Characterization of ReadiGRAFT® BLX Fibers, an Optimal Scaffold for Bone Healing

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ORIGINAL RESEARCH ARTICLE

Bone formation requires three elements - osteoconductivity, osteoinductivity, and osteogenicity. ReadiGraft BLX Fibers is a specifically engineered 100% bone allograft matrix that provides an osteoconductive scaffold with increased surface area for cell attachment and proliferation and contains osteoinductive natural growth factors to recruit host bone forming cells. ReadiGraft BLX Fibers are a loose bone void filler that can be mixed with autologous bone or bone marrow aspirate (BMA), which would provide an osteogenic component. The osteoconductivity and osteoinductive potential of the fiber and the graft matrix was characterized through *in vitro* and *in vivo* testing and is presented here.

Data from *in vitro* tests illustrated that ReadiGraft BLX Fibers supported the attachment of viable cells. Cell-mediated matrix deposition was also observed in these tests, a further indicator of sustained cellular activity and allograft biocompatibility. In addition, hemocompatibility tests showed that ReadiGraft BLX Fibers rehydrated with blood produced fibrin clots, which may contribute additional growth factors on the surface of the scaffold. Osteoinductive (OI) potential of the graft matrix was demonstrated in an athymic rat model in which implanted material was associated with the growth of new bone elements such as cells and neo-vascularization. Finally, implant-host bone fusion was achieved in an athymic rat posterolateral fusion (PLF) model which showed that ReadiGraft BLX graft matrix supported the generation of new bone growth that successfully bridged and fused spinal processes. Taken altogether, these characteristics demonstrate that ReadiGraft BLX Fibers afford osteoinductive and osteoconductive properties necessary for proper bone healing.

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KEY WORDS: demineralized bone matrix fibers; osteoinductive (OI); hemocompatibility; cellular activity; cellular viability; cell attachment; bone growth; bone fusion

Introduction

The ability of demineralized bone matrix (DBM) to help facilitate bone healing has been known in clinical settings for over a century [1]. However, the factors contributing to this phenomenon were unknown until 1965 when Dr. Marshall Urist characterized specific active proteins trapped in the matrix of bone that endow it with this property [2, 3]. These protein factors are called bone morphogenetic proteins (BMPs) while the property of directing bone formation is referred to as osteoinduction. Since this discovery, bone void fillers containing DBM sourced from donated human tissue have been used in orthopedic and spinal fusion surgery to facilitate healing and new bone formation. LifeNet Health has developed proprietary demineralization technology to expose BMPs, growth factors, and other proteins trapped in the bone matrix to enhance its therapeutic potential in driving bone healing and the regeneration process [4-8]. In addition to containing active proteins which can direct bone formation, the porosity of bone grafts is essential for vascularization of and cell infiltration into the bone graft. This provides enough space between bone fibers for blood vessels to form within the bone fiber matrix and for the patient's own cells to migrate into and proliferate in the scaffold [9-11]. The purpose of this study was to characterize and assess the osteoconductive properties and osteoinductive potential of ReadiGraft BLX Fibers and graft matrix.

Methodology Overview

a. In vitro Cellular Activity, Viability, and Attachment [12]

ReadiGraft BLX Fibers from three separate donor lots were placed in triplicate in non-treated plastic cell culture plates and seeded with human bone marrow-derived mesenchymal stem cells (BM-MSCs; Life Technologies, Carlsbad, CA) recovered from donor bone marrow. The BM-MSCs were seeded to assess whether the ReadiGraft BLX Fibers created a biohospitable environment that supported cellular functions. BM-MSCs were seeded at 25,000 BM-MSCs per 25 ± 2.5 mg fiber sample on day 0 and cultured over 7 days with the appropriate growth media. At days 1, 3, 5, and 7 in culture, medium was changed and Alamar Blue® reagent (Bio-Rad Laboratories, Raleigh, NC) was

added to each sample. After incubation, medium containing the Alamar Blue reagent was removed from the samples and analyzed to assess sustained cellular activity and viability using a fluorescence plate reader. Fluorescence was recorded using relative fluorescence units (RFUs) and values were averaged for each donor lot and normalized to an appropriate control.

To visually assess cell attachment, ReadiGraft BLX Fibers were seeded with 4x as many BM-MSCs as was used in the Alamar Blue assay (100,000 BM-MSCs per 25 ± 2.5 mg fiber sample), and samples were imaged using scanning electron microscopy (SEM) at 1 hour, 1 day, and 7 days after seeding.

b. In vitro Hemocompatibility [12]

ReadiGraft BLX Fibers were plated and hydrated with clotting blood (Biological Specialty Corporation, Colmar, PA) to coat the entire sample. Hydrated samples were allowed to incubate for 20 min and subsequently fixed in two stages followed by dehydration and imaged using scanning electron microscopy (SEM).

c. In vivo Osteoinductive Potential (OI) [13, 14]

1. Osteoinductive potential (OI) of the ReadiGraft BLX Fibers graft matrix and the generation of new bone was assessed *in vivo* utilizing a 5 week athymic rat muscle pouch model of OI with histological analysis using an implant of 0.45cc.

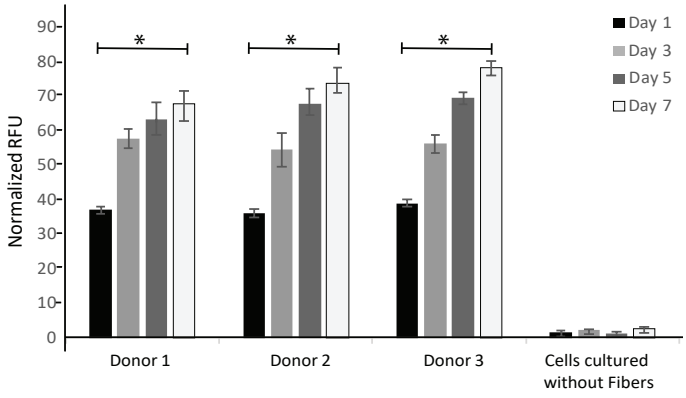
Additionally, the *in vivo* OI potential of ReadiGraft BLX Fibers graft matrix (with a mineralized cancellous bone component) was also assessed in an 8 week athymic rat posterolateral fusion (PLF) model where bilateral implantation of 0.2cc of ReadiGraft BLX Fibers graft matrix was applied between L4 and L5 transverse spinal processes. Fusion was analyzed by radiographs, micro CT, and histology at the conclusion of the 8 week study.

Results

Stem Cells Demonstrate Robust Cell Attachment, Sustained Cellular Activity, and Cellular Viability

Mesenchymal stem cells (MSCs) were seeded and cultured on ReadiGraft BLX Fibers generated from the three different donor lots to test the biocompatibility of the scaffolds over 7 days. The cellular activity and cellular viability of the MSCs were determined by Alamar Blue Assay. MSCs seeded on ReadiGraft BLX Fibers had a statistically higher cellular activity compared to MSCs grown on non-treated cell culture plates beyond 5 days in culture **(Figure 1).** Overall, the cellular activity of the MSCs was shown to steadily increase over the course of the 7 day investigation. These data suggested the ReadiGraft BLX Fibers offered both a biocompatible and biohospitable environment for cells to colonize. Furthermore, MSC attachment to ReadiGraft BLX Fibers was confirmed by SEM at one hour, 1 day and 7 days after seeding **(Figure 2).** After 1 hour, cells immediately showed robust attachment. A closer examination of the ReadiGraft BLX Fibers showed a rough surface topography which facilitates initial cell attachment **(Figure 3).** After 1 day in culture, cells began transitioning from a spherical to a flattened morphology and had also begun secreting extracellular matrix (ECM) **(Figure 2).** By day 7, the cells had reached confluence and covered the fibers of the scaffold with uniform flattened sheets of highly networked cells with multiple junctions and points of contact.

Figure 1. Cellular activity of MSCs seeded on three donor lots for ReadiGraft BLX Fibers increased over the course of seven days.



The graph shows the average relative fluorescence unit (RFU) values (bars reflect Standard Deviation) for each set of triplicate test samples that were normalized to the average RFU of the corresponding control group (cells cultured without fibers) for all three donors. * $P < 0.05$ between day 1 and 7.

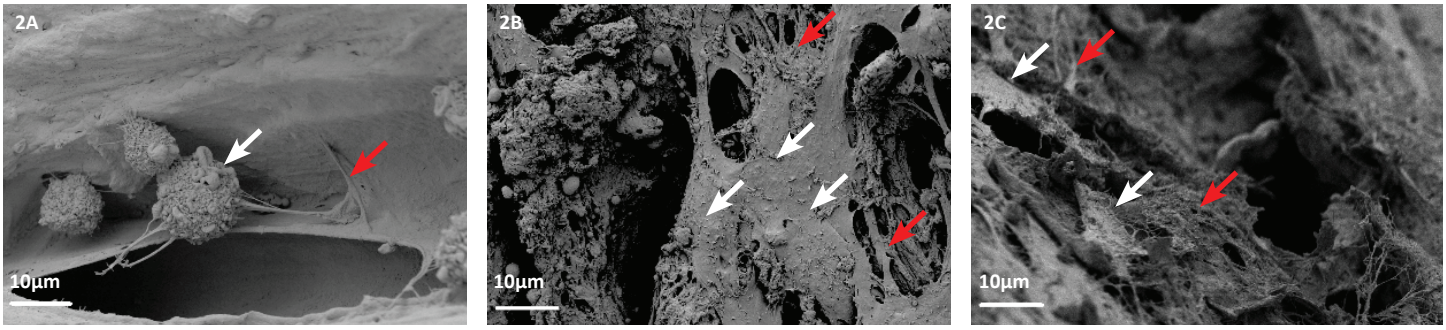


Figure 2. Cells robustly attach as early as one hour after seeding followed by matrix deposition that persists over time.

SEM images demonstrate the progression of extracellular matrix (ECM) deposition by MSCs at 1 hour (2A), 1 day (2B), and 7 days (2C) of incubation. ECM deposition as early as 1 hour is advantageous for bone healing as ECM provides a scaffold on which bone cells can grow and attach, thus providing a biohospitable environment for bone growth and healing (Figure 2A). In Figure 2B, cells are evident 1 day after initial incubation and possess a flattened morphology (white arrows) that suggests attachment to ReadiGraft BLX Fibers and subsequently deposited ECM (red arrows). In Figure 2C, cells persist in culture over 7 days and still appear flattened (white arrows) and attached to ReadiGraft BLX Fibers and ECM (red arrows). This flattening facilitates the ability of MSCs to migrate and proliferate, thus promoting bone healing. Images taken with a Zeiss SEM at 3,000x magnification.

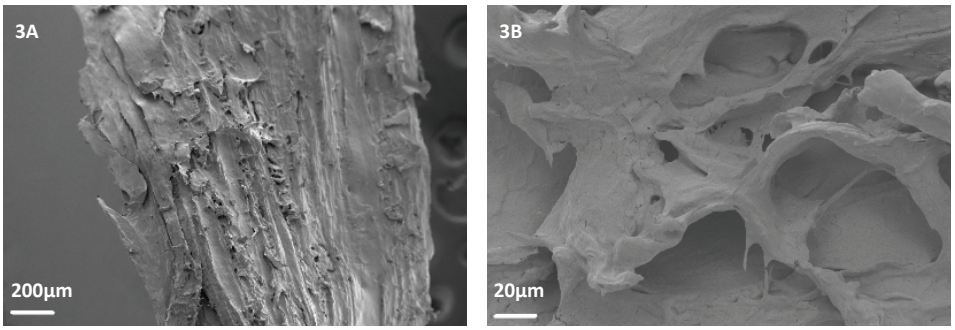


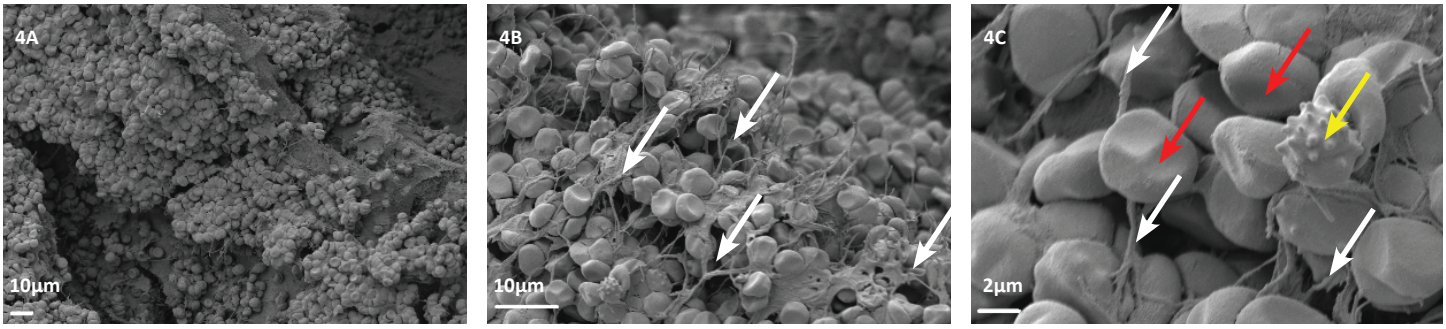
Figure 3. Fiber surface roughness facilitates robust cell attachment.

The surface topology of ReadiGraft BLX Fibers is rough and affords for multiple touch points for attachment of seeded MSC thereby affording for rapid attachment and colonization by cells. Images taken with a Zeiss SEM at (3A) 100x and (3B) 1000x.

Hemocompatibility

When clotted blood was added to the ReadiGraft BLX Fibers *in vitro*, it was readily absorbed. SEM experiments utilizing clotting blood suggested hemocompatibility by the presence of intact red blood cells and activated platelets closely associated with the scaffold **(Figure 4).** Copious amounts of fibrin-like fibers were present. The researchers speculated that their presence could contribute additively to the growth factors present on the scaffold [15-17]. In summary, these experiments showed that the ReadiGraft BLX Fibers possessed the appropriate infrastructure to support the colonization of cells and also act as a hemocompatible scaffold.

Figure 4. The interaction of clotting blood with ReadiGraft BLX Fibers shows presence of fibrin fibers.



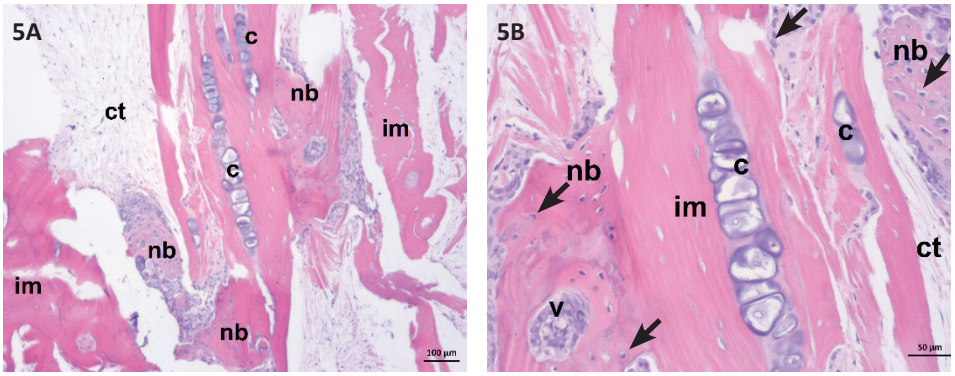
Depiction of blood clots (4A) applied to ReadiGraft BLX Fibers and imaged by scanning electron microscopy (SEM). The clots formed the fibrin-like fibers (white arrows) which may potentially aid in holding ReadiGraft BLX Fibers together and carry both cells and growth factors to the implant site. The fibrin-like fibers (white arrows) are clearly evident as well as the typical morphology of erythrocytes (red arrows) and activated platelets (yellow arrow) (4B and 4C). Importantly, the presence of fibrin fibers indicates an activated clotting cascade. Image taken with Zeiss SEM at 100x (4A), 3000x (4B), and 10000x (4C) magnification.

Generation of New Bone *In Vivo*

The previous study established that the ReadiGraft BLX Fibers provided a conducive environment *in vitro* to support cell attachment and colonization. The next two experiments assessed bone formation *in vivo*. ReadiGraft BLX Fibers graft matrix were implanted inter-muscularly in an athymic rat to physiologically assess the OI potential of the graft. At the conclusion of the 5 week implantation study, the analysis of hematoxylin & eosin (H&E) stained sections of the explants demonstrated that ReadiGraft BLX Fibers graft matrix maintained its integrity. Closer histological analysis after 5 weeks also revealed new bone, the formation of new blood vessels, and prominent new bone elements like osteoblasts and chondrocytes around and within the implanted scaffold **(Figure 5A and B).**

Following an assessment of OI potential, the *in vivo* performance of this new bone formation driven by ReadiGraft BLX Fibers graft matrix (with a mineralized cancellous bone component) was tested in an athymic rat posterolateral intertransverse process spinal fusion (PLF) model. ReadiGraft BLX Fibers graft matrix was implanted bilaterally between L4 and L5, and bone fusion was assessed at 8 weeks. At the conclusion of the study, the data showed that fusion (unilateral or bilateral) was achieved in 100% of the cases as assessed by radiography and microcomputed topography (micro CT) **(Figure 6)** thereby showing functional bridging and bony fusion across spinal processes in both a physiological and clinically relevant setting [18-19].

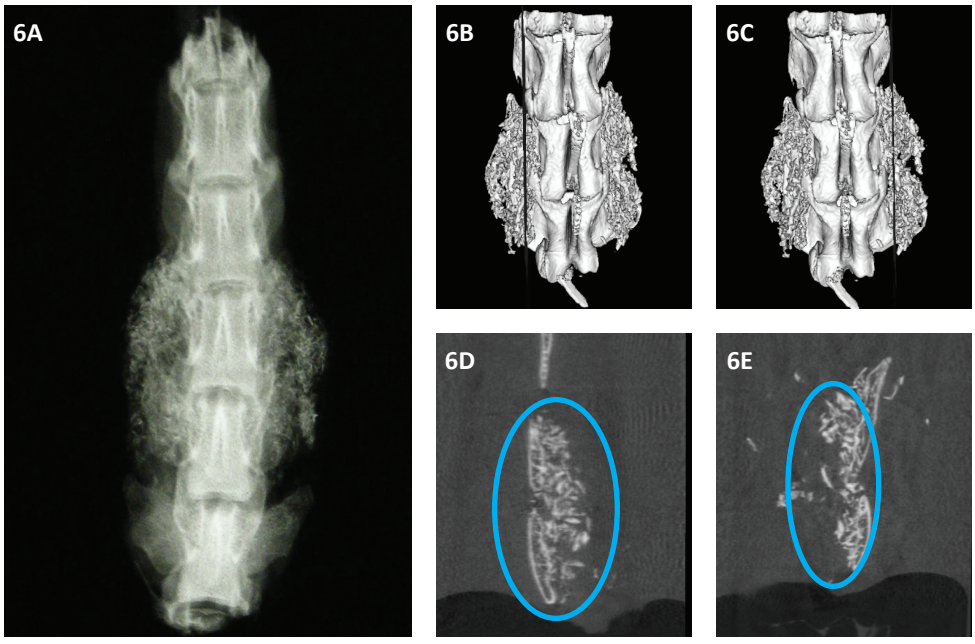
Figure 5. Bone formation present at 5 weeks with ReadiGraft BLX Fibers graft matrix in an athymic rat model of OI.



The ReadiGraft BLX Fibers graft matrix is shown after 5 weeks in life, with new bone forming elements prominently noted in the 4x magnified representative image (5A). A higher magnification clearly shows new bone cells (black arrows) among the new bone, cartilage, new osteoid, and blood vessels associated with the implanted ReadiGraft BLX Fibers graft matrix (5B). Abbreviations: black arrows = bone cells (possible osteoclast, osteocyte, pre-osteoblasts, osteoblasts), c = cartilage/chondrocytes/chondroblasts, ct = connective tissue, im = DBM implant, nb = new bone, and v = blood vessels. Images are H&E taken at 4x, 100µm scale bar (5A) and 10x, 50µm scale bar (5B) respectively.

Figure 6. Bone fusion is successfully achieved with ReadiGraft BLX Fibers graft matrix (with mineralized cancellous bone component) in an athymic rat PLF model.

Representative radiograph of successful fusion at the points of implantation between L4/L5 (6A). This is further confirmed with micro-computed topography images shown in 6B and 6C, and respective imaging of the cross sections (charcoal line in 6B and 6C) of bilateral bony fusion bridging the transverse spinal processes (circled in blue) in panels 6D and 6E.



CONCLUSION

ReadiGraft BLX Fibers support cell attachment, infiltration, and colonization, all qualities facilitated by its rough surface topology and open, fibrous structure. Human blood was able to fully hydrate the ReadiGraft BLX Fibers. Additionally, this hemocompatibility allows for better handling characteristics over traditional particulate DBM and further enriches the growth factors present on the scaffold with the deposition of fibrin. Finally, the bone forming potential of ReadiGraft BLX graft matrix (with a mineralized cancellous bone component) was demonstrated by the presence of new bone elements in an athymic rat model and bridging and bone fusion in a clinically relevant athymic rat PLF model. ReadiGraft BLX Fibers and graft matrix are capable of providing the appropriate osteoconductive and osteoinductive signaling necessary for new bone formation as supported by both *in vitro* and *in vivo* data.

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