonstrated no significant differences in expression of tendon healing genes between groups at 1 week (Figure 5J). Presence of IBP was undetectable by ELISA in the blood serum of the 4- and 8-week animals.

3.4 | Biomechanical testing

Stiffness, maximum load, modulus, and maximum stress of the untreated tendons (No_BiLDS) were significantly greater than either of the BiLDS and BiLDS_IBP treated tendons at 4 weeks. However, these differences were not observed at 8 weeks between the No_BiLDS untreated tendons and BiLDS_IBP treated tendons (Figure 6A-D). In contrast, modulus and maximum stress of the BiLDS treated tendons were significantly lower compared to the No_BiLDS untreated tendons at 8 weeks (Figure 6C,D). Stiffness, maximum load, and maximum stress significantly increased for all groups over time (Figures 6A,B and 6D). The percent stress relaxation of the BiLDS_IBP treated tendons at 8 weeks was statistically greater than at 4 weeks (Figure 6E). There were no significant differences in stiffness and maximum load at 8 weeks (Figure 6A,B) or in tendon cross-sectional area (Figure 6f) at either 4 or 8 weeks.

4 | DISCUSSION

Scaffold-based delivery systems that locally release IBP at the optimal controlled rate are a promising strategy to improve rotator cuff repair outcomes. The purpose of our study was to evaluate the in vitro and in vivo biological and mechanical implications of a BiLDS for temporal release of IBP. We hypothesized that IBP would be released at a faster rate in physiological-like conditions and would not have an adverse effect on cellular biocompatibility in vitro. We also hypothesized that the release of IBP from the BiLDS would improve tendon healing in the rat rotator cuff by decreasing the expression of proinflammatory cytokines and improving tendon remodeling and mechanics.

The in vitro release profiles of IBP from Labrafil-modified poly(lactic-co-glycolic) acid microspheres in serum and PBS and from BiLDS constructs in serum were investigated. The total amount of IBP released in vitro over 2 weeks was inversely related to the concentration of Labrafil oil used to create the microspheres. These findings were consistent with other studies where the addition of Labrafil into polymeric microspheres lowered the initial IBP burst and prolonged the drug release rate. This is due to the amphiphilic characteristics of the Labrafil compound, which possesses both hydrophilic and lipophilic properties. Therefore, although Labrafil is derived from PEG, the presence of fatty acids makes the molecule hydrophobic and delays degradation and release rate. Furthermore, the release of IBP was increased in serum, but the initial release was delayed and sustained when the microspheres were enclosed in the BiLDS. These results confirm the prolonged drug delivery potential of BiLDS in physiologically relevant conditions and confirm our hypothesis. The addition of fresh serum over time, resembling in vivo conditions of serum exchange, increased the release of IBP from the BiLDS. This could be due to the frequent pH changes in the environment affecting the scaffold degradation or release kinetics. The findings from these studies supported our choice to use the microspheres with 300 µL of Labrafil, L300, for the subsequent in vitro cell and
sustainability studies, and in vivo study due to the ideal sustained release profile.

As we hypothesized, in vitro cytocompatibility studies proved the components of the BiLDS did not have an adverse effect on tendon cell metabolism over time. This was to be expected because PCL is an inert and compatible polymer used in many Food and Drug Administration approved devices. In a simultaneous study, we also demonstrated cytocompatibility with rat biceps tendon cells from young and aged animals seeded on the BiLDS constructs. The increase in metabolic activity of the cells seeded on the tissue culture plate in comparison to the cells on the BiLDS construct is possibly due to the differences in measuring metabolite concentration in 2D and 3D systems. In the indirect cell study, the BiLDS group is statistically decreased in comparison to the other groups at day 1, but this difference was not observed at the other timepoints. We interpret this as a delay...
in cellular growth and not a direct response to the conditioned media. Additionally, the high concentration of IBP in the conditioned media potentially led to the early growth lag, but the metabolic activity of the cells treated with the BiLDS_IBP conditioned media recovered by day 7. The cells were confluent by day 10, and therefore, the decrease in metabolic activity is likely caused by contact inhibition of proliferation. The cell seeding density in our studies was approximately 10,000 cells/cm$^2$. Similar in vitro studies concluded the proliferative potential of tendon-derived cells is greatest at lower seeding densities around 5,000 cells/cm$^2$. Future studies will further elucidate the in vitro cellular behavior using PicoGreen assay for DNA content as well as at a lower seeding density.

The biological and mechanical effects of the implantation of a BiLDS were evaluated in a rat supraspinatus acute injury and repair model for up to 8 weeks postsurgery. Histological analysis demonstrated that there were no differences in cellularity or nuclear shape in the insertion zone with the delivery of IBP, which is consistent with our previous work, where Food and Drug Administration supraspinatus tendon detachment and repair, and similar studies by other researchers. BiLDS, with and without IBP delivery, led to a decrease in expression of proinflammatory cytokine TNF-$\alpha$ over time. This observation supports our hypothesis regarding an anti-inflammatory effect. Studies have shown that inhibition of TNF-$\alpha$ can improve the biomechanical strength of tendon-bone healing in a rat rotator cuff model at early timepoints. Nonetheless, the implantation of the BiLDS with and without IBP led to a slight increase in TGF-$\beta$1, which is associated with the scarring process. Other studies observed a significant increase in TGF-$\beta$1 messenger RNA and protein in the healing tissue 10 days after supraspinatus repair, which then decreased over time. Therefore, it is possible that we did not observe a significant effect of the BiLDS treatments on the anti-inflammatory cytokine expression because we did not measure the expression at timepoints earlier than 4 weeks and the expression may have returned to baseline by 4 weeks. The delivery of IBP from the BiLDS led to a decrease in inflammatory cells within the tendon tissue. Specifically, CD45+ positive cells were reduced, whereas these cells are known to be elevated in response to tendon injury. The significant increase in type III collagen for the BiLDS_IBP group at 1 week and over time for the BiLDS group was unexpected. In a study by Galatz et al., immunohistochemistry demonstrated the presence of a large amount of type III collagen in early rat rotator cuff healing in the initial disorganized scar material at the insertion site. Therefore, it is possible the BiLDS constructs promoted scar formation at the insertion site. However, this increase over time is not seen with the BiLDS_IBP treatments. Future studies will test this hypothesis and evaluate the protein expression of these pro- and anti-inflammatory cytokines at earlier timepoints.

Contrary to our hypothesis, the tendons repaired and treated with the BiLDS constructs were associated with inferior structural and material properties at the earlier healing stage observed at 4 weeks. Early tendon healing is the critical time period when the strength of the tendon and bone interface ordinarily increases substantially following surgery. The poor mechanical properties we
observed might have resulted from the thickness of the BiLDS construct causing tissue damage due to subacromial impingement in the tight acromial space in the rat model. Previous work revealed no significant differences in structural properties or deleterious effect on healing with the implantation of a single layer PCL scaffold in the same animal model. Nonetheless, the increase in stiffness, maximum load, and maximum stress over time indicates the tissue quality may have recovered by 8 weeks. The delivery of IBP led to an increase in stress relaxation over time, indicating a mild decrease in scar tissue and improved healing over time. NSAIDs, such as IBP, have been proven to prevent peritendinous adhesions by inhibiting COX-1 and COX-2 and reducing the inflammatory response, thus reducing tissue adhesions and improving stress relaxation in flexor tendon studies.23,35–39 Although supraspinatus tendons are not surrounded by a sheath and have a different healing mechanism than sheathed tendons, such as flexor tendons, there appears to be a correlation between the delivery of IBP modulating the inflammatory response and the increase in stress relaxation. Future studies are necessary to elucidate the effect of IBP on COX activity and the healing response in non-sheathed tendons.

This study is not without limitations. Although we observed differences in cytokine expression levels and stress relaxation with the delivery of IBP, we were unable to accurately measure the presence of IBP in the tissue due the sensitivity limitations of the ELISA kit. Therefore, we were also unable to confirm the delayed and sustained release of IBP in vivo and the drug loading and encapsulation efficiency of the microsphere used in this study were unknown. Another limitation is the varying IBP metabolism process between rats and humans and the affect this may have on tendon healing. Lastly, the acute rotator cuff injury model used in this study does not resemble pathologies seen with degenerative chronic conditions in humans.

5 | CONCLUSION

The objective of this study was to investigate a biocompatible nanofibrous BiLDS for localized delivery of IBP to mitigate inflammation in vitro and in vivo in a rat rotator cuff repair model. Although addition of the BiLDS at the repair site was not therapeutically beneficial for rat rotator cuff healing in terms of improving mechanical properties, the implants remained intact and mitigated the inflammatory response over time. Additionally, repairs treated with an IBP-releasing BiLDS recovered their structural properties by 8 weeks. Future studies are required to elucidate the mitigatory effects of the BiLDS in a rat model beyond 8 weeks or in a larger animal model with a larger subacromial space.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the article, including research conception and design (BLT, JH, LJS, RLM, AFK, and JB), data acquisition (BLT, DHK, JH, SJB, SNW, CAN, and HAR), data analysis (BLT), and critically editing the article (BLT, DHK, JH, SNW, CAN, HAR, LJS, RLM, AFK, and JB). All authors have read and approved the final submitted manuscript.

ORCID

Brittany L. Taylor http://orcid.org/0000-0002-1886-2261
Dong Hwa Kim http://orcid.org/0000-0001-6618-3055
Julianne Huegel http://orcid.org/0000-0002-7051-861X
Louis J. Soslowsky http://orcid.org/0000-0001-9325-7662
Robert L. Mauck http://orcid.org/0000-0002-9537-603X
Joseph Bernstein http://orcid.org/0000-0001-9052-2897

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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