In-vivo evaluation of a next-generation, nanocrystalline hydroxycarbanoapatite & collagen advanced bone graft in a posterolateral fusion model

Abstract

A single-level rabbit posterolateral spinal fusion model was used to evaluate the bone-forming ability of Agilon® Strip compared to autograft. Agilon Strip is a next-generation bone graft product incorporating TrelCor™ technology. TrelCor comprises a porous calcium carbonate scaffold with a nanocrystalline hydroxycarbanoapatite surface that actively promotes bone healing. In this study, Agilon Strip was hydrated with bone marrow aspirate, combined 50/50 with autograft, and implanted in the rabbit posterolateral spine. Iliac crest autograft was used as a positive control. Bone formation was evaluated at six, nine, and twelve week time points using radiographic, biomechanical, histological, and histomorphometric methods. Evaluation of plain film, Faxitron, and microCT images all revealed new bone formation and implant resorption, as indicated by the reduction of implant resolution with time. At twelve weeks, the radiographs for six of the eight (75%) Agilon Strip animals and five out of eight (63%) autograft control animals were scored as bilaterally fused. These results correlated well with the manual palpation results, where seven out of eight (88%) Agilon Strip and four out of eight (50%) autograft treated animals were scored as bilaterally fused. Quantitative 6-degree-of-freedom biomechanical testing confirmed the manual palpation results, as seen by decreased spinal motion compared to an unoperated control. The histological assessment of Agilon Strip explants revealed bone formation directly on the surface and throughout the TrelCor granule porosity. This biological response was similar to the autograft group. Histomorphometric analysis (HMA) of new bone showed an initial increase in bone formation at six weeks with similar amounts at nine and twelve weeks. HMA also showed progressing implant resorption over the twelve-week implantation period. Overall, the study demonstrated that Agilon Strip is a safe and effective bone graft extender in the rabbit posterolateral spine, with a healing response and fusion rates similar to autograft.

Introduction

Off-the-shelf bone grafting products are designed to act as scaffolds to guide the natural, biological bone regeneration processes. At a minimum, these products must be biocompatible, osteoconductive, and easy-to-use during surgery. These features are found in various first-generation bone graft products that have been commercially available for the past two decades. In the synthetic bone graft category, products have typically been based on calcium phosphate materials such as hydroxyapatite (HA), tri-calcium phosphate (TCP), and combinations of the two (biphasic calcium phosphate – BCP). These bone graft materials are typically porous structures available in granular form, putty form (granules suspended in a moldable carrier), or in strip/sheet form (granules suspended in collagen scaffolds).

Once implanted, these osteoconductive materials passively allow bone formation on the material's surface and throughout the graft's porosity. For calcium phosphate materials, the healing process is driven by cellular activity. This involves an initial osteoclast phase where the graft surface is prepared for eventual bone formation. Following the preparation phase, bone-forming cells (osteoblasts) attach, proliferate, and begin forming mineralized bone tissue over the graft surface. Although first-generation synthetic bone grafts have been proven to be effective osteoconductive scaffolds, the materials themselves do not actively stimulate a cellular response. Contemporary research has focused on identifying material properties capable of promoting a cellular response that improves bone regeneration. As a result, researchers have identified specific material properties, such as surface topography and composition, that play an active role in intrinsic cellular bone formation processes.

One area of particular interest has been materials with nanoscale surface morphology. Surface features at the submicron, nanoscale level have been shown to positively impact cell function and differentiation due to the cellular interaction with materials' topography [1-5]. These studies have demonstrated that nanostructured materials can increase osteoblast function and trigger stem cell differentiation. This response is attributed to a mechanotransduction effect where cell attachment to the nanostructured surface causes a physical effect on cells, leading to increased osteoblast proliferation and function, and stem cell differentiation.

In addition to nanostructured surfaces, the material composition was also found to influence the bone formation process. First-generation bone graft products focused on materials based on

the calcium phosphate component of bone mineral, resulting in various products composed of HA, TCP, and BCP. However, human bone mineral is not pure hydroxyapatite. It also contains carbonate and additional trace elements. In recent years, several studies have specifically examined the bone formation ability of carbonate substituted apatite (hydroxycarbanoapatite – HCA) compared to standard calcium phosphate materials [6-10]. These studies found that HCA materials have a shortened osteoclast preparation phase compared to HA, TCP, and BCP ceramics due to HCA's similarity to naturally carbonated bone mineral. In vivo, the shortened cellular preparation phase resulted in faster bone formation. One study showed that bone formation was significantly faster and more robust on HCA scaffolds compared to those made from TCP or HA. At the early four-week time point, the average percentage of new bone for the HCA implants was ~4X higher than TCP and ~14X higher than HA [8].

In recent years, there has been a shift from first-generation products that solely function as an osteoconductive scaffold to materials that also actively facilitate the cellular healing process. Biogennix (Irvine, CA) has specifically developed its TrelCor technology to include these advanced bone graft properties. The TrelCor material consists of a porous calcium carbonate scaffold with a thin nanocrystalline, hydroxycarbano apatite surface region. The nanocrystalline surface morphology and HCA composition of TrelCor actively promote cellular healing and provide an enhanced osteoconductive surface.

Additionally, regulation of the slower-resorbing HCA surface thickness on the faster-resorbing calcium carbonate core enables precise control over the material's resorption. Unlike standard single-phase, calcium phosphate bone graft materials, the dual-phase TrelCor composition purposefully provides an optimal resorption profile that acts in synchrony with the natural bone regeneration process. Consequently, TrelCor continues its function as a scaffold to support bone growth until bone has fully formed throughout its porosity. Once this process is complete, TrelCor is fully resorbed, leaving natural host tissue in its place.

In addition to its enhanced osteoconductive surface, TrelCor also possesses a pore architecture that closely resembles human cancellous bone. The TrelCor structure is approximately 65% porous with 500μm diameter (nominal) pores that are 100% interconnected. This optimized architecture encourages bone and supporting tissue in-growth throughout the entire graft area.

TrelCor based bone graft products are available in multiple forms. Agilon Strip is a collagen/TrelCor device recently introduced by Biogennix that is available in a highly absorbent sheet form. It contains 1-2mm sized TrelCor granules embedded in a porous Type 1, bovine collagen matrix. This advanced bone graft material becomes flexible and conformable following the collagen carrier's hydration with bone marrow aspirate. The TrelCor granules in Agilon Strip provide an enhanced osteoconductive surface while the collagen fibers provide structural support for intraoperative manipulation. The collagen also keeps the TrelCor granules in a manageable form, and maintains the granules close to each other while allowing the product to be mixed with other materials such as autograft.

The objective of this study was to evaluate the bone formation response of Agilon Strip when used as a bone graft extender in a well-characterized animal spine model. A validated rabbit posterolateral spine fusion model designed to evaluate graft fusion without the use of ancillary instrumentation was used in the study [11-16].

Materials and Methods

Study Location and Animal Committee Approval

This study was conducted at the University of New South Wales, Australia, Surgical & Orthopaedic Research Laboratory (William Walsh, Ph.D. – Director) following protocol approval by the University Institutional Animal Care and Use Committee.

Treatment Groups

The experimental group was implanted with Agilon Strip (Biogennix, Irvine, CA), bone marrow aspirate, and autograft, while the control group was implanted with autograft alone. Agilon Strip was provided sterile to the surgical laboratory in a 2cc size. Iliac crest bone marrow (2cc) was obtained by standard marrow aspiration techniques. Agilon Strips were soaked in the bone marrow aspirate until the material was fully saturated and pliable. Iliac crest autograft was unilaterally harvested (2cc total), morselized, and combined with the hydrated Agilon Strip. The resulting 4cc mixture was equally divided and implanted bilaterally (2cc per side).

The control group had 2cc of iliac crest bone harvested bilaterally (total of 4cc), morselized, and implanted 2cc per side. As a result, the Agilon Strip treated animals used half as much autogenous bone as the positive controls (2cc vs. 4cc).

Implantation

All animals were procured, acclimated, housed, and treated by the veterinary staff following university procedures. Rabbits were radiographically verified for full maturity (i.e., greater than seven months old with closed epiphyses) and randomized into treatment and control groups. After general anesthesia, two adjacent, intertransverse processes in the lumbar spine were identified and bilaterally decorticated. Graft materials (4cc per treatment group) were evenly split, with each side of the spine receiving a total of 2cc of graft material. Following graft placement, standard soft tissue closure techniques were used. No stabilization hardware was used.

A total of 36 adult New Zealand white rabbits was used in the study. The animals were sacrificed at intervals of six, nine, and twelve weeks. The number of animals for each group and for each time interval is provided in **Table 1.**

Treatment Group	Total # of Animals	6 Weeks	9 Weeks	12 Weeks
Agilon Strip	18	5	5	8
Autograft	18	5	5	8
Totals	36	10	10	16

Table 1. Allocation of animals in the study

Clinical Assessment

During post-operative care, the animals were evaluated by blood chemistry and clinically observed for behavior, eating habits, and wound appearance.

Radiography

For the longer term nine and twelve week animals, in-life plain film radiographs were taken at the six and nine week time points, as appropriate. Upon completion of each timepoint, both high resolution radiographs (Faxitron) and micro-computed tomography (microCT) was performed on all explants. High resolution X-ray imaging was performed using an MX-20 X-ray unit (Faxitron, AZ, USA). MicroCT imaging was performed using an Inveon microcomputer tomography scanner (Siemens Medical, PA, USA). Image reconstructions (Inveon™ Research Workplace IRW) produced 44μm and 0.5mm thick images in the axial, sagittal and coronal planes. These scans were evaluated for adverse events, healing, and implant resorption by blinded reviewers. Faxitron and microCT images of the spine fusion areas were also subjectively judged into four categories by blinded, independent reviewers: "definitely fused", "probably fused", "probably not fused", and "definitely not fused".

Biomechanical Testing

Explanted spines were biomechanically assessed by manual palpation for fusion immediately after harvest. Blinded reviewers bilaterally evaluated the implanted levels for lateral bending and flexion-extension compared to the adjacent, untreated levels. Each side was graded as either "fused" or "not fused." Following manual palpation, quantitative, non-destructive mechanical testing was also performed to provide multi-directional flexibility analysis that mimicked the manual palpation loading [17]. Specimens were potted, and various loading modes were applied to the fused segment (flexion-extension and lateral bending). Maximum applied pure moments of 0.27 Nm were used for each loading mode at a rate of 0.3 degrees/second. Motion during the loading modes was measured. Intact, non-operated adult rabbit spines that had no previous surgery were also tested as a baseline motion comparison.

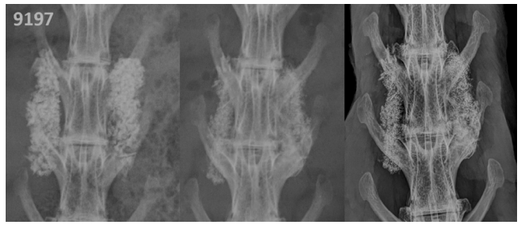
Histology/Histomorphometry

Immediately following mechanical testing, spines were fixed in phosphate-buffered formalin. Specimens were cut in the sagittal plane through the middle of the vertebral body. One side was allocated for paraffin (decalcified) histology, while the contralateral side was allocated for PMMA (non-decalcified) histology. At least four blocks were sectioned from each fusion mass. The decalcified, paraffin-embedded spines were sectioned across the entire fusion mass and stained with hematoxylin and eosin (H&E) and tetrachrome stains. Qualitative histology was assessed by blinded reviewers for biocompatibility (according to ISO 10993-6) and for intertransverse bony bridging in the sagittal plane. The PMMA sections were stained with methylene blue and basic fuchsin were analyzed by automated, computer detected histomorphometry (HMA). On the HMA images, a region of interest (ROI) was identified to determine the area percentage of the graft materials, bone tissue (new bone and marrow), and other soft tissues present in each section. The graft materials and bone tissue (mineralized bone and bone marrow elements) were identified by pixel color and morphology.

Results

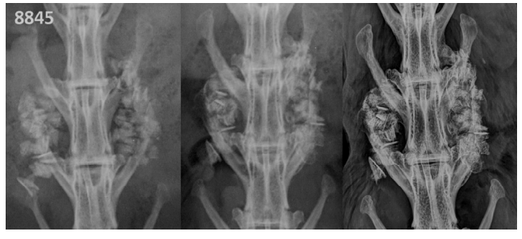
All surgeries concluded without incident. The bone marrow aspirate transformed the rigid Agilon Strip into a flexible bone graft that was readily mixed with autograft (1:1 ratio). Surgeon observation indicated that Agilon Strip handled well and was easily packed into the posterolateral, intertransverse spinal space. Routine blood work was normal before implantation and at each harvesting interval. There were no adverse local tissue or distant organ reactions at any time point.

Radiographic assessment of both the high-resolution radiographs **(Figure 1)** and the microCT scans **(Figure 2)** revealed newly formed bone at each time point in the Agilon Strip group. This was seen as early as six weeks after implantation.



6 Weeks (Plain film) *9 Weeks (Plain film)* *12 Weeks (Faxitron)*

Agilon Strip (animal 9197)



6 Weeks (Plain film) *9 Weeks (Plain film)* *12 Weeks (Faxitron)*

Autograft (animal 8845)

Figure 1. Radiographs of the Agilon Strip group (top) and autograft group (bottom)

Progression of healing from six to twelve weeks was seen in the radiographs, as characterized by a loss of detail in the TrelCor granules and concurrent increasing radiopacity of new bone on the coronal and sagittal planes. In several specimens, bridging bone was seen across the operative segment. Radiographic images were used by blinded reviewers to independently assess the degree of fusion. The results of this evaluation are shown in **Table 2**. The results showed that the Agilon Strip group has a similar radiographic fusion outcome as the autograft group.

Timepoint	Fusion Score	Agilon Strip	Autograft
6 Weeks	Definitely Fused	0	0
	Probably Fused	5	5
	Probably Not Fused or Not Fused	0	0
9 Weeks	Definitely Fused	2	4
	Probably Fused	5	1
	Probably Not Fused or Not Fused	0	0
12 Weeks	Definitely Fused	6	5
	Probably Fused	2	3
	Probably Not Fused or Not Fused	0	0

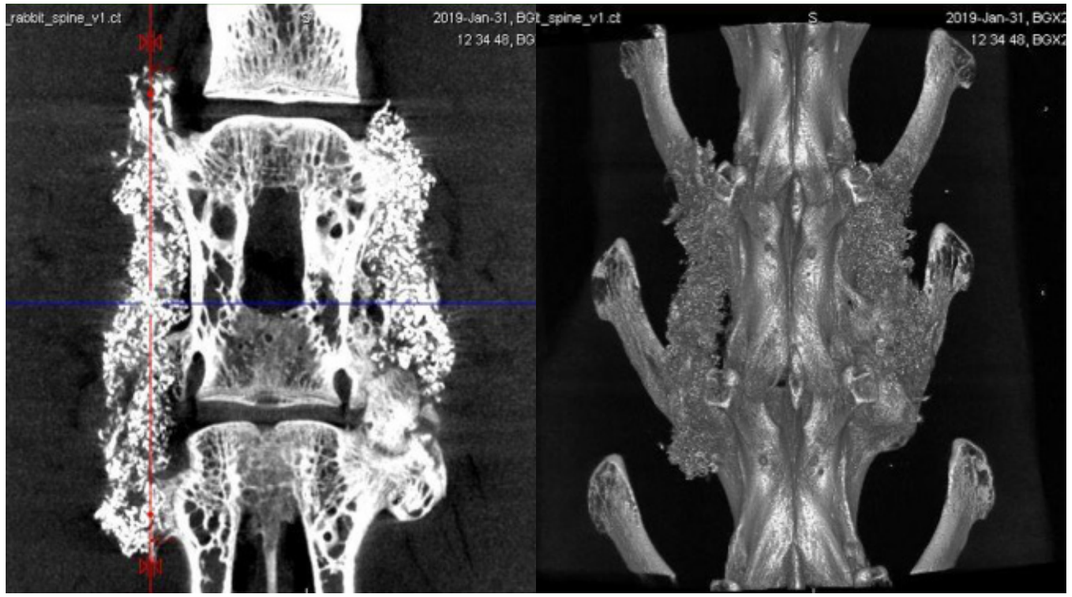
Table 2. Semi-quantitative radiographic fusion scores

In addition to the radiographic assessment, a qualitative biomechanical spine fusion evaluation was conducted using a manual palpation method. Blinded observers classified the specimens as "bilaterally fused" or "not fused" based on the stiffness of the operated segment in bending and flexion-extension. **Table 3** summarizes the manual palpation results for the Agilon Strip group and the autograft control group.

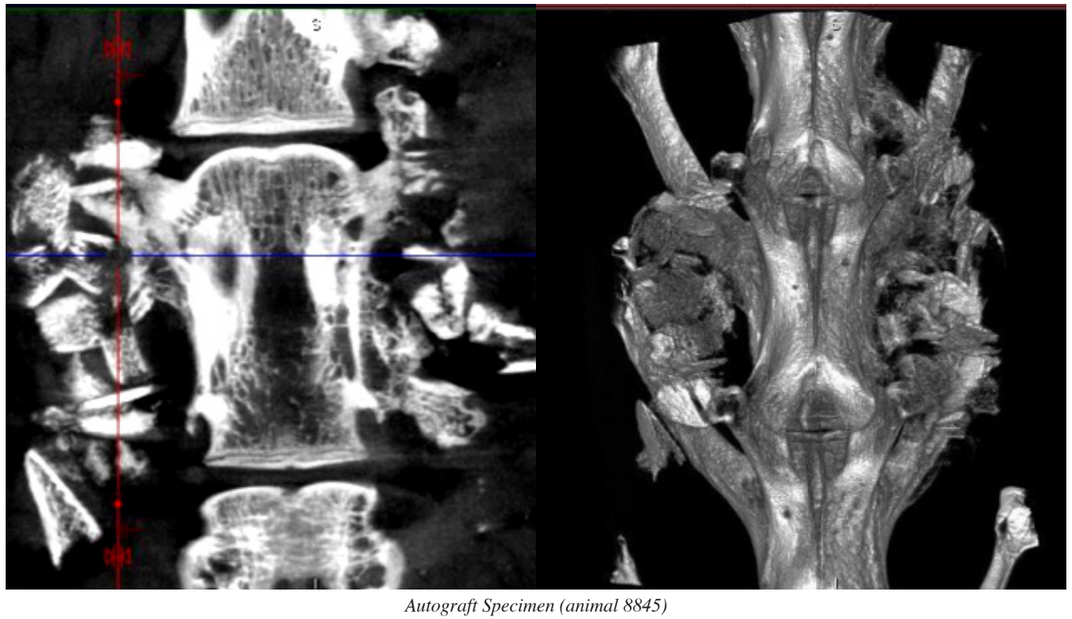
Group	Fusion Rate at 6 Weeks	Fusion Rate at 9 Weeks	Fusion Rate at 12 Weeks
Agilon Strip	40% fused (2 out of 5)	40% fused (2 out of 5)	40% fused (2 out of 5)
Autograft	40% fused (2 out of 5)	40% fused (2 out of 5)	40% fused (2 out of 5)

Table 3. Manual palpation fusion results

The Agilon Strip group showed a 40% fusion rate at six weeks, 60% at nine weeks, and 88% at twelve weeks. The autograft group showed fusion rates of 60%, 80%, and 50% for the same respective time points.



Agilon Strip Specimen (animal 9197)



Autograft Specimen (animal 8845)

Figure 2. MicroCT imaging (0.5mm section – left; 3-D image -right) of 12-week Agilon Strip specimen (top) and autograft specimen (bottom) showing bilateral, bridging bone at the fusion site

An additional 6-degree of freedom biomechanical testing was also conducted using the same flexion-extension (FE), and lateral bending (LB) motions qualitatively assessed in the manual palpation evaluation. Motion at the operative segments for each group was compared to an unoperated control spine. The results are graphically shown in **Figure 3**. The biomechanical data showed a similar reduction in both the Agilon Strip and autograft groups' motion compared to an unoperated, intact spine (control). This was seen in both modes of motion (FE and LB).

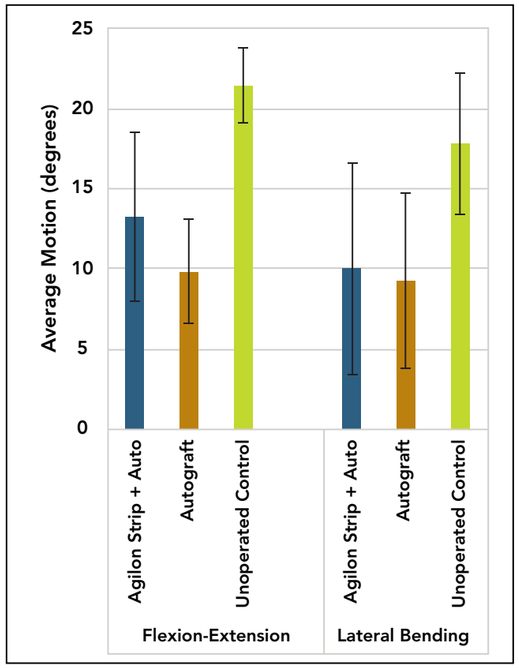


Figure 3. Biomechanical data showing average motion

Following the non-destructive biomechanical testing, the explants were processed for histology. Specimens were processed using decalcified paraffin and non-decalcified PMMA methods. Qualitative histological analysis showed that Agilon Strip, when mixed with bone marrow aspirate and autograft, provided a robust woven bone response at 6 weeks, which remodeled to lamellar bone over time. Representative images of the histological response are shown in **Figure 4.**

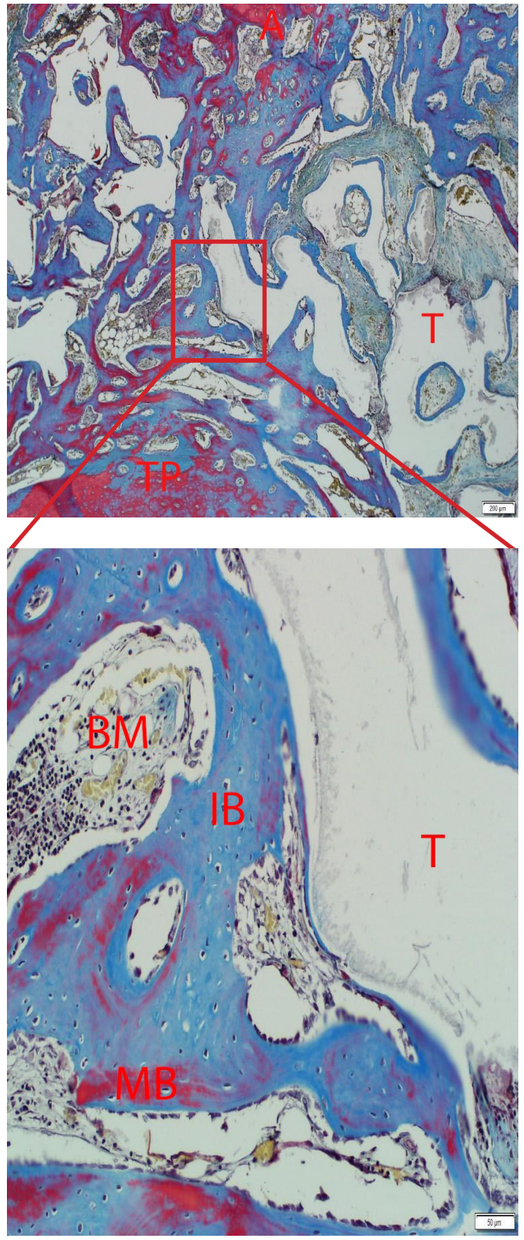


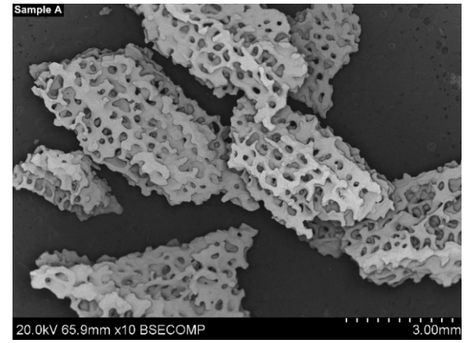
Figure 4. Decalcified histology after treatment with Agilon Strip + bone marrow + autograft at 6 weeks (low mag. – top; medium mag. - bottom; T=TrelCor, BM=bone marrow, MB=mature bone, IB=immature bone, A=autograft, TP=transverse process)

Figure 4 images show a representative histological response to the Agilon Strip product. Abundant regenerated bone (blue or red) is seen growing directly on the TrelCor granules (white) within the Agilon Strip graft. Bone is also seen on the surface layer of the porosity. Some resorption of the Agilon Strip material was noted by twelve weeks, along with remodeling of the early formed bone and the formation of marrow spaces. Inflammation at all time intervals was minimal or absent. There was a normal amount of fibrovascular tissue formation within and around the graft material at all time intervals. Histological evaluation showed no adverse reactions. Based on the results, Agilon Strip was considered biocompatible and a non-irritant according to ISO 10933-6.

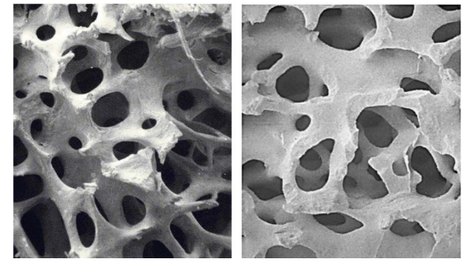
Histomorphometry (HMA) was performed on the methylene blue and basic fuchsin stained, non-decalcified, PMMA images to determine the area percentage of bone tissue, marrow space, graft material, and connective tissue. The average volume fraction of bone in the Agilon Strip group was 29%, 30%, and 27 % at six, nine, and twelve weeks, respectively. Comparatively, the autograft treated animals showed average bone amounts of 34%, 40%, and 41%, respectively, that were higher than the Agilon Strip group. However, since HMA cannot differentiate between autograft particles initially present at the implant site and newly formed bone, the autograft group will have an artificially higher new bone percentage reported in the HMA data. The HMA also showed that Agilon Strip was resorbing as seen by a decrease in graft material (24% at 6 weeks, 21% at 9 weeks, and 18% at 12 weeks). The histological and HMA data showed that Agilon Strip performed as expected by providing an osteoconductive scaffold, participating in bone healing, slowly resorbing, and forming new bone that remodeled over time. The results showed a similar biological response between the Agilon Strip and autograft groups.

Discussion

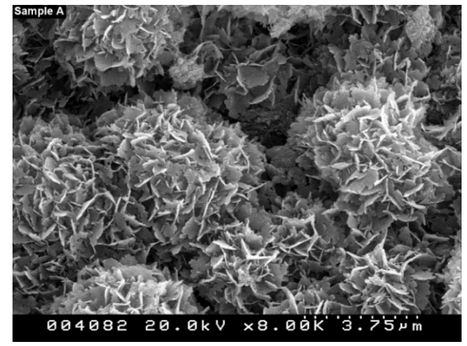
This study demonstrated that Agilon Strip effectively regenerated bone in the rabbit spine model. Fusion rates and the biological bone formation response were similar to autograft controls. The success of Agilon Strip was attributed to its composition and use of TrelCor granules. Agilon Strip has a high TrelCor content that is 98% granules and 2% collagen. The morphology and structure of TrelCor granules and Agilon Strip are shown in **Figure 5.**



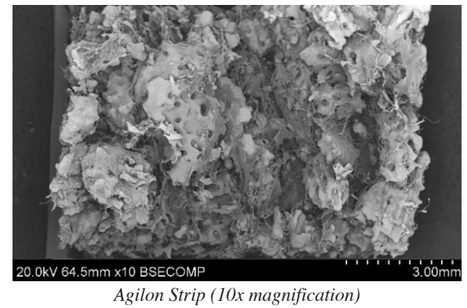
TrelCor Granules (10x magnification)



Cancellous Bone (left) and TrelCor (right)



Nanocrystalline HCA Surface (8,000x magnification)



Agilon Strip (10x magnification)

Figure 5. Electron microscope images of TrelCor granules, the HCA surface, and Agilon Strip

These images show that the biomimetic structure of the TrelCor granules is similar to cancellous bone and that TrelCor has a nanocrystalline surface morphology. Additionally, the Agilon Strip image shows how the TrelCor granules are suspended in the porous collagen carrier. As seen from the images, both TrelCor granules and the Agilon Strip structure are designed to function as an enhanced scaffold for bone formation. The nanostructured surface topography, HCA composition, and porosity combine to create an optimal cellular bone formation environment.

The combination of Agilon Strip with bone marrow aspirate and autograft fully capitalizes on the benefits of the TrelCor surface and composition. By wicking bone marrow aspirate into Agilon Strip's porosity, stem cells are deposited directly onto the TrelCor nanocrystalline HCA surface. Additionally, the combination of autograft with Agilon Strip places viable host bone with live osteoblast cells in direct contact with TrelCor granules. Since TrelCor was designed to play an active role in cellular bone healing, the combination of Agilon Strip with cellular tissues (BMA and autograft) provides an optimal graft combination to regenerate bone. This result was confirmed in this study, which showed radiographic and histological bone formation in a well-established posterolateral spine fusion model in rabbits. This model has also been shown to be clinically relevant because the fusion rates using autogenous bone graft are similar to the rates in humans when using rigid internal instrumentation.

As shown in this study, Agilon Strip effectively absorbed bone marrow aspirate due to the capillary action of the porosity and the absorbance of the collagen fibers. Bone grew onto the TrelCor granules from bone marrow aspirate and the pre-existing bone, either from the lateral transverse processes or from the autograft mixed with the TrelCor granules and BMA. The regenerated bone was visible on radiographs as well as the CT scans, as evidenced by the consolidation of the relatively radiopaque TrelCor granules over time with progressive diffusion and graying. The histology substantiated the radiographic data by clearly showing that bone directly contacted the TrelCor granule surface. Most importantly, the biomechanical analysis was consistent with both the radiographic and histologic results. Although some fusions occurred in this model after only six weeks, the fusion rate was maximal by twelve weeks at 88%. In contrast, the autograft controls were 50% at twelve weeks. This is particularly important because the group treated with Agilon Strip used half as much bone as the control group; thus, Agilon Strip served effectively as a bone graft extender.

Conclusions

The performance of Agilon Strip in the rabbit spine was assessed at multiple time points using a variety of analytical methods and assessments. The results showed that Agilon Strip was an effective bone graft extender. It readily absorbed bone marrow aspirate and was easy to mix with autograft. The overall bone healing response measured by radiographic imaging, histology/HMA, and biomechanical testing showed increased bone over time and fusion results similar to autograft.

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This study was conducted by Biogennix at an outside, independent laboratory. Report and data on file.