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Electrospun PLGA Nanofiber Scaffolds Release Ibuprofen Faster and Degrade Slower after *In Vivo* Implantation

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Abstract

While delayed delivery of non-steroidal anti-inflammatory drugs (NSAIDs) has been associated with improved tendon healing, early delivery has been associated with impaired healing. Therefore, NSAID use is appropriate only if the dose, timing, and mode of delivery relieves pain but does not impede tissue repair. Because delivery parameters can be controlled using drug-eluting nanofibrous scaffolds, our objective was to develop a scaffold for local controlled release of ibuprofen, and characterize the release profile and degradation both *in vitro* and *in vivo*. We found that when incubated *in vitro* in saline, scaffolds containing ibuprofen had a linear release profile. However, when implanted subcutaneously *in vivo* or when incubated *in vitro* in serum, scaffolds showed a rapid burst release. These data demonstrate that scaffold properties are dependent on the environment in which they are placed and the importance of using serum, rather than saline, for initial *in vitro* evaluation of biofactor release from biodegradable scaffolds.

Keywords

NSAID; Tendon Healing; Polymer Scaffold

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Conflict of Interest

The authors have no relevant disclosures related to this study.

Introduction

More than 28 million patients in the US experience a tendon or ligament injury each year,¹⁴ and the use of non-steroidal anti-inflammatory drugs (NSAIDs) for pain management after injury or surgery is common.¹ To be sure, the dose of NSAID must be kept low enough to avoid side systemic effects, yet high enough to maintain adequate local concentrations. This constraint may be overcome through the development of better delivery systems for the drugs using implanted biomaterials. Biomaterials allow for the controlled-release of drugs only in the desired areas, and thereby can reduce systemic side effects. Additionally, local delivery might provide therapeutic concentrations of the drug in areas of lower blood flow, such as dense connective tissues.¹

When using degradable polymeric materials as carriers, molecules can be released by both diffusion of the drug out of the material, as well as degradation of the polymer. Therefore, the type of polymer, the polymer formulation, and its geometric features can be specifically selected to adjust the timing and dosage of drug delivery.¹ A number of natural and synthetic biomaterial options exist for local drug delivery applications. Poly(lactic-co-glycolic acid) (PLGA) is one of the most commonly used materials, and is FDA approved for multiple therapeutics. PLGA is biodegradable and widely biocompatible.²⁷ It undergoes hydrolytic degradation in aqueous environments, providing a consistent degradation profile.²⁷ PLGA can be fabricated into microspheres, microcapsules, microparticles, nanocapsules, nanospheres, nanofibers, and nanomeshes, among others.²⁷ Each shape can be uniquely matched to specific drug-delivery applications, with the surface area to volume ratio and chemical properties of the drug influencing polymer degradation and biofactor delivery.

PLGA and its related polymer variants have been used for the delivery of NSAIDs, and specifically ibuprofen (IBP).^{25, 33} Numerous investigators have altered the release rate of IBP from polymer scaffolds for particular applications. Incorporation of poly(ethylene glycol)-g-chitosan (PEG-g-CHN)²⁶ or Labrafil¹⁹ into PLGA attenuates the release of IBP, creating a more sustained release profile. Similarly, addition of modified mesoporous silica (MMS) reduces the initial burst release of IBP and increases the duration of release in poly-L-lactide (PLLA) electrospun fibers.²⁴ The chemical linking of IBP to α,β -poly(N-2-hydroxyethyl)-DL-aspartamide-graft-poly(lactic acid) (PHEA-g-PLA) fibers also alters the release profile compared to the physical incorporation of the drug into fibers.³⁶

While NSAIDs are effective for post-operative analgesia, there have been reports of potentially negative effects of systemic NSAIDs on wound healing. The undesirable influence of NSAIDs on bone metabolism and fracture healing has been well documented in experimental studies.^{3, 5, 15, 21, 28, 31} Clinical reports also link fracture nonunion to NSAIDs.²² However, the effects of NSAIDs on tendon healing are considerably less well-studied. The administration of NSAIDs is associated with decreased repair strength in various injury models.^{9, 13, 20, 30} *In vitro* studies demonstrate that NSAIDs inhibit tendon cell migration and proliferation, decrease prostaglandin release and DNA synthesis, and increase protein synthesis in tendon fibroblasts.^{2, 40, 41} These data suggest a negative effect on tendon healing in the early proliferative phase but a beneficial one in the remodeling phase.^{9, 13, 20, 30} Two *in vivo* studies tested this hypothesis, and found that early treatment of NSAIDs resulted in

decreased mechanical properties, while late treatment resulted in either decrease in cross-sectional area and increased maximum stress, or no change in healing outcome.^{10, 43} Although these studies demonstrate the importance of timing for NSAID delivery, they have not evaluated the effect of local delivery of IBP or the timing of this local delivery on repair outcomes.

Therefore, the objective of this study was to develop and characterize a nanofibrous PLGA scaffold for the localized and timed release of IBP during tendon healing. Specifically, we pursued three specific aims. Because electrospun scaffold implantation does not alter the tendon healing response,⁴ our first aim was to determine if an electrospun PLGA scaffold loaded with IBP produced a continuous, linear release profile of IBP that could be tailored to deliver the drug at specific time points after injury. Additionally, because the addition of small molecules can influence the properties of biomaterials, our second aim was to determine if the addition of IBP altered the degradation and mechanical properties of the PLGA scaffold. Finally, because the incubation environment can impact release from biomaterials, our final aim was to determine if *in vivo* implantation altered IBP release profile and degradation of the PLGA scaffold compared to *in vitro* incubation.

Materials and Methods

PLGA Scaffold Fabrication

PLGA polymer solutions were created by mixing 35% w/v 75:25 PLGA (Durect Corporation, Birmingham, AL) in a 1:1 solution of N,N-dimethylformamide (DMF) and tetrahydrofuran (THF) (Fisher Scientific, Asheville, NC). Polymer solutions were formulated either with 5% w/w ibuprofen (IBP) (Sigma-Aldrich, St. Louis, MO) or without the incorporation of IBP (blank). A total of 7.5mL of polymer solution was used to form each scaffold. Scaffolds were formed by electrospinning, as previously described.³⁴ The solution was ejected through a charged metal spinneret at a rate of 2.5mL/hr, the needle charged to 12kV. Fibers were collected onto a grounded collection mandrel, placed 10cm from the charged needle, rotating with a surface velocity of 10m/s to produced aligned sheets.

Mechanical Analysis of Fabricated Scaffolds

For mechanical analysis, scaffolds were cut to 60×5mm strips, with the long axis oriented along the fiber direction. Scaffold cross-sectional area was determined using a custom laser device,¹⁸ and gripped in testing fixtures with 10mm in each grip to create a 40mm gauge length. Scaffolds were pre-loaded to 0.01N and tested in tension at 0.5%/sec until failure. From the load vs. displacement curve, the stiffness, yield load, yield displacement, failure load, and failure displacement were determined. From the stress vs strain curve, the modulus, yield stress, yield strain, failure stress, failure strain, and toughness were determined.

IBP Release in PBS

Scaffold segments were cut into 5×10mm strips (~10 mg), placed in 1.5mL PBS, and incubated at 37°C on a shaker. IBP release was evaluated at 5 and 10 hours, and 1, 2, 3, 7,

10, 14, and then weekly to 63 days. At each time point evaluated, the PBS solution was removed, and 1.5mL of fresh PBS was added to the sample to continue the release. The supernatant at each time point was centrifuged for 5 min at 3220rcf and IBP release was measured using UV/Vis spectrophotometry (Nanodrop 1000, Thermo Scientific) at a wavelength of 223nm (IBP absorption peak value) in triplicate.^{6, 38} Standard solutions of IBP in PBS were used to create a standard curve of absorption vs. IBP concentration to determine the concentration of IBP in each sample.

Quantification of IBP Remaining in Scaffold

In order to quantify the amount of IBP remaining in the scaffolds, scaffolds were dissolved in dimethyl sulfoxide (DMSO) and the solution was centrifuged for 5 min at 3220rcf. The supernatant was measured using UV/Vis spectrophotometry (Nanodrop 1000, Thermo Scientific) at a wavelength of 265nm.²⁹ While ibuprofen has a peak in absorbance at 223nm regardless of solution, the DMSO interferes with the reading at that wavelength. Therefore, we used 265nm, which is the peak absorbance outside of the interference region of DMSO, for all readings performed in DMSO. Known standards of IBP DMSO solutions were used to create a standard curve of absorbance vs. IBP concentration to determine the amount of IBP remaining in each sample.

IBP Release in Serum

Scaffolds were cut into 5×10mm strips, and placed in 1.5mL of rat serum (EMD Millipore Corp, Billerica, MA) for 0.5, 3, or 7 days or PBS. Half of each scaffold was used to quantify the IBP remaining in the scaffold, and the other half of the scaffold was placed into PBS for evaluation of continued release. IBP release from PBS was evaluated at 0.5, 3, 5, 7, 10, and 12 days, and weekly to 42 days. The release profile was generated by first determining the amount of IBP released in the serum solution by subtracting the measured amount of IBP remaining in the scaffold (as described in “Quantification of IBP Remaining in Scaffold”) from the starting total IBP in the fabricated scaffold measured by dissolving a non-incubated scaffold in DMSO and quantifying the IBP content. Then, the release of IBP in the PBS solution was determined for each day (as described in “IBP Release in PBS”) and measured in triplicate. Cumulative release was calculated by adding the measured release values for each day, starting with the serum release calculation.

Subcutaneous Implantation

Animal studies were performed in compliance with the Corporal Michael J. Crescenz Veterans Affairs Medical Center Institute for Animal Care and Use Committee (IACUC) protocols. Scaffolds were cut into 8mm diameter pieces using a biopsy punch, and were implanted subcutaneously in 4 month old Sprague Dawley rats (4 scaffolds/animal). Scaffolds were placed on the dorsal side of the rat, two on the caudal and two on the cranial aspects, each on either side of the rat midline. Animals were sacrificed at 0.5, 3, 7, and 14 days after implantation, and scaffolds retrieved for environmental scanning electron microscopy (ESEM) imaging (n=1), histological analysis (n=1), quantification of IBP left in the scaffold (n=3), and assessment of continued release of IBP from explanted scaffolds in PBS (n=3).

Histology

After sacrifice using CO₂ inhalation, according to AVMA guidelines, scaffold samples along with any adherent tissue were immediately harvested and placed in formalin. Samples were soaked in a 30% sucrose solution, followed by flash freezing in embedding compound. Samples were sectioned to 10µm and stained with hematoxylin and eosin (H&E). Images were acquired at 25X and 100X total magnification (2.5× and 10× objectives with 10× magnification in the camera).

Environmental Scanning Electron Microscopy

ESEM was used to qualitatively assess electrospun fiber morphology and organization at the nanoscale. Micrographs were taken under low vacuum with a 15 kV electron beam using a Quanta 600 FEG ESEM (Fei Company; Hillsboro, OR). Samples were imaged at 5000X magnification. Samples were imaged in the plane of and perpendicular to the fiber direction to obtain top-down and cross-sectional views, respectively.

Mechanical Analysis of Incubated Scaffolds

IBP and blank scaffolds were cut into 60×5mm strips prior to incubation in 7mL of either serum or PBS for 0.5, 3, 7, 14, and 21 days. Cross sectional area was calculated after incubation by measuring the width and thickness at multiple points along the scaffolds using calipers and calculating the average area assuming a rectangular cross-section (the curvature along the length of the scaffolds prevented measurement using the custom laser device used in the fabricated scaffold case). Scaffolds contracted to about 1/3 of their original length, generating gauge lengths of only ~6mm. Scaffolds were speckle-painted with black spray to create small dots on the scaffold surface for optical tracking of strain. Optical tracking was performed using a custom MATLAB program where regions at the 4 corners of the scaffold were hand-selected, and then automatically tracked using a tracking algorithm to calculate local strains.^{12, 32, 37} The scaffolds were mechanically tested using a tensile ramp to failure at 0.5%/sec as described in the “Mechanical Analysis of Fabricated Scaffolds” section. Due to high percentage of grip failure in the incubated samples, yield and failure data could not be determined, and so only modulus and stiffness are reported.

Change in Scaffold Weight

After 0.5, 3, 7, 14, and 21 days of incubation in either PBS or serum, IBP and blank scaffolds were collected and immediately flash frozen (and stored at –80°C) until further analysis. After thawing, each scaffold was weighed three times to establish the average wet weight. Scaffolds were all lyophilized and weighed again three times to establish the average dry weight. Non-incubated scaffolds were weighed from both the IBP and blank groups, and changes in wet and dry weight were calculated as a difference from these average values.

Statistical Analysis

All of the release studies, weight measurements, and mechanical tests were performed with n=3 scaffolds/group. Mechanical properties of dry scaffolds were evaluated using Student's t-tests comparing IBP vs. Blank scaffolds. Mechanical properties of incubated scaffolds were evaluated using 2-way ANOVA comparing between groups (IBP and Blank) and across

time for both modulus and stiffness measures. Change in scaffold weight was evaluated using a 2-way ANOVA comparing between groups (Blank + PBS, Blank + Serum, IBP + PBS, and IBP + Serum) and across time. Significance was set at $p < 0.05$ for all analyses, and all plots are presented as mean $\pm$ standard deviation.

Results

Scaffold Fabrication and In Vitro Release in PBS

Both blank and IBP scaffolds had an aligned fibrous structure under ESEM imaging, and fiber thickness was not significantly altered by the incorporation of IBP. However, the IBP scaffold had slightly more “wavy” fibers, with cross-sectional fibers fused together when observed in cross section (Figure 1A–B).

The release of IBP from the PLGA scaffold demonstrated an attenuated release profile, consisting of 3 phases: (1) a small burst release from days 0–3, (2) a lag phase of little to no release from days 3–10, and (3) a linear release during which roughly 1 μg IBP/mg scaffold/day was released after 10 days (Figure 1C).

Scaffold Mechanical Analysis

The addition of IBP altered the properties of the scaffold (Figure 2A). While the stiffness of the scaffolds did not change with the addition of IBP (Figure 2A,B), the modulus of IBP scaffolds was significantly lower than the blank group (Figure 2B). Additionally, the yield load and stress were significantly lower in the IBP group, while the displacement and strain at yield were not. The maximum failure load was not different between groups, though the maximum failure stress was significantly lower in the IBP group. Further, the maximum failure strain (Figure 2B) and displacement were significantly greater in the IBP group. Finally, the toughness was significantly increased in the IBP group due to the greatly increased failure strain (Figure 2B).

In Vivo Implantation of Scaffolds

We observed that scaffolds implanted *in vivo* in subcutaneous pockets contracted from an 8mm diameter to a 6 $\times$ 3mm oval shape within 12 hours of implantation (Figure 3A). Little tissue adhered to the scaffold within the first week, whereas by day 14, the surrounding tissue had adhered to, but not infiltrated, the scaffold (Figure 3A,C). ESEM images demonstrated the maintenance of fiber structure at all time points, with little fiber swelling or degradation apparent (Figure 3B).

We next quantified the amount of IBP remaining in these scaffold at various time points after implantation. Unlike the *in vitro* degradation profile, these *in vivo* incubated scaffolds showed that 80–90% of the total IBP had released from the scaffold within the first 0.5 days (Figure 3D). Scaffolds retrieved from *in vivo* implantation and placed in PBS for continued release confirmed this finding, with no significant additional release of IBP seen out to 100 days (Figure 3E). Additionally, scaffolds that were transitioned from *in vivo* implantation to *in vitro* incubation in PBS did not macroscopically degrade. Instead, these scaffolds became

hard and brittle, as shown by the cracked scaffold after 100 days in PBS following subcutaneous retrieval (Figure 3F).

Release of IBP In Vitro in Serum

Given the *in vivo* findings, we assessed the release of IBP and scaffold degradation in serum. Scaffolds placed in serum solution *in vitro* demonstrated the same response as those implanted subcutaneously *in vivo*. That is, there was an initial burst response of 80% of total IBP within the first 0.5 days, with all of the IBP released by 7 days when placed in serum (Figure 4).

Degradation of IBP and Blank Scaffolds in PBS and Serum

While the IBP-containing scaffolds macroscopically degraded when placed in PBS, these same IBP scaffolds did not show any signs of degradation when placed in serum, similar to the *in vivo* group (Figure 5A). Similarly, ESEM showed clear fiber swelling by day 14, and loss of all fiber structure by day 21 for IBP scaffolds incubated in PBS. Conversely, IBP-containing scaffolds incubated in serum showed no swelling or degradation for up to 21 days (Figure 6B). Additionally, when evaluating the cross-section ESEM view, the PBS incubated IBP-containing scaffolds showed fiber merging by day 0.5, whereas there was still differentiation of fibers through day 21 in the serum incubated IBP-containing scaffolds (Figure 6D).

Neither the PBS nor the serum incubated blank scaffolds showed macroscopic signs of degradation (Figure 5B). However, the ESEM images of the PBS incubated blank scaffolds showed fiber swelling at the surface (Figure 6A), and fibers merging together in the cross-sectional images (Figure 6C), demonstrating ongoing degradation, though at a slower rate than in the IBP-containing scaffold group. Similar to the IBP group, blank scaffolds incubated in serum showed no microscopic signs of degradation (Figure 6A,C).

Mechanical Analysis of Scaffolds with In Vitro Incubation

Scaffolds that were sectioned into 60×5mm strips for mechanical evaluation primarily contracted along their long axis (in the direction of the fibers) to about 1/3 of their original length in all groups (Figure 7A). When evaluating the change in wet weight, there were significant changes between groups ($p<0.0001$), as well as over time ($p=0.03$). Blank scaffolds increased in weight after incubation, whereas the IBP-containing scaffolds decreased in weight after incubation (Figure 7A), indicating increased uptake of water into the blank scaffolds. For both the blank and IBP groups, incubation in serum resulted in increased weight compared to groups incubated in PBS, however, weights of scaffolds generally decreased over time (Figure 7B). When evaluating the dry weight of the scaffolds, the IBP-containing scaffold groups showed a larger decrease compared to the blank scaffolds. The IBP + PBS group showed the largest change, which decreased further by day 21 (Figure 7C). Mechanical analysis showed that the PBS incubated blank scaffolds had a significantly higher modulus ($p<0.0001$) and stiffness ($p<0.0001$) than the serum incubated blank scaffolds (Figure 7D–F). There was also a significant effect of time for both modulus ($p=0.03$) and stiffness ($p<0.0001$). Due to high percentage of grip failure in the incubated samples, yield and failure data could not be determined. The IBP scaffolds could not be

tested due to warping during incubation and fracture upon loading into the grips. Additionally, the day 21 PBS scaffolds were too stiff (and thus broke in the grips) in the blank scaffold PBS incubation group, so data are only shown through day 14.

Discussion

In this study, we developed an IBP-loaded nanofibrous PLGA scaffold and characterized its properties *in vitro* in PBS, *in vitro* in serum, and *in vivo* through subcutaneous implantation. Although we demonstrated a favorable IBP release profile when incubated in PBS, this result was not replicated *in vivo*. The release profile and morphological changes observed *in vivo* were also seen *in vitro* when scaffolds were incubated in serum rather than PBS. These findings suggest that for IBP-containing scaffolds, serum interactions influence both the release and fiber degradation.

We have demonstrated that an electrospun PLGA scaffold incorporating IBP produced a favorable release profile when incubated *in vitro* in PBS consisting of three phases: (1) an initial burst release from days 0–3 of about 20% of the IBP, (2) a lag phase of little to no release from days 3–10, and (3) a linear release phase during which roughly 1 µg IBP/mg scaffold/day was released after day 10. Assuming *in vivo* release mirrors these results, this profile allows the use of the scaffold to deliver IBP at two distinct time points *in vivo*. To achieve delivery immediately following implantation, the scaffold can be washed for about 10 days *in vitro* prior to implantation until it reaches its linear phase. For delayed release of drug, the scaffold can be washed about 3 days to remove the burst release.

Having these two distinct profiles would provide a tool to study the effect of the local administration of IBP during different phases of healing. We have previously shown that early systemic administration of IBP may impair healing, but there is evidence to suggest that delayed administration is not harmful, and may be beneficial for healing. This system would allow for both local administration and temporal control of IBP delivery to study its effect on tendon healing.

To determine how IBP affects scaffold properties, we performed a mechanical evaluation of dry scaffolds, with and without IBP. We found that the incorporation of IBP significantly altered the mechanical properties of the scaffold, reducing the modulus and increasing the failure strain and toughness. However, the stiffness and maximum failure load were not different between groups. Additionally, ESEM images demonstrated a similar fiber structure between groups. While we could conclude that the incorporation of IBP alters the scaffold properties, it was not apparent from these results how these changes would affect degradation or release performance of the scaffold in an *in vivo* setting.

Finally, we determined that degradation and release properties might vary in different environments, finding that there were notable changes in scaffolds when incubated *in vivo* and *in vitro* in serum compared to the *in vitro* PBS results. Specifically, scaffolds *in vivo* or in serum failed to degrade over time (up to 100 days), whereas scaffolds in PBS degraded steadily over the course of a few weeks. Additionally, instead of the slow linear release profile seen in PBS, the *in vivo* and serum groups demonstrated an initial burst release of

100% of the IBP within the first few days of incubation. Additionally, the mechanical and degradation properties not only changed with the addition of IBP, but also changed depending on the incubation environment. In summary, unlike the PBS results, the *in vivo* and serum results are not favorable for a drug delivery application due to an altered scaffold degradation and IBP release profile.

Overall, these data demonstrate that analysis of release only in a PBS *in vitro* environment can provide an inaccurate representation of what will actually occur *in vivo*. If we had not continued our evaluation *in vivo* or *in vitro* incubation in serum, we would have considered our system to be favorable for our intended application, when in fact it is clearly not due to the *in vivo* results. These findings are important to the field since many researchers only evaluate their biomaterials in PBS to mimic the release *in vivo*.

There have been other reports that modifications to *in vitro* environment (such as change in pH,^{17, 45} ionic strength,¹⁷ and mechanical stress⁴⁴) as well as scaffold properties (such as scaffold shape,²³ composition,³⁵ and drug/biofactor³⁹) can alter the release kinetics and polymer degradation rate. In this work, we demonstrate that *in vivo* implantation of these scaffolds produces a distinctly different drug release profile and degradation properties versus *in vitro* incubation in PBS. Furthermore, whereas prior studies found faster release of biofactors with faster degradation,^{44, 45} our study demonstrated an inverse relationship: the *in vivo* environment was associated with a slower polymer degradation, but a faster drug release.

While this result might be considered surprising in context of the literature on both PLGA biomaterials for drug delivery as well as local IBP administration, it should be noted that there is little to no evidence of a full evaluation of scaffold properties in different environments with the addition of IBP. That is, many prior studies have only evaluated scaffold properties and IBP release in PBS at 37°C.^{7, 19, 25, 26, 33} And while some previous studies have tested outcome measures with *in vivo* application, those studies did not generally evaluate degradation, mechanical, or release properties in serum or *in vivo* prior to completing *in vivo* healing studies.^{24, 25, 33} Many studies likewise did not evaluate long time points (on the order of months) *in vivo*, which may explain why some of the issues highlighted in the present findings are not apparent in previous work. One study evaluated how the incorporation of IBP altered scaffold properties, and found similar to our study, that it accelerated the degradation of the scaffold in water. However, this analysis was not performed in serum.⁷ Finally, another study included IBP release studies in human serum, and found very similar results to our study, with a burst release of 100% of the IBP within 24 hours. However, the evaluation of degradation in that study occurred in PBS, but did not assay degradation in serum.³⁶ Our findings suggest that both the release properties of IBP and the degradation of the polymer differ in PBS versus serum containing solutions.

While limited data exists to support significant alterations in release of IBP from PLGA *in vivo*, studies evaluating the release of other small molecules or factors have performed release analyses both *in vitro* and *in vivo*. For example, the delivery of VEGF from PLGA constructs demonstrated a faster release rate *in vivo* than *in vitro*.¹⁶ However, despite the change in rates, both release environments created a release behavior consisting of an initial

burst followed by a slow linear release. Additionally, a group evaluating the delivery of antibiotics from PLGA scaffolds demonstrated differences in release kinetics *in vivo* compared to *in vitro*.^{8, 42} However, while they observed these differences, they were still able to obtain sustained release of their drug *in vivo*, making it a successful delivery method for their application. In our study, it is possible that IBP itself, as well as the addition of a nanofiber scaffold form, causes a distinct change in release in these different environments with the outcome of an unfavorable *in vivo* release profile.

Although this study was not designed to elucidate the mechanism for differential activity in PBS compared to in serum or *in vivo*, we hypothesize that proteins and other macromolecules in serum may be responsible. It is known that IBP binds highly to serum albumin in blood circulation,¹¹ which could explain why it released so quickly from the scaffold in the presence of serum or implanted subcutaneously. However, this does not explain the altered degradation properties of the PLGA scaffold, both with and without the incorporation of IBP in serum and *in vivo*. It is possible that the form of the PLGA may play a large role in how environmental interactions influence degradation. Macro- vs. micro- vs. nano-scale lengths of polymer architecture could potentially alter how the scaffold interacts with other surrounding molecules. Likewise, since the incorporation of IBP alters the dry scaffold mechanics, it is possible that the polymer structure is altered during fabrication, and therefore degrades differently with the addition of IBP, even after all of the IBP is released.

This study has several limitations. First, we evaluated only a limited number of time points for the *in vivo* incubation study. Hence, we cannot determine whether further degradation of the scaffold would occur *in vivo* that does not occur *in vitro* in serum, potentially due to enzymatic or mechanical degradation. However, we were able to detect major changes *in vivo* that were not present in PBS, and were replicated in serum, which serves the purpose of this study.

Another limitation is that mechanical properties could not be evaluated in the IBP-containing scaffold groups or the day 21 blank-scaffold groups due to scaffold perturbation after incubation. An alternate method of incubation that restricts the curling of the scaffold while it contracts or a different fixture system that allows for the gripping of the scaffold after it becomes brittle, would be necessary to evaluate these properties.

Our observation of macroscopic changes in degradation suggests that the mechanical properties would be different between the IBP serum and PBS groups. Further, it is interesting to note the changes between PBS and serum in the scaffold in the absence of IBP: the addition of IBP to the scaffold alone is not fully responsible for the changes between serum and PBS, although it may exacerbate the changes.

In addition, we were not able to perform mechanical analysis on the *in vivo* incubated scaffolds. It would be interesting to evaluate any potential changes between the *in vivo* and serum groups. However, due to the significant contraction of the scaffolds, it would be difficult to implant a scaffold piece large enough for mechanical evaluation after harvest. A more thorough evaluation of the scaffold composition after incubation in these different environments would likewise provide a more comprehensive understanding of the

mechanism behind these changes. Therefore, future work will include the evaluation of the scaffolds using gel permeation chromatography (GPC) to determine the polydispersity index and molecular weight distribution as each polymer group degrades.

An analysis of how IBP incorporation affects the glass transition temperature (T_g) of the scaffold could provide insight into a possible explanation for the changes in mechanics and degradation seen with the incorporation of IBP. More work to analyze the protein interactions with the drug and the scaffold could also provide insight into the mechanism of the observed release and degradation changes. Finally, we plan to investigate how PLGA materials of different length-scales (such as microspheres instead of nanofibers) respond to these *vitro* vs. *in vivo* environmental conditions.

Although PLGA is widely used as a biomaterial for drug delivery applications, its degradation properties can vary drastically depending on the method of fabrication of the material, the incorporation of a small molecule or drug, and the incubation conditions. In this study, we determined that a nanofibrous PLGA formulation is capable of producing a linear release profile of IBP when incubated *in vitro* in a PBS solution. However, when these same scaffolds were placed *in vivo*, the profile changed to a burst release and scaffold degradation slowed dramatically. Importantly, this phenomenon was reproduced *in vitro* by incubating the scaffold in a serum solution. This study therefore highlights the importance of the alterations in the biomaterial fabrication or application, as well as the importance of thoroughly characterizing a scaffold system in the most relevant environmental conditions prior to completing more long-term *in vivo* studies.

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Figure 1. Scaffold Fabrication and In Vitro Release in PBS

(A) Schematic of the orientation of the PLGA scaffolds for ESEM imaging. (B) Representative images of blank and IBP scaffolds imaged at the surface and in cross-section (scale bars = 20μm, 5000× magnification). (C) The release profile of IBP from scaffolds incubated in PBS shows an initial burst release, followed by a lag phase, and a linear release phase.

Figure 2. Scaffold Mechanical Analysis

(A) Representative load vs. displacement curves for blank and IBP scaffolds tested in tension. The inset shows a zoom in of the linear region of the curve (dashed box). (B) The addition of IBP did not alter scaffold stiffness, but significantly decreased scaffold modulus and increased scaffold failure strain and toughness (* $p < 0.05$).

Figure 3. In Vivo Implantation of Scaffolds

(A) Macroscopic and (B) ESEM images (5000x magnification) of scaffolds after fabrication (day 0) and after subcutaneous implantation for 0.5, 3, 7, and 14 days demonstrating marked changes in macroscopic shape, little tissue adherence to the scaffold, and maintenance of fiber structure (scale bar = 5mm (A) and 20 μ m (B)). (C) H&E histological image at 25X total magnification (scale bar = 400 μ m) and 100X total magnification (scale bar = 200 μ m) of a scaffold after 14 days *in vivo*, demonstrating little cellular infiltration into the scaffold. (D) The quantification of IBP remaining in scaffolds after 0.5, 3, 7, and 14 days *in vivo* demonstrated an immediate release of 80–90% of the total IBP. (E) IBP release profile from scaffolds incubated in PBS after retrieval from subcutaneous implantation confirms the immediate release of IBP *in vivo*. (F) Image of implanted scaffold after further incubation in PBS for 100 days becoming very brittle (scale bar = 2mm).

Figure 4. Release Profiles of IBP Scaffolds

Scaffolds were incubated (1) *in vitro* in serum for 0.5, 3, or 7 days (blue), (2) *in vivo* subcutaneous implantation (red), and (3) *in vitro* PBS incubation (green). While PBS incubation provides a linear release of IBP, both subcutaneous and serum incubation results in an immediate burst release of 80–100% of total IBP from the scaffolds.

Figure 5. Macroscopic images

(A) IBP-containing and (B) blank scaffolds incubated in PBS and serum. IBP-containing scaffolds incubated in PBS show clear signs of degradation over the course of 21 days. No other group shows any macroscopic degradation over this same time course (scale bar = 1cm).

Figure 6. ESEM images

(A, C) Blank and (B, D) IBP-containing scaffolds after 0.5, 14, and 21 days incubation in either PBS or serum. Both the IBP and blank scaffolds incubated in PBS show fiber swelling (surface: A, B) and loss of fiber structure (cross-section: C, D). The IBP-containing scaffold degrades faster, with no fiber structure present at day 21 (* indicates the same picture used for surface and cross-section). There was little to no swelling or degradation in either serum group (scale bar = 20 μ m, 5000 $\times$ magnification).

Figure 7. Mechanical Analysis of Scaffolds with In Vitro Incubation

(A) Image of pre-incubation and post-incubation IBP-containing scaffold. All scaffolds shrunk to about 1/3 of their initial length, regardless of scaffold type or incubation solution.

(B) Change in wet weight shows that blank scaffolds increased, and IBP scaffolds decreased in weight after incubation. Serum incubation increased the weight of both blank and IBP scaffolds (2-way ANOVA significant for both time and group).

(C) Change in dry weight shows that all scaffolds decrease in weight after incubation, with IBP scaffolds showing the largest change (2-way ANOVA significant for both time and group).

(D) Representative image of scaffold speckle painted for optical tracking and tested in tension.

(E) Modulus and (F) stiffness of blank scaffolds show increases in properties with PBS incubation both compared to serum incubation, and over time (2-way ANOVA significant for both time and group).