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Materials composed of the *Drosophila* Hox protein Ultrabithorax are biocompatible and nonimmunogenic

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Abstract: Although the *in vivo* function of the *Drosophila melanogaster* Hox protein Ultrabithorax (Ubx) is to regulate transcription, *in vitro* Ubx hierarchically self-assembles to form nanoscale to macroscale materials. The morphology, mechanical properties, and functionality (via protein chimeras) of Ubx materials are all easily engineered. Ubx materials are also compatible with cells in culture. These properties make Ubx attractive as a potential tissue engineering scaffold, but to be used as such they must be biocompatible and nonimmunogenic. In this study, we assess whether Ubx materials are suitable for *in vivo* applications. When implanted into mice, Ubx fibers attracted few immune cells to the implant area. Sera from mice implanted with Ubx contain little to no antibodies capable of recognizing Ubx. Furthermore, Ubx fibers cultured with macrophages *in vitro* did not lyse or activate the macrophages, as measured by TNF-α and NO secretion. Finally, Ubx fibers do not cause hemolysis when incubated with human red blood cells. The minimal effects observed are comparable with those induced by biomaterials used successfully *in vivo*. We conclude Ubx materials are biocompatible and nonimmunogenic. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 103A: 1546–1553, 2015.

Key Words: protein-based materials, Ultrabithorax, biocompatible, macrophage, hemolysis

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INTRODUCTION

Protein-based materials have many advantages for use as tissue-engineering scaffolds. Many of these materials are biocompatible and have low immunogenicity.[1,2] Protein forms a major component of the extracellular matrix, and protein-based materials can have chemical and mechanical properties similar to the natural cell environment, which is important for the correct specification of cell behavior.[3,4] Recombinant protein monomers can also be readily functionalized by gene fusion, in which DNA encoding a functional peptide or protein is fused to DNA encoding the self-assembling protein.[5,6] Finally, recombinant production of proteins provides a renewable and uniform supply of monomers for assembly whose sequences, and hence properties, can be easily engineered.[2] However, recombinant production and *in vitro* assembly in harsh physicochemical conditions can sometimes damage the mechanical properties of the resulting materials and preclude incorporation of fused proteins in their native and active state.

The *Drosophila melanogaster* transcription factor Ultrabithorax (Ubx) binds DNA and proteins *in vivo* to regulate gene transcription.[7–13] Recently, we discovered that Ubx also rapidly self-assembles in mild conditions *in vitro* to form hierarchically structured materials.[14,15] Ubx fibers have tunable mechanical properties which can mimic those of natural elastin.[4] Since Ubx self-assembles rapidly in gentle buffers, full-length, folded proteins can be easily incorporated in their active state and even patterned within the materials (Fig. 1).[5]

Cells respond to both the mechanical properties of their environment and to proteins, which bind to cell receptors to transmit signaling information. Consequently, the ability of Ubx materials to both mimic an extracellular matrix protein and display active proteins is a key advantage for application of these materials as tissue engineering scaffolds. However, these materials must also be biocompatible to be useful for tissue engineering. Ubx materials are compatible

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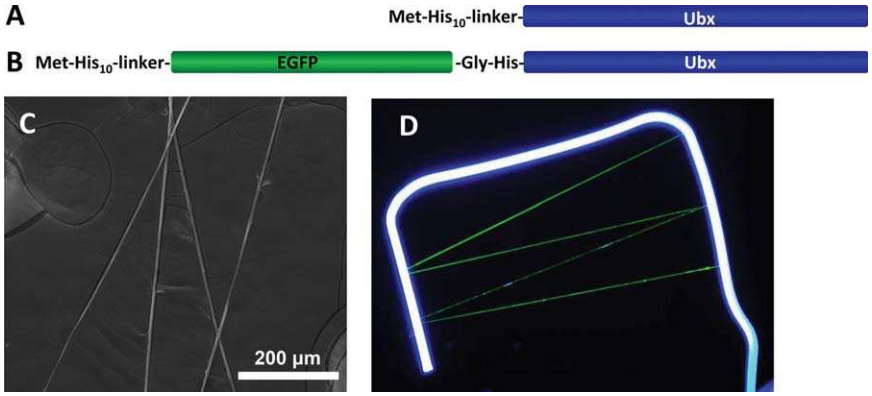

FIGURE 1. Variants of Ubx used to make materials. A: Sequence schematic of his-tagged Ubx splicing variant Ia, which was used for most of the experiments in this study. B: Sequence schematic of the his-tagged EGFP-Ubx fusion protein used for hemolysis experiments. C: Scanning electron microscopy of four Ubx fibers. D: Fluorescent photomicrograph of an EGFP-Ubx fiber (green) supported by a plastic-coated wire (white). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with a variety of cell types *in vitro*, including endothelial cells, smooth muscle cells, and pericytes.[16] Ubx materials do not leach toxins, and no signs of cytotoxicity were observed for cells in direct contact with fibers. Cells also readily attach to Ubx fibers, which are sufficient for their support.

In general, *in vitro* cytocompatibility correlates well with *in vivo* biocompatibility.[17] However, if an immune response should occur it can cause inflammation or even necrosis of host tissues.[18] Furthermore, the extent to which a biomaterial stimulates an inflammatory response can also affect the immune response of the host to cells transplanted with the materials in a tissue-engineered construct.[19] Consequently it is important to determine whether Ubx fibers can trigger an inflammatory or immune response *in vivo*.

In this paper, we examine the biocompatibility and immunogenicity of Ubx materials. We find that Ubx fibers attract very low levels of immune cells when implanted subcutaneously in mice. Similarly, mice challenged with Ubx fibers produced little to no antibodies capable of recognizing Ubx in response to implantation. Ubx fibers neither stimulate macrophage apoptosis, nor activate macrophages in cell culture, indicating they are not inflammatory. Finally, Ubx fibers do not cause hemolysis. All responses are comparable to other biomaterials, such as silk fibroin, which have been used successfully *in vivo*.[18,20–23] We conclude that Ubx materials are biocompatible and nonimmunogenic.

MATERIALS AND METHODS

Production of Ubx materials

Monomers of his-tagged Ultrabithorax splicing isoform Ia were produced in *E. coli* from the pET19b-UbxIa vector and purified as previously described.[16] Ubx fibers were produced by the tray/buffer reservoir system, also as previously described.[5,15,16]

Immunogenecity studies in mice

Preparation of sponges, fiber assembly and harvest were carried out inside a Level II Safety cabinet to maintain aseptic conditions. PVA sponges (10 mm diameter, 3 mm thick disks) were obtained through PVA Unlimited (Warsaw, IN). Sponges were prepared according to the method described by Molecular Imaging Research (Standard Operating Procedures, Sponge Granuloma in Rats, August 2008). Briefly, sponges were soaked in 70% EtOH overnight, rinsed in sterile distilled water (Gibco Ultrapure, DNase/RNase free), and then placed in boiling sterile water for 10 min. Sterilized sponges were transferred to a sterile conical tube containing sterile water, and stored at 4°C overnight and used the following day.

Hydrated sterile sponges were transferred to a sterile Petri dish. The sponge disk was pierced radially with a sterile 16 gauge needle. The handle of a sterile, plastic inoculating loop was then inserted into the punctured side of the sponge disk to form a handle. Fibers were collected from the buffer tray, wound around the sponge (~8 wraps of fiber per sponge), and allowed to air dry for 10 min such that the sponges were not touching any surface other than the plastic handle. The fiber-coated sponge was removed and transferred into a sterile 15 mL conical centrifuge tube containing 200 μL of sterile DPBS-CMF (calcium/magnesium free) (Gibco). Tubes were sealed with parafilm and stored at 4°C until use.

In vivo studies of fiber-wrapped sponges, including histologic assessment by a board certified veterinary pathologist, were performed by Charles River Laboratories Discovery Research Services. In brief, sponges were implanted into 7–9 weeks C57/BL/6 female mice in the subcutaneous space on the dorsal surface between the scapulae of each mouse. Four mice received fiber-wrapped sponges, and four additional mice received plain sponges as vector controls. After 29 days, each animal was euthanized and the sponge was retrieved, formalin-fixed, bisected into hemispheres, and paraffin embedded. Two 4 μm sections, at 200 μm step levels, were cut from each hemisphere and analyzed by microscopy.

Testing for anti-Ubx antibodies

Blood was collected by terminal cardiac puncture under CO_2 anesthesia and frozen. Total protein concentration for the mouse serum samples was determined using the Bio-

Rad protein assay. Positive controls, in which primary antibody is known to bind Ubx, include 6× anti-his tag antibody (Qiagen) and FP3.38 anti-Ubx homeodomain antibody (Developmental Studies Hybridoma Bank, University of Iowa).[24] In order to maintain a constant concentration of primary antibodies for western blotting, total protein concentrations, and SDS-PAGE was used to compare the quantity of antibody in mouse serum samples (Supporting Information Fig. 1) with anti-His and FP3.38 antibody concentrations. Much higher concentrations of antibody in mouse sera were used than for the positive control antibodies to increase the probability of detecting anti-Ubx antibodies in the mouse sera.

Western blotting was subsequently used to determine whether mouse sera contained antibodies that could specifically recognize purified Ubx and Ubx in *E. coli* whole cell lysate. For Western blots, sera from four different mice exposed only to sponges were mixed together for the negative control primary antibody. Likewise, sera from four mice challenged with sponges and Ubx fibers were also mixed together. The purified mouse anti-His antibody (Qiagen, USA) was diluted 1:10,000 to a final concentration of 20 pg/μL, and FP3.38 anti-Ubx antibody[24] was diluted 1:200 to a final concentration of 250 pg/μL. Unchallenged mouse serum, and challenged mouse serum (both at 248 pg/μL) were used in separate western blots as the primary antibody solutions to probe against purified His-tagged Ubx Ia protein and *E. coli* whole cell lysate containing His-tagged Ubx Ia protein. Goat antimouse IgG conjugated with horseradish peroxidase (HRP) was used as secondary antibody at a concentration of 50 pg/μL. Blots were detected by reaction with ECL chemiluminescent substrate (Invitrogen) followed by 10 min film exposure.

ELISA assays were also used to test for anti-Ubx antibodies in mouse serum. The wells of an Immulon 1B microtiter plate (Thermo Scientific, USA) were coated with Ubx by incubating 100 μL/well of purified Ubx (1 μg/mL) in bicarbonate/carbonate buffer (28 m*M* Na_2CO_3 and 72 m*M* $NaHCO_3$, pH 9.6) in the wells at room temperature for 2 h. The wells were washed three times with 200 μL PBST (16 m*M* Na_2HPO_4, 2.6 m*M* KCl, 1.2 m*M* K_2HPO_4, 68 m*M* NaCl, and 0.05% v/v Tween20, pH 7.4) and then blocked by incubating in 200 μL blocking buffer (2% BSA in PBS) overnight at 4°C.

After washing three times with 200 μL PBST to remove excess blocking buffer, the wells were incubated with 100 μL of primary antibody (FP3.38/unchallenged mouse serum/challenged mouse serum) diluted 1:100, 1:1000, and 1:10,000 in blocking buffer at room temperature for 2 h before washing the wells three times with 200 μL PBST to remove excess primary antibody. After a 2 h incubation in 100 μL of 20 pg/μL donkey antimouse IgG conjugated with horseradish peroxidase (HRP) in blocking buffer, wells were washed three times with 200 μL PBST, the signal was developed by adding 100 μL of Sigmafast-OPD (Sigma-Aldrich, USA, one tablet dissolved in 20 mL of ddH_2O) to each well. The reaction was stopped by adding 100 μL of 2*M* sulfuric acid to each well. The absorbance of each well at 490 nm was measured using a VICTOR™ X Multilabel Plate Reader (Perkin Elmer, USA).

Cytokine production assays

pET19b-UbxIa fibers, produced by the buffer tray method,[15] were harvested onto large vinyl-coated paperclips, using the buffer tray method. Fibers were transferred to Eppendorf tubes containing 900 μL of PBS pH 7.4 solution. Fibers were then subjected to either mechanical disruption with a pestle, resulting in larger fiber fragments, or bath sonication (15 min), resulting in small fragments and a more 'homogenous' solution. Purified protein monomer (0.18 mg/mL) was included as control.

Murine macrophage-like J774.A1 (ATCC TIB-67) cells were used to assess the biocompatibility/activation of Ubx fiber fragments. Macrophage assays were performed as previously described, with some modifications.[25] Briefly, macrophages were cultured in Dulbecco's modified Eagle medium with 10% (vol/vol) fetal bovine serum, 1 m*M* L-glutamine, and 1 m*M* nonessential amino acids. Monolayers of macrophages containing 2.5×10^5 cells per well were incubated with different concentrations of Ubx fibers. At 48 h postinfection, culture supernatants were collected and analyzed for nitric oxide production and TNFα production. Nitric oxide concentrations were measured using the Griess reagent (Sigma) following the manufacturer's protocols. Lipopolysaccharide (LPS) at 0.5 μg/mL was used as a positive control for both nitric oxide and TNF-α assays. All assays were performed in triplicate and repeated at least three times.

Macrophage cytotoxicity assay

Ubx fiber fragments were prepared and cultured with J774A.1 macrophages as described above. LDH release into cell culture supernatants was detected using the CytoTox 96 nonradioactive cytotoxicity assay as previously described.[25] Cell death was expressed as the percentage of LDH release, which was calculated using the following formula: percentage of LDH release = 100 × (test LDH release − spontaneous release)/(maximum release − spontaneous release). The maximum release was determined following dissolution of cell monolayers using 1% (vol/vol) Triton X-100.

Cytokine ELISA

Tumor necrosis factor alpha (TNF-α) levels in the culture supernatants were determined 48 h poststimulation using sandwich ELISA kits (PeproTech, Rocky Hill, NJ) according to the manufacturer's instructions as previously described.[26]

Ubx fiber proteolysis

Ubx fibers were wrapped around a glass slide and allowed to dry for 1 h on the lab bench under a kimwipe. A custom imaging chamber was created in which a coverslip formed the bottom surface and a 1 mm nylon washer formed the walls. The trypsin reaction (2 mg/mL trypsin in 0.3*M* Tris, pH 8.0, 200 m*M* KCL, 1 m*M* $CaCl_2$, 1 m*M* DTT) was added to this chamber, and the fiber wrapped slides were placed on top to initiate proteolysis. Ubx fiber proteolysis was observed by acquiring DIC Z-stack images every 30 min for 15 h on a Nikon Eclipse Ti confocal microscope with NIS Elements AR 4.10.01 software.

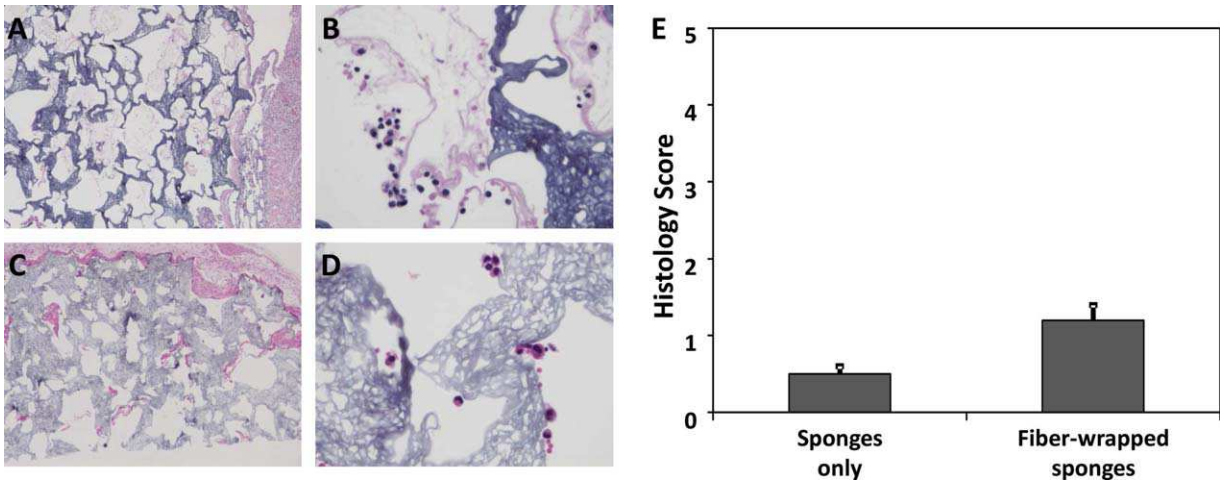

FIGURE 2. Histological sections of Ubx fiber/sponge implants after 29 days. A–D Representative micrographs of histological sections. A, B: Sections of sponges implanted without Ubx fibers. C, D: Sections of sponges wrapped in Ubx fibers. E: All data represented as histology scores, ranging from 0 to 5, as defined in Table I. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Hemolysis assays

EGFP-Ubx1a fibers were pulled on to large vinyl-coated paperclips, using the buffer reservoir method. Fibers were harvested from the paperclips, and placed in 1.5 mL Eppendorf tubes and on 35 mm glass bottom dishes (MatTek Corporation, MA). Fibers were washed with PBS (pH 7.4) three times before adding red blood cells (RBCs). The hemolysis assays were performed as described.[20,27] Briefly, human whole blood purchased from Gulf Coast Regional Blood Center (Houston, TX) was washed with PBS three times and centrifuged at 1500*g* for 5 min to obtain RBCs. The RBCs were then diluted with PBS to produce 1% RBC solution, 100 μL of which was added to tubes with Ubx fibers and incubated for 20 or 70 min at 37°C. This approach is similar to that previously used to test other biomaterials.[20] The intact RBCs and cell debris were removed by centrifuging at 1500*g* for 5 min. The supernatants were transferred to 96-well plate and the absorbance was measured at 450 nm to detect the release of hemoglobin with a plate reader (GloMax®-Multi+ Detection System, Promega, WI). RBCs incubated in tubes without proteins were tested to determine the background hemolysis, and RBCs incubated with 0.1% Triton X-100 served as positive controls (100% hemolysis). All experiments were repeated five times. The dishes with fibers were incubated with 200 μL of 1% RBCs and placed on a heating stage at 37°C for 20 or 70 min as indicated. Imaging was performed by an inverted epifluorescence microscope (Model IX81; Olympus, Center Valley, PA) equipped with Rolera-MGI Plus back-illuminated EMCCD camera (Qimaging, Surrey, BC, Canada). The images were acquired from bright field channel and FITC fluorescence filter (Ex = 482 ± 35 nm/Em = 536 ± 40 nm).

RESULTS AND DISCUSSION

Ubx materials do not induce widespread inflammation *in vivo*

In order for Ubx fibers to be used as a tissue engineering scaffold, they must be biocompatible *in vivo*. To determine whether Ubx materials are safe for *in vivo* applications, we implanted Ubx fibers into C57/BL/6 mice. Because (i) fibers would be difficult to locate at the end of the procedure, and (ii) Ubx fibers are strong and tend to pull out of sliced sections, Ubx fibers were wrapped around sterile PVA sponges prior to subcutaneous implantation. Even though Ubx fibers can be difficult to visualize in slices, the sponges trap any immune cells attracted by the fibers. Sponges with wrapped fibers were implanted subcutaneously in four female C57/BL/6 mice aged 7–9 weeks. As a negative control, sponges without Ubx fibers were implanted in four additional mice.

Sponges with or without Ubx fibers were implanted in mice for 29 days. This duration is both sufficient to develop antibodies and an immune response and simultaneously long enough for surgery-induced inflammation to subside.[28,29] For these reasons, many studies have used similar exposure times to assess immunogenicity.[28–32] The body weight of mice in the control and treated groups were not significantly different during the study (Supporting Information Fig. 2). Microscopy of the sponges revealed a small number of macrophages, lymphocytes, or neutrophils associated with the Ubx fibers (Fig. 2). For samples both with and without Ubx fibers, fibroblasts, and collagen were present along the sponge border and in the matrix of some sponge pores. Widely scattered inflammatory cells were more frequently present in Ubx fiber-wrapped sponges. When present, these cells were more likely to be located near the sponge surface. Micrographs were scored according to Table I. The slight increase in the number of immune cells in the presence of Ubx fibers was significant. However, the overall quantity of immune cells was both low and comparable to the quantity attracted by other nonimmunogenic protein-based materials *in vivo*,[18–21] including materials that have been successfully used in medical applications.[18]

Ubx materials stimulate little to no antibody production *in vivo*

To determine whether this low level of inflammation reflects activation of the immune system, we first used

TABLE I. Definition of Histopathology Scores

Score	Definition
0	Normal
0.5	Very widely scattered inflammatory cells in sponge surface or spaces
1	Minimal increase in inflammation with at least 3% of the sponge surface/spaces having infiltrates of macrophages, lymphocytes, and/or neutrophils
2	At least 10% of the sponge surface/spaces having infiltrates of macrophages, lymphocytes, and/or neutrophils
3	At least 20% of the sponge surface/spaces having infiltrates of macrophages, lymphocytes, and/or neutrophils
4	At least 40% of the sponge surface/spaces having infiltrates of macrophages, lymphocytes, and/or neutrophils
5	At least 75% of the sponge surface/spaces having infiltrates of macrophages, lymphocytes, and/or neutrophils

western blots to test whether sera from the mice contained antibodies that could recognize Ubx. Because western blots detect denatured protein, this method can detect antibodies that recognize buried portions of Ubx monomers that are either exposed during materials assembly or exposed through proteolysis of the materials *in vivo*. For each western blot, gel lanes contained either clarified lysate from *E. coli* expressing Ubx or purified Ubx. Extremely high concentrations of *E. coli* cell lysate were used to increase the opportunity for detecting binding by any anti-Ubx antibodies present in the mouse serum. However, the resulting overloaded lanes appear streaky. The total concentration of antibody in the challenged and unchallenged mouse sera were similar (Supporting Information Fig. 1). Both anti-His tag antibody and FP3.38, an antibody that specifically recognizes the Ubx DNA-binding domain[24] [Fig. 3(A,B)] served as positive controls. As expected, sera from negative control mice implanted only with sponges did not contain antibodies capable of recognizing Ubx [Fig. 3(C)]. Sera from mice implanted with sponges wrapped with Ubx fibers also lacked antibodies recognizing Ubx [Fig. 3(D)]. Because Western blots are less sensitive than ELISA assays, we also used ELISA assays to test the mouse sera. Similarly, sera from three Ubx-challenged mice showed no evidence of anti-Ubx antibodies, although a very weak response was observed for one mouse [Fig. 3(E)]. Therefore, we conclude that Ubx fibers are unlikely to elicit a humoral (acquired) immune response *in vivo*.

Ubx materials do not stimulate release of pro-inflammatory cytokines

Macrophages infiltrate and rapidly respond to biomaterial implantation.[33] Ubx fibers could damage the immune system by being toxic to macrophages, or trigger an innate immune response by activating macrophages. To test these possibilities, we first examined whether Ubx fibers, fragmented into small (pipetable) pieces by mechanical disruption with a pestle and sonication, could kill or activate

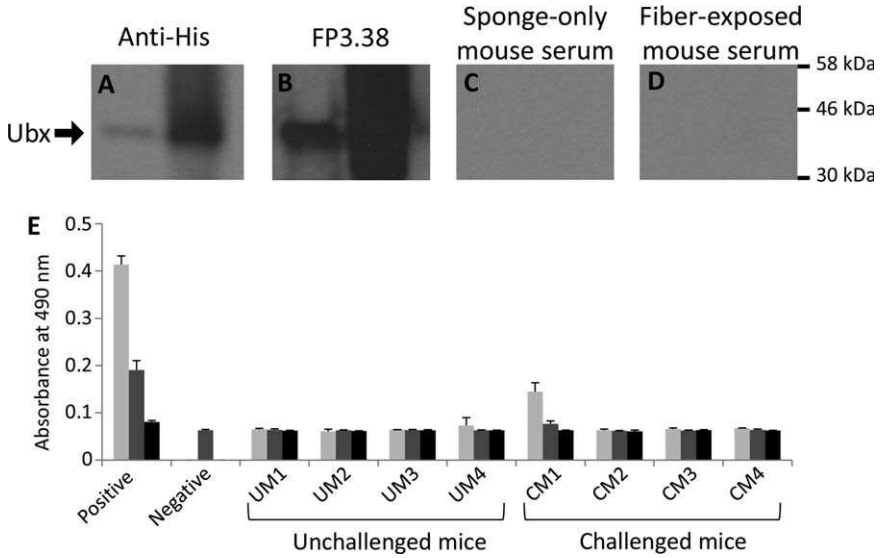

FIGURE 3. Ubx fibers do not elicit a significant antibody response. Each panel show a western blot using a different source for primary antibodies to determine whether antibodies are present that can detect Ubx. For each blot, lanes are 1: purified his-tagged Ubxla, 2: crude *E. coli* cell lysate containing his-tagged Ubxla. Panels A (6× anti-his) and B (FP3.38) depict Western blots using primary antibodies known to react with Ubx and serve as positive controls. Panel C is serum from the negative control mice implanted only with sponges, and panel D is serum from mice implanted with fiber-wrapped sponges. The Ubx band is marked with an arrow to the left of panel A, and the positions of nearby molecular weight markers are indicated to the right of panel D. No anti-Ubx antibodies were detected in the fiber-implanted mice. In panel E, ELISA assays show no detectable level of antibody in mice implanted with Ubx materials. The purified anti-homeodomain antibody (FP3.38) was used for positive control and purified donkey antimouse IgG HRP antibody was used for negative control. G1(#1–#4) represent unchallenged mouse and G2(#1–#4) represent challenged mouse serum. Dilution factors for primary antibody or mouse serum are 1:100 (light gray), 1:1000 (dark gray), and 1:10,000 (black).

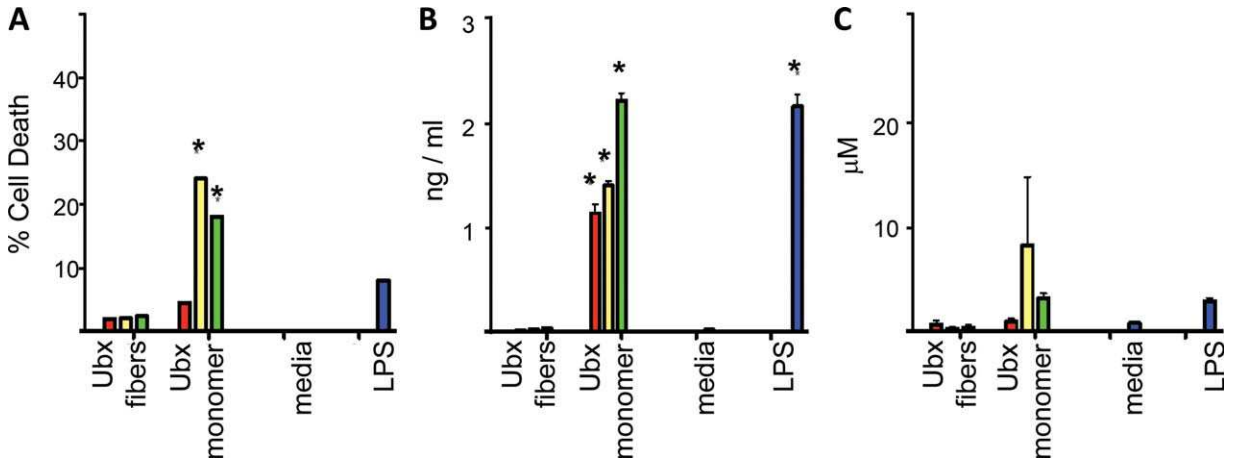

FIGURE 4. Ubx fibers are not toxic and do not activate macrophages in cell culture. A: LDH release. B: TNF-α production. C: Nitric oxide production. In each experiment, media was used as a negative control and lipopolysaccharide as a positive control. Ubx monomer and fiber concentrations were 0.45 μg/mL (red bar), 4.5 μg/mL (yellow bar), and 9 μg/mL (green bar). Lipopolysaccharides (LPS) served as a positive control, and media lacking any additives as a negative control. While Ubx monomers showed a positive response, Ubx fibers did not affect macrophages. The significance of differences between groups was assessed by ANOVA followed by Tukey's *post hoc* multiple comparison test. For ANOVA, $p < 0.05$ was considered statistically significant. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

J774.A1 macrophages in cell culture. Macrophages play a crucial role in immune regulation. Macrophage apoptosis (and subsequent lysis) was tracked by monitoring the release of a cytosolic enzyme, lactate dehydrogenase, into the media. Although Ubx *monomers* induce macrophage lysis, in the presence of Ubx *fibers*, lysis is minimal [Fig. 4(A)].

TNF-α, which is secreted by macrophages and other cell types in response to foreign agents, serves as a pro-inflammatory marker for acute and chronic inflammation.[22,34] Low levels of TNF-α release suggest a low overall immunogenicity of a material.[35] Ubx monomers do stimulate TNF-α secretion. However, the levels of TNF-α secretion induced by Ubx fibers were similar to the negative control, and significantly different from the LPS positive control [Fig. 4(B)]. Silks, which have been used *in vivo* for thousands of years,[18] have a similar differential response in which particles, but not fibers, stimulate TNF release.[22,23]

Activated macrophages also produce high levels of the cytotoxic molecule nitric oxide (NO), providing a second molecular measure for macrophage stimulation.[33] NO production in the presence of Ubx monomers and fibers was used to confirm the TNF-α results. This metric also shows that Ubx monomers, but, importantly, not Ubx fibers, stimulate NO production [Fig. 4(C)]. Thus, we conclude that Ubx fibers are immunologically inert.

Ubx fibers are protease-resistant

Because Ubx *monomers* do activate macrophages, one concern is whether degradation of Ubx fibers might release toxic monomers over very long time scales. However, Ubx monomers are unusually susceptible to proteases, due in a large part to the fact that most of the protein lacks stable secondary structure.[7] Indeed, even at extremely low protease concentrations, Ubx monomers are proteolyzed in just 5 min. In contrast, Ubx fibers are remarkably resistant to proteases. Protease concentrations 1000-fold higher require several hours just to create cavities in the fiber, with much of the fiber structure remaining intact (Fig. 5). Therefore, the levels of protease activity required to degrade Ubx fiber are far higher than those required to degrade Ubx monomer, and Ubx monomer should not accumulate *in vivo*. Indeed, gel electrophoresis of products from a partially digested fiber demonstrates that Ubx monomer and large fragments of the Ubx protein do not accumulate (data not shown). These results are very similar to those observed for spider silks. Although silk monomers elicit an immune response, and antibodies to silk monomers can be produced, silk fibers are immunologically inert and can be safely degraded *in vivo*.[36–38]

Ubx materials are not hemolytic

We can generate Ubx fibers with mechanical properties comparable to natural elastin, and elastin is a major component of the extracellular matrix of vasculature.[39,40] In addition, Ubx fibers are compatible with a variety of vascular cell types.[16] These traits suggest Ubx materials may make useful vascular scaffolds. However, for this application to be successful, Ubx materials must not only be biocompatible—they also cannot be hemolytic. We tested whether Ubx materials induce hemolysis by incubating fibers with human red blood cells. Because this data includes microscopy, we used fibers produced from a fusion of enhanced green fluorescent protein (EGFP)-Ubx[5] to insure we could visualize the fibers [Fig. 6(A,C)]. No hemolysis induced by EGFP-Ubx was observed after incubating for either 20 or 70 min [Fig. 6(B,D,E)].

CONCLUSIONS

Since Ubx materials, unlike elastin and collagen, are not naturally found *in vivo*, it is particularly important to determine

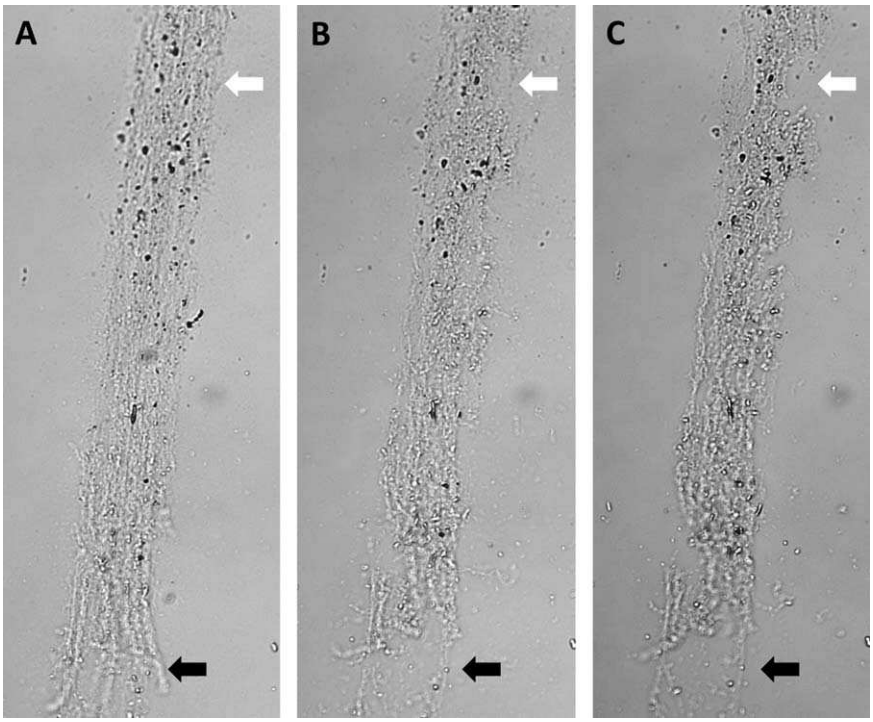

FIGURE 5. Ubx fibers are resistant to protease degradation. DIC microscopy of a Ubx fiber during digestion with 2 mg/mL trypsin for A: 0.3 h, B: 8.3 h, and C: 15.3 h. Although large (black arrow) and small (white arrow) cavities appear, the fiber is still largely intact even after a long exposure to 1000-fold more trypsin than is required to digest Ubx monomer in 5 min.[7] Consequently, toxic Ubx monomers are unlikely to accumulate *in vivo*.

whether Ubx materials are biocompatible to evaluate their potential for *in vivo* applications. Although Ubx fibers attract very low levels of immune cells when implanted subcutaneously in mice, the mice did not produce antibodies capable of recognizing Ubx in response to implantation. Ubx fibers neither stimulate macrophage apoptosis, nor activate macrophages in cell culture. Ubx protein monomers or protein fragments do not accumulate during proteolysis of Ubx fibers. Furthermore, Ubx fibers did not cause hemolysis or inappropriately bind red blood cells. We conclude that Ubx materials are biocompatible, nonimmunogenic, noninflammatory, and nonhemolytic.

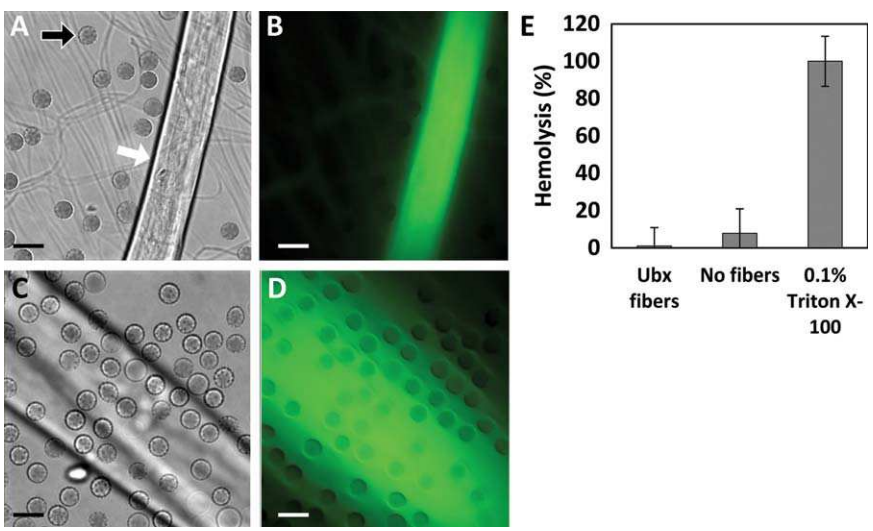

FIGURE 6. Hemolysis assay. A: A 100× photomicrograph showing red blood cells (arrow) and B: the corresponding fluorescence micrograph (not re-focused) confirming the fiber is composed of EGFP-Ubx after a 20 min incubation. C: A photomicrograph and D: a fluorescence micrograph of an EGFP-Ubx fiber incubated with red blood cells after 70 min. E: Graph depicting hemolysis data acquired after 70 min incubation. Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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