Demineralized bone matrices (DBMs) have been used in a wide variety of clinical applications involving bone repair. An ideal DBM provides both osteoinductive and osteoconductive properties, while offering versatile handling capabilities. Many commercial DBMs are composed of demineralized bone combined with an inert carrier which is used to improve handling. The proportion of the osteoinductive element of the graft - the demineralized bone - varies widely by manufacturer.

In response to this need, LifeNet Health has developed PliaFX Prime, an advanced demineralized bone graft that is comprised of 100% bone fibers and offers both osteoinductive and osteoconductive properties as well as optimized handling.

Data from in vitro tests illustrate that PliaFX Prime supports the cellular function and attachment of bone marrow-derived mesenchymal stem cells (BM-MSCs). Scanning electron microscopy (SEM) imaging showed the presence of flattening cells and cell-to-cell interactions after 7 days in culture. Bone morphogenetic protein (BMP)-2 and BMP-7 were found at levels that were similar to those reported in literature.

In vivo data from an athymic mouse model demonstrated the formation of new bone elements, suggesting the osteoinductive potential of PliaFX Prime. Altogether, these results suggest that PliaFX Prime provides osteoconductivity and osteoinductive potential to facilitate bone regeneration.

**KEY WORDS:** demineralized bone matrix; osteoinductive; osteoconductive potential; growth factors; metabolic activity; cell attachment; bone growth

**Introduction**

The ability of demineralized bone matrix (DBM) to facilitate bone healing has been known in clinical settings for over a century. However, it was not until 1965, when Dr. Marshall Urist characterized specific proteins trapped within the bone matrix, that it was understood that bone morphogenetic proteins (BMPs) contributed to the osteoinductive property of DBMs. Since the discovery of BMPs, other proteins have also been found to contribute to the process of bone healing and regeneration. DBMs, which use acid demineralization to expose these growth factor proteins, have been used successfully for decades in a variety of complex bone repair procedures. In addition to containing active proteins, optimal surface characteristics of the DBM are essential for supporting cellular attachment and proliferation. It is crucial to provide a surface topography that allows for the patient’s own cells to migrate into and proliferate on the scaffold.

LifeNet Health has developed PliaFX Prime, which consists of 100% bone fibers. These long cortical fibers provide a rough surface with multiple protrusions allowing many points for cellular attachment. The contiguous surface of interconnected fibers allows the cells to spread and make cell-to-cell connections. PliaFX Prime is demineralized using LifeNet Health’s patented PAD® technology. Literature suggests that DBMs with different degrees of residual calcium show significant differences in osteoinductivity. The proprietary PAD demineralization process results in an optimized level of residual calcium, allowing proteins trapped in the bone matrix to become available to facilitate mineral deposition. These proteins include growth factors, such as BMPs, which facilitate the osteoinductive potential of the DBM.

Many manufacturers add synthetic, xenograft, or allograft carriers to improve the handling capabilities of their DBMs; however, it has been reported that some carriers may inhibit osteoinductive potential. The optimized handling characteristics of PliaFX Prime are achieved without the use of a carrier. The length and width of PliaFX Prime fibers are designed to encourage malleability, while microhooks allow surrounding fibers to interlock, thereby maintaining placement in the implant site.

PliaFX Prime gives surgeons optimized handling capabilities, undiluted osteoinductive potential, and a hospitable scaffold for cellular attachment. This paper reviews the in vitro and in vivo tests that were used to assess the osteoconductive and osteoinductive potential of PliaFX Prime.
Methodology Overview

1. Fiber Generation:
Human cortical long bones were recovered from 6 donors with research authorization through LifeNet Health’s organ and tissue procurement service. The bones were debrided, and the marrow and trabecular bone were removed. The resulting tissue was processed into demineralized fibers using proprietary procedures developed by LifeNet Health. The demineralized fibers were freeze-dried and sterilized via low dose, low temperature gamma irradiation.10,11

2. Fiber Analysis:
   a. In vitro Metabolic Activity of Seeded BM-MSCs
      Bone marrow-derived mesenchymal stem cells (BM-MSCs) seeded on PliaFX Prime fibers were measured for metabolic activity using the alamarBlue® assay over the course of 7 days. BM-MSCs without fibers were also measured and served as the control. This assay was used to determine whether PliaFX Prime fibers served as a hospitable environment to support cellular functions. PliaFX Prime samples from 6 donors were placed in triplicate in low-attachment 24-well cell culture plates and seeded with BM-MSCs. BM-MSCs were seeded at 62,500 cells per 25 ± 1 mg of fiber sample on Day 0 and cultured over 7 days with the appropriate growth media. At 1, 4 and 7 days in culture, media was aspirated and replaced with 1 mL of 10% alamarBlue reagent. After 2 hours incubation, the alamarBlue solution was collected and analyzed to assess sustained cellular viability and proliferation using a fluorescence plate reader. Fluorescence was recorded using relative fluorescence units (RFUs), and values were averaged for each donor lot and normalized to its time-matched control. A one-way ANOVA in conjunction with a Tukey post-hoc was used for statistical analysis.

   b. BM-MSC Attachment and Morphology
      Scanning electron microscopy (SEM) was used to qualitatively evaluate the attachment and morphology of cells. BM-MSCs were seeded 100,000 BM-MSCs per 25 ± 1mg fiber sample and imaged at 0.5 hour, 1 hour, 1 and 7 days in culture. Samples were from 2 donors with 4 seeded replicates and 1 fibers-only control for each sample.8

   c. In vitro Growth Factor Analysis
      BMP-2 and BMP-7 were quantified in samples of PliaFX Prime from 6 donors. Samples were digested with collagenase enzymatic solution and assayed for both growth factors. The resulting solutions were analyzed in triplicate using an enzyme-linked immunosorbent assay (ELISA) from R&D Systems, Minneapolis MN. The measured BMP content was averaged across all 6 donors and results were reported in ng protein/g of demineralized fibers.

   d. In vivo Osteoinductive Potential (OI)
      PliaFX Prime was assessed in vivo for osteoinductive potential and new bone formation using an athymic mouse intermuscular pouch model. Fiber samples (0.5cc) from 6 donors with research consent (4 replicates for each sample) were prepared and then implanted in the biceps femoris and superficial gluteal muscle of athymic mice. The implants were recovered 5 weeks post-implantation and fixed, sectioned, and stained with hematoxylin and eosin (H&E) for histological assessment.
Results

**PliaFX Prime supports attachment of mesenchymal stem cells and sustained cellular activity**

Overall, the cellular activity of the BM-MSCs was shown to steadily increase during the course of the 7 day investigation. The results indicated that BM-MSCs seeded on PliaFX Prime fibers showed a significant increase in proliferation between days 4 (51.3 ± 1.2 RFU) and 7 (59.5 ± 1.5 RFU) compared to day 1, (21.3 ± 0.8 RFU) (Figure 1). These data suggest that PliaFX Prime provides a hospitable environment for BM-MSCs. Furthermore, SEM images confirmed BM-MSCs attached within 30 minutes of seeding (Figure 2, A). At Day 1, imaging showed flattened cells with multiple adhesion points and cellular extensions as well as secretion of extracellular matrix (ECM), which provides a scaffold on which cells can grow and attach (Figure 2, B). Additionally, after 7 days in culture, BM-MSCs infiltrated between fibers and demonstrated cell-to-cell interactions, which allow cells to communicate with each other and are critical to the development and function of tissues (Figure 2, C). The ability of cells not only to quickly attach to the matrix but also to maintain a healthy morphology throughout the duration of culture provides evidence of the osteoconductive qualities of PliaFX Prime.

**PliaFX Prime contains important growth factors and demonstrates osteoinductive potential**

The ELISA results indicated the presence of growth factors in PliaFX Prime samples. The levels of BMP-2 and -7 were consistent with values reported in the literature. In our study, the average BMP-7 concentration was 85.78 ± 6.84 and the BMP-2 concentration was 11.24 ± 1.49 ng per gram DBM (Figure 3). Previous studies have reported a wide span of BMP-7 and BMP-2 levels in demineralized bone: BMP-2 (6.6-110 ng/g DBM) and BMP-7 (44-125 ng/g) DBM.12-14 The experiment demonstrates that PliaFX Prime retains growth factors.

In the athymic mouse muscle pouch model, histological analysis revealed new bone elements around and within the implanted scaffold at the time of sacrifice (5 weeks; Figure 4). Panel A shows a set of merged images that illustrate new bone elements present in the explant (4x objective). Panels B and C highlight the presence of new bone elements such as cartilage, chondroblasts/cytes, bone marrow, new blood vessels, and new bone.

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**Figure 1.** Proliferation of BM-MSCs attached to PliaFX Prime over 7 days. The average relative fluorescence units (RFU) values for each set of triplicate test samples were normalized to the average RFU of the corresponding control group (fibers of the respective donor cultured without cells) for all six donors. Asterisks represent statistically significant differences from Day 1 proliferation activity.

**Figure 2:** Representative SEM images illustrating the morphology of cells attached to PliaFX Prime. Following culture for 30 minutes (A), 1 day (B) or 7 days (C), the samples were fixed in 2.5% glutaraldehyde and processed for scanning electron microscopy. Images are representative of all samples evaluated and were taken at 3000x magnification. Images were pseudo-colored in Photoshop to distinguish the cells (in yellow) from the fibers.

**Figure 3.** BMP-2 and BMP-7 content in PliaFX Prime. PliaFX Prime produced from 6 different donors was digested in collagenase for 16-18 hours. Using ELISAs, the resulting digestion solution was tested for BMP-2 and BMP-7 content in triplicate (mean ± SE).
Figure 4. H&E staining of explants from athymic nude mouse implant with PliaFX Prime. A) Merged set of H&E images showing new bone elements present in the entire explant at 35 days post-implantation (4x objective). B) and C) H&E images showing the presence of new bone elements such as cartilage (^), chondroblasts/cytes (#), bone marrow ($), new blood vessels (&), and new bone (+) at 35 days.

Conclusion

In vitro and in vivo data shows that PliaFX Prime exhibits osteoinductive and osteoconductive properties. PliaFX Prime also facilitates BM-MSC attachment, viability, and proliferation. The presence of BMP-2 and BMP-7, important growth factors for bone formation, suggest that PliaFX Prime contains osteoinductive factors. In vivo data also confirm the osteoinductive potential of PliaFX Prime, with the explants showing new bone elements including cartilage, bone marrow, and new blood vessels.

The results presented here demonstrate that PliaFX Prime exhibits the osteoinductive potential and osteoconductive properties needed to promote bone formation. The interlocking fibers allow the graft to become moldable upon rehydration without the use of a carrier.

References

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