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Localized Delivery of Ibuprofen via a Bilayer Delivery System (BiLDS) for Supraspinatus Tendon Healing in a Rat Model

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Abstract

The high prevalence of tendon re-tear following rotator cuff repair motivates the development of new therapeutics to promote improved tendon healing. Controlled delivery of non-steroidal anti-inflammatory drugs (NSAIDs) 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 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 ibuprofen from Labrafil-modified poly(lactic-co-glycolic) acid (PLGA) microspheres within sintered poly(ϵ -caprolactone) (PCL) electrospun scaffolds. Biocompatibility of the BiLDS was demonstrated with primary Achilles tendon cells *in vitro*. Implantation of the ibuprofen-releasing BiLDS at the repair site in a rat rotator cuff injury and repair model led to decreased expression of pro-inflammatory cytokine, TNF- α , and increased anti-inflammatory cytokine, TGF- β 1. 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 bilayer delivery system 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 eight weeks or in a larger animal model.

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Keywords

biomaterials; tendon; tissue engineering

Introduction

Musculoskeletal injuries and conditions affect over 28 million people in the United States annually [1]. Rotator cuff tendon tears due to overuse or degeneration are common shoulder injuries and result in pain, instability, or decreased shoulder function [2]. 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 re-tear rate [3]–[8]. The mechanistic relationship between NSAIDs and tendon healing remains unclear but it is hypothesized that the inhibition of COX decreases prostaglandin (PGE2) levels which hinders tendon-to-bone healing [5], [6], [9], [10]. Several studies utilizing the rat rotator cuff injury and repair model demonstrated that early systemic delivery of NSAIDs impaired healing during the proliferative phase while delayed delivery improved remodeling by decreasing inflammation [3]. 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 [11].

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 [12]. 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 [6].

To this end, we developed a bilayer delivery system (BiLDS), which dually offers a three-dimensional 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 -capalactone) (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 [13], 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 1) evaluate the release profile of IBP from microspheres alone and within the BiLDS, 2) elucidate the effect of IBP on primary tendon cells *in vitro*, and 3) 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 pro-inflammatory cytokines, thus improving tendon remodeling and mechanical properties.

Methods

Microspheres and BiLDS Fabrication

Poly(lactic-co-glycolic) acid (75:25 PLGA, MW = 70kDa, 0.15g/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 (DCM) mixture in 30, 300 or 600µL 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 [14]–[17]. 30mg/mL of ibuprofen (IBP, MW = 206.28) was also added to the mixture for the IBP-loaded microspheres and stirred for 4 hours at room temperature. Based on the sustained linear release of IBP from the L300 microspheres *in vitro*, BiLDS were created by entrapping 10mg of the L300 microspheres in phosphate buffer saline (PBS), with and without IBP (BiLDS_IBP and BiLDS respectively), between two heat sintered 6×8mm electrospun poly(ϵ -capalactone) (PCL) scaffolds to create approximately 100µm 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.

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 5mL 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 ELISA and UV spectrophotometer ($\lambda = 223\text{nm}$). In the second release study, BiLDS and BiLDS_IBP were incubated on a shaker at 37°C in 5mL serum for up to 14 days. In one group, the total 5mL 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) 2mL of serum was collected at the same time points 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 (400–450g). The tendon was incubated on tissue culture polystyrene (TCP) in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% 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 one 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 6×8mm 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 one 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 were cells cultured in normal culture media. MTT assay was employed to quantify cell metabolic activity.

Rotator Cuff Surgical Procedure

This study was approved by the Philadelphia Veteran Affairs Medical Center Animal Component of Research Protocol (ACORP) Committee. Ninety adult male Sprague-Dawley rats (400–450g) underwent bilateral supraspinatus detachment with immediate repair. Animals were randomly divided into three groups: repair without BiLDS (No_BiLDS), repair with 3×5mm BiLDS which did not contain microspheres or IBP (BiLDS), and repair with 3×5mm BiLDS containing 170µg of IBP-loaded L300 PLGA microspheres in 1µL of PBS (BiLDS_IBP) (n=30/group). The L300 IBP-loaded microspheres with 30mg/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 270µg of cumulative IBP to the injury site over an 8 week period [12].

Animals were anesthetized via inhalation of 5% isoflurane and maintained at 2–3% throughout surgery. Supraspinatus detachment was performed as previously described using sterile technique [18], [19], and the BiLDS were overlaid on the repair site and attached by suturing the BiLDS proximally to the tendon and distally to the 0.5mm bone tunnel drilled through the greater tuberosity. Rats were allowed ad lib cage activity and administered buprenorphine every 12 hours for 3 days post-operation. 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 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.

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. 5µm thick sections were stained with hematoxylin and eosin 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 CD45 (1:400, Abcam, ab31630), CD163 (1:400, Abcam, ab182422), and CD68 (1:300, Abcam) antibodies to evaluate the inflammatory response, and collagen types I (1:200, Sigma-Aldrich, AB755P),

III (1:500, Sigma-Aldrich, C7805), and matrix metalloproteinase-13 (MMP13) (1:250, Abcam, ab39012) to assess matrix remodeling. Antigens were retrieved with enzymatic digestion using 0.5mg/ml of hyaluronidase for COL I and MMP13 and 0.4mg/ml of Proteinase K for COL III stained sections. Heat induced antigen retrieval in 10mmol sodium citrate buffer (pH 6.0) was performed for the CD45 (10 minutes at 75°C), CD68 (10 minutes at 95°C), and CD163 (30 minutes at 80°C) stained sections. Sections were digitally scanned using a Zeiss Axio Scan.Z1 slide. Regions of interest (ROI) in the tendon insertion area were taken using Zen lite software with a 10X zoom for analysis and were either blindly semi-quantitatively graded from 1 (low expression) to 3 (high expression), or quantified using a custom MATLAB code for percent positive area.

Gene and Protein Expression

Total RNA from pulverized one-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, USA). The reverse transcription reaction was carried out with High Capacity cDNA synthesis kit with RNase inhibitor (Applied Biosystems by Life Technologies) in 20µl reactions containing 1.0µg RNA. All of the primers were purchased from Thermo Fisher Scientific as selected markers for gene expression of inflammation, tendon remodeling, and tendon repair: tumor necrotic factor- α (TNF- α), interleukins -1b (IL-1b), -6 (IL-6), and -10 (IL-10), Prostaglandin E2 (PGE2), clusters of differentiation -68 (CD68) and -163 (CD163), transforming growth factors -1 (TGF- β 1) and -3 (TGF- β 3), basic fibroblast growth factor 1 (bFGF1), vascular endothelial growth factor B (VEGF B), collagen types -1 (COL I), -3 (COL III), and -4 (COL IV), matrix-metalloproteinases- 2 (MMP2), -3 (MMP3), -9 (MMP9) and -13 (MMP13), tenascin-C (TNC), tenomodulin (Tnmd), 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 (GAPDH, Rn01775763_g1). The fold change was calculated by normalizing the treatment groups to the control group (No_BiLDS).

ELISAs for TNF- α (Sigma-Aldrich, RAB0480, St. Louis, USA), IL-6 (Sigma-Aldrich, RAB3012, St. Louis, USA), and TGF- β 1 (Abcam, ab119668, Cambridge, USA) were performed on protein isolates from the excised tendons. Competitive ELISA for IBP (Neogen, Lexington, USA) 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.

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 2mm, 4mm, and 8mm 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 0.5 N for 10 cycles, followed by ramp to 5% strain at 5% per second and stress relaxation for 10mins. The tendons were then ramped to failure at 0.3% strain per second. Stress relaxation, modulus, stiffness, maximum load, and maximum stress were computed.

Statistical Analysis

Two-way ANOVA and normality tests were performed on all datasets. One-way ANOVAs with Tukey's post hoc test or Kruskal-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 $p < 0.05$ (*). Data is presented as mean $\pm$ standard deviation or median $\pm$ interquartile range when specified in figure legend.

Results

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 μ m. ESEM images of the BiLDS confirmed the pocket of microspheres between the two sintered 6 $\times$ 8mm nanofibrous scaffolds (Figures 2B & 2C). The IBP released more rapidly from the L0 and 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 μ g/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 (Figures 3A & 3B). The BiLDS with IBP in the continuous group (BiLDS_IBP_Cont) released significantly more total IBP between day 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).

Histologic Analysis and Immunohistochemistry

Semi-quantitative grading from images from the insertion were made between groups at each timepoint. Representative images of samples stained for H&E, MMP13, CD68, and CD183 are shown in Figures 4 A–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 Supplement (Figure S1). There were no statistically significant differences between groups at any time point in cellularity (Figure 4E), nuclear shape (Figures 4F), expression of MMP13 (Figures 4G), CD68 (Figures 4H), and CD163 (Figures 4I). Percent positive area 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 Figures 5 A–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 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 collagen III 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).

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 TGF- β 1 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.

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 (Figures 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 (Figure 6A–B & 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.

Discussion

Scaffold-based delivery systems that locally release ibuprofen 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 bilayer-delivery system (BiLDS) for temporal release of ibuprofen. We hypothesized that ibuprofen 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 pro-inflammatory 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 two 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 ibuprofen burst and prolonged the drug release rate [16]. 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 [16]. Furthermore, the release of ibuprofen 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 [12], [20], [21]. The findings from these studies support our choice to use the microspheres with 300µL 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 FDA-approved devices. In a simultaneous study, we also demonstrated biocompatibility 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 time points. 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/cm². Similar *in vitro* studies concluded the proliferative potential of tendon-derived cells is greatest at lower seeding densities around 5,000 cells/cm² [22]–[25]. 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 bilayer delivery system were evaluated in a rat supraspinatus acute injury and repair model for up to 8 weeks post-surgery. Histological analysis demonstrated that there were no differences in cellularity or nuclear shape in the insertion zone with the delivery of ibuprofen, which is consistent with our

previous work [11], where ibuprofen was administered orally following supraspinatus tendon detachment and repair, and similar studies by other researchers [26]. BiLDS, with and without IBP delivery, led to a decrease in expression of pro-inflammatory cytokine TNF- α overtime. This observation supports our hypothesis regarding an anti-inflammatory effect. Studies have shown that inhibition of TNF- α can improve the biomechanical strength of tendon-bone healing in a rat rotator cuff model at early time points [27], [28]. Nonetheless, the implantation of the BiLDS with and without IBP led to a slight increase in TGF- β 1, which is associated with the scarring process [26], [29]–[31]. Other studies observed a significant increase in TGF- β 1 mRNA and protein in the healing tissue 10 days after supraspinatus repair, which then decreased over time [26], [31]. 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 [32], [33]. 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. [26], immunohistochemistry demonstrated a 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 four weeks. Early tendon healing is the critical time period when the strength of the tendon and bone interface ordinarily increases substantially following surgery [34]. 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 [19]. 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 ibuprofen led to an increase in stress relaxation over time, indicating a mild decrease in scar tissue and improved healing over time. NSAIDs, such as ibuprofen, have been proven to prevent peritendinous adhesions by inhibiting cyclooxygenase –1 and –2 (COX-1 and COX-2) and reducing the inflammatory response, thus reducing tissue adhesions and improving stress relaxation in flexor tendon studies [7], [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 ibuprofen modulating the inflammatory response and the increase in stress relaxation. Future studies are necessary to elucidate the effect of ibuprofen on cyclooxygenase 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 ibuprofen, we were unable to accurately measure the presence of ibuprofen 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 ibuprofen 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.

Conclusion

The objective of this study was to investigate a biocompatible nanofibrous bilayer delivery system (BiLDS) for localized delivery of ibuprofen 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Edelstein L, Thomas SJ, Soslowky LJ. 2011 Rotator cuff tears: what have we learned from animal models. *J Musculoskelet Neuronal Interact* 11:150–162. [PubMed: 21625052]
2. Cofield RH. 1985 Rotator cuff disease of the shoulder. *J Bone Joint Surg Am* 67:974–979. [PubMed: 4019548]
3. Virchenko O, Skoglund B, Aspenberg P. 2004 Parecoxib Impairs Early Tendon Repair but Improves Later Remodeling. *Am J Sports Med* 32:1743–1747. [PubMed: 15494342]
4. Ferry ST, Dahners LE, Afshari HM, Weinhold PS. 2007 The Effects of Common Anti-Inflammatory Drugs on the Healing Rat Patellar Tendon. *Am J Sports Med* 35:1326–1333. [PubMed: 17452512]
5. Dimmen S, Engebretsen L, Nordsletten L, Madsen JE. 2009 Negative effects of parecoxib and indomethacin on tendon healing: an experimental study in rats. *Knee Surgery, Sport Traumatol Arthrosc* 17:835–839.
6. Cohen DB, Kawamura S, Ehteshami JR, Rodeo SA. 2006 Indomethacin and Celecoxib Impair Rotator Cuff Tendon-to-Bone Healing. *Am J Sports Med* 34:362–369. [PubMed: 16210573]
7. Su B, O'Connor JP. 2013 NSAID therapy effects on healing of bone, tendon, and the enthesis. *J Appl Physiol* 115:892–899. [PubMed: 23869068]

8. Galatz LM, Ball CM, Teefey SA, et al. 2004 The Outcome and Repair Integrity of Completely Arthroscopically Repaired Large and Massive Rotator Cuff Tears. *J Bone Jt Surg* 86:219–224.
9. Simon AM, Manigrasso MB, O'Connor JP. 2002 Cyclo-Oxygenase 2 Function Is Essential for Bone Fracture Healing. *J Bone Miner Res* 17:963–976. [PubMed: 12054171]
10. Dimmen S, Nordsletten L, Engebretsen L, et al. 2009 The effect of parecoxib and indometacin on tendon-to-bone healing in a bone tunnel. *J Bone Joint Surg Br* 91-B(2):259–263.
11. Connizzo BK, Yannascoli SM, Tucker JJ, et al. 2014 The Detrimental Effects of Systemic Ibuprofen Delivery on Tendon Healing Are Time-Dependent. *Clin Orthop Relat Res* 472:2433–2439. [PubMed: 23982408]
12. Riggan CN, Qu F, Kim DH, et al. 2017 Electrospun PLGA Nanofiber Scaffolds Release Ibuprofen Faster and Degrade Slower After In Vivo Implantation. *Ann Biomed Eng* 45:1–12. [PubMed: 27632024]
13. Kim DH, Taylor BT, Huegel J, et al. (2019, 2). Improving Supraspinatus Tendon Healing using an FGF-Loaded Bilayer Delivery System in a Rat Model. Poster presented at the Orthopaedic Research Society Annual Meeting, Austin, TX.
14. Devani M, Ashford M, Craig DQM. 2004 The emulsification and solubilisation properties of polyglycolysed oils in self-emulsifying formulations. *J Pharm Pharmacol* 56:307–316. [PubMed: 15025855]
15. Yoo HS. 2006 Preparation of biodegradable polymeric hollow microspheres using O/O/W emulsion stabilized by Labrafil®. *Colloids Surfaces B Biointerfaces* 52:47–51. [PubMed: 16930956]
16. Fernández-Carballido A, Herrero-Vanrell R, Molina-Martínez IT, Pastoriza P. 2004 Biodegradable ibuprofen-loaded PLGA microspheres for intraarticular administration. Effect of Labrafil addition on release in vitro. *Int J Pharm* 279:33–41. [PubMed: 15234792]
17. Gao ZH, Crowley WR, Shukla AJ, et al. 1995 Controlled release of contraceptive steroids from biodegradable and injectable gel formulations: in vivo evaluation. *Pharm Res* 12:864–868. [PubMed: 7667191]
18. Huegel J, Kim DH, Cirone JM, et al. 2017 Autologous tendon-derived cell-seeded nanofibrous scaffolds improve rotator cuff repair in an age-dependent fashion. *J Orthop Res* 35:1250–1257. [PubMed: 27500782]
19. Beason DP, Connizzo BK, Dourte LM, et al. 2012 Fiber-aligned polymer scaffolds for rotator cuff repair in a rat model. *J Shoulder Elb Surg* 21:245–250.
20. Zolnik BS, Burgess DJ. 2007 Effect of acidic pH on PLGA microsphere degradation and release. *J Control Release* 122:338–344. [PubMed: 17644208]
21. Estey T, Kang J, Schwendeman SP, Carpenter JF. 2006 BSA Degradation Under Acidic Conditions: A Model For Protein Instability During Release From PLGA Delivery Systems. *J Pharm Sci* 95:1626–1639. [PubMed: 16729268]
22. Colter DC, Class R, DiGirolamo CM, Prockop DJ. 2009 Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc Natl Acad Sci* 97:3213–3218.
23. Rui Y-F, Lui PPY, Li G, et al. 2010 Isolation and Characterization of Multipotent Rat Tendon-Derived Stem Cells. *Tissue Eng Part A* 16:1549–1558. [PubMed: 20001227]
24. Dezawa M, Ishikawa H, Itokazu Y, et al. 2005 Bone Marrow Stromal Cells Generate Muscle Cells and Repair Muscle Degeneration. *Science* 309:314–317. [PubMed: 16002622]
25. Tan Q, Lui PPY, Rui YF, Wong YM. 2012 Comparison of Potentials of Stem Cells Isolated from Tendon and Bone Marrow for Musculoskeletal Tissue Engineering. *Tissue Eng Part A* 18:840–851. [PubMed: 22011320]
26. Galatz LM, Sandell LJ, Rothermich SY, et al. 2006 Characteristics of the rat supraspinatus tendon during tendon-to-bone healing after acute injury. *J Orthop Res* 24:541–550. [PubMed: 16456829]
27. Gulotta LV, Kovacevic D, Cordasco F, Rodeo SA. 2011 Evaluation of Tumor Necrosis Factor α Blockade on Early Tendon-to-Bone Healing in a Rat Rotator Cuff Repair Model. *Arthrosc J Arthrosc Relat Surg* 27:1351–1357.
28. Blaine TA, Kim Y-S, Voloshin I, et al. 2005 The molecular pathophysiology of subacromial bursitis in rotator cuff disease. *J Shoulder Elb Surg* 14:S84–S89.

29. Würgler-Hauri CC, Dourte LM, Baradet TC, et al. 2007 Temporal expression of 8 growth factors in tendon-to-bone healing in a rat supraspinatus model. *J Shoulder Elb Surg* 16:S198–S203.
30. Gulotta LV 2009 Growth Factors for Rotator Cuff Repair. *Clin Sports Med* 28:13–23. [PubMed: 19064162]
31. Dahlgren LA, Mohammed HO, Nixon AJ. 2005 Temporal expression of growth factors and matrix molecules in healing tendon lesions. *J Orthop Res* 23:84–92. [PubMed: 15607879]
32. Wong JKF, Alyouha S, Kadler KE, et al. 2010 The cell biology of suturing tendons. *Matrix Biol* 29:525–536. [PubMed: 20600895]
33. Abrams GD, Luria A, Carr RA, et al. 2016 Association of synovial inflammation and inflammatory mediators with glenohumeral rotator cuff pathology. *J Shoulder Elb Surg* 25:989–997.
34. Rodeo SA, Arnoczky SP, Torzilli PA, et al. 1993 Tendon-healing in a bone tunnel. A biomechanical and histological study in the dog. *J Bone Joint Surg Am* 75:1795–1803. [PubMed: 8258550]
35. Liu S, Hu C, Li F, et al. 2013 Prevention of Peritendinous Adhesions with Electrospun Ibuprofen-Loaded Poly(l -Lactic Acid)-Polyethylene Glycol Fibrous Membranes. *Tissue Eng Part A* 19:529–537. [PubMed: 23013368]
36. Lee JH, Go AK, Oh SH, et al. 2005 Tissue anti-adhesion potential of ibuprofen-loaded PLLA-PEG diblock copolymer films. *Biomaterials* 26:671–678. [PubMed: 15282145]
37. Strick MJ, Filan SL, Hile M, et al. 2004 Adhesion formation after flexor tendon repair: a histologic and biomechanical comparison of 2- and 4-strand repairs in a chicken model. *J Hand Surg Am* 29:15–21. [PubMed: 14751097]
38. Tan V, Nourbakhsh A, Capo J, et al. 2010 Effects of Nonsteroidal Anti-Inflammatory Drugs on Flexor Tendon Adhesion. *J Hand Surg Am* 35:941–947. [PubMed: 20513575]
39. Szabo RM, Younger E. 1990 Effects of indomethacin on adhesion formation after repair of zone II tendon lacerations in the rabbit. *J Hand Surg Am* 15:480–483. [PubMed: 2348070]

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.

Figure 1.

(A) Schematic of the bilayer delivery system (BiLDS). (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.

Figure 2.

Representative ESEM images of (A) L300 microspheres (500X), (B) top view of the sintered BiLDS (darker area to the right of the black dotted line is the sintered region) (50X), and (C) cross-section of the BiLDS with L300 microspheres denoted by black arrow (100X).

Figure 3.

(A) Release of IBP from free microspheres in serum (dashed line) and PBS (solid line) over 14 days showed a more rapid initial release of IBP from the L0, L30, and L300 microspheres in serum (dash line) than the microspheres with the same concentration of Labrafil oil in PBS (solid line). The L300 and L600 microspheres exhibited a more sustained IBP release profile over time in serum and PBS. Statistical comparisons were made between the release in serum and PBS at each time point (denoted by the ***). (B) The release of IBP from L300 microspheres within the BiLDS was significantly greater with serum exchange (BiLDS_IBP_Cont) between day 7 and 14 than the IBP-releasing BiLDS in static serum conditions (BiLDS_IBP). Statistical comparisons at day 7 are shown in the text box. Statistical significance at day 14 is denoted on the graph which indicates significant difference compared to the other groups at the same time point. Metabolic activity of primary Achilles tendon cells (C) directly cultured on tissue culture polystyrene (control), BiLDS alone (BiLDS), BiLDS with empty microspheres (BiLDS_MS), or BiLDS with IBP-loaded microspheres (BiLDS_IBP), and (D) indirectly cultured with media pre-conditioned with BiLDS, BiLDS_MS, and BiLDS_IBP constructs for one week prior did not adversely affect biocompatibility over time. Data presented as mean $\pm$ or + S.D. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 4.

Representative histological images from the No_BiLDS, BiLDS, and BiLDS_IBP groups at 1, 4 and 8 weeks stained for (A) H&E and (B) MMP13, (C) CD68, and (D) CD163. These images were semi-quantitatively graded for cellularity and cell shape or positive expression. Semi-quantitative grading results for (E) cellularity, (F) nuclear shape, and expression of (G) MMP13, (H) CD68, and (I) CD163 between groups at each timepoint (top row). Cellularity significantly decreased over time for all groups. Scale bar = 100 μ m. Data presented as median $\pm$ IQR. (* p <0.05, ** p <0.01, *** p <0.001).

Figure 5.

Representative histological images from the No_BiLDS, BiLDS, and BiLDS_IBP groups at 1, 4 and 8 weeks stained for (A) COL 1, (B) COL 3, and (C) CD45, (D) Saf-O. These images were quantitatively graded using a custom MATLAB code. Calculated percent positive area of immunohistological images stained for (D) type I collagen, (E) type III collagen, and (F) CD45. Cytokine expression measured using ELISA of (G) TNF- α , (H) TGF- β 1, and (I) IL-6. (J) Gene expression (normalized fold change) of selected tendon healing markers. Scale bar = 100 μ m. Data presented as mean + S.D. (*p<0.05, **p<0.01, ***p<0.001).

Figure 6:

Calculated (A) stiffness, (B) maximum load, (C) modulus, and (D) maximum stress of untreated tendons (No_BiLDS) were significantly greater at 4 weeks than the treated tendons (BiLDS and BiLDS_IBP), but these differences were not observed at 8 weeks. (E) Stress relaxation increased over time for the IBP-treated tendons (BiLDS_IBP) and there were no differences in (F) cross-sectional area between groups. Data presented as mean + S.D. (*p<0.05, **p<0.01, ***p<0.001).

Table 1:

Microsphere and BiLDS group

Abbreviation	Description
<i>In Vitro</i> Release Studies	
L0	PLGA microspheres with 0 μ L Labrafil and 30mg/mL IBP
L30	PLGA microspheres with 30 μ L Labrafil and 30mg/mL IBP
L300	PLGA microspheres with 300 μ L Labrafil and 30mg/mL IBP
L600	PLGA microspheres with 600 μ L Labrafil and 30mg/mL IBP
BiLDS	BiLDS construct without microspheres or IBP in static serum conditions
BiLDS_Cont	BiLDS construct without microspheres or IBP in serum exchange conditions
BiLDS_IBP	BiLDS construct with L300 IBP-loaded microspheres in static serum conditions
BiLDS_IBP_Cont	BiLDS construct with L300 IBP-loaded microspheres in serum exchange conditions
<i>In Vitro</i> Cell Studies (Direct and Indirect)	
Control	Cells seeded on tissue culture polystyrene (TCP) or in normal culture media
BiLDS	Cells seeded on empty BiLDS constructs (no microspheres or IBP) or cultured in BiLDS-conditioned media
BiLDS_MS	BiLDS constructs with empty microspheres (no IBP) or cultured in media conditioned with BiLDS_MS constructs
BiLDS_IBP	BiLDS constructs with L300 IBP-loaded microspheres or cultured in media conditioned with BiLDS_IBP constructs
<i>In Vivo</i> Rotator Cuff Study	
No_BiLDS	Repaired tendon without BiLDS
BiLDS	Repaired tendon with empty BiLDS constructs (no microspheres or IBP)
BiLDS_IBP	Repaired tendon with L300 IBP-loaded microspheres

Table 2:

Taqman Assays used for qRT-PCR

Gene	Assay ID	Gene	Assay ID	Gene	Assay ID
TNF- α	RN99999017_m1	IL-1b	Rn00580432_m1	IL-6	Rn01410330_m1
IL-10	Rn01483988_g1	PTGER2	Rn00579419_m1	CD68	Rn01495634_g1
CD163	Rn01492519_m1	TGF- β 1	Rn00572010_m1	TGF- β 3	Rn00565937_m1
bFGF1	Rn00689153_m1	VEGF B	Rn01454585_g1	COL I	Rn01463848_m1
COL III	Rn01437681_m1	COL IV	Rn01482928_m1	MMP2	Rn01538170_m1
MMP3	Rn00591740_m1	MMP9	Rn00579162_m1	MMP13	Rn01448194_m1
TNC	Rn01454948_m1	Tnmd	Rn00574164_m1	ACAN	Rn00573434_m1