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THESIS APPROVAL PAGE FOR MASTER OF SCIENCE IN ORAL BIOLOGY

Title of Thesis: Comparison on Porosity, Hardness, and Indentation Modulus of 3D Electrospun PCL Scaffolds, OsteoGen, and Bio-Oss Collagen for Alveolar Ridge Preservation

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June 12, 2023

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Comparison on Porosity, Hardness, and Indentation Modulus of 3D Electrospun PCL Scaffolds, OsteoGen, and Bio-Oss Collagen for Alveolar Ridge Preservation

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Short Running Title: 3D PCL Scaffolds

Summary (1 sentence): Hardness and indentation modulus of 3D electrospun PCL scaffolds, with collagen alone or with collagen and hydroxyapatite, were similar to OsteoGen and "spongy" aspect of Bio-Oss Collagen but not to the cortical aspect, while the porosity was similar to Bio-Oss Collagen but less than OsteoGen.

Abstract

Socket grafting is an effective procedure to preserve the alveolar ridge post-extraction, reducing the loss of horizontal and vertical dimensions. This study evaluated the mechanical properties of three electrospun PCL scaffolds (e.g., PCL only, PCL-collagen, and PCL-collagen-hydroxyapatite) and compared them with two commercially available socket grafting materials: OsteoGen Plug (Impladent) and Bio-Oss Collagen (Geistlich Pharma). A spherical copper mesh was used as a collector to prepare the 3-D PCL scaffolds by electrospinning. The scaffolds were then functionalized with collagen and mineralized with hydroxyapatite (HA). Micro-CT was used to measure scaffold porosity and internal structures. The modulus and hardness of the electrospun scaffolds and grafting materials were characterized by nanoindentation. For each group, a mean and standard deviation (SD) was calculated for modulus, hardness, and porosity. Significant differences were determined using one-way analysis of variance (ANOVA) testing and Tukey's post hoc test. The porosity of the PCL scaffolds (56.85 - 61.51%) were comparable to Bio-Oss Collagen (52.22%) but less than OsteoGen (97.16%). The hardness and indentation moduli were comparable to OsteoGen and the "spongy" aspect of Bio-Oss Collagen. This may translate to PCL scaffolds having similar clinical handling properties to OsteoGen and cellular response similar to Bio-Oss Collagen.

INTRODUCTION

The alveolar bone proper is a tooth-dependent structure, and tooth extraction often leads to irreversible vertical and horizontal resorption of the residual ridge. Implant therapy is currently considered the standard of care for post-extraction to restore the missing dentition. However, having sufficient bone volume and a favorable ridge morphology is necessary for implant success.[1,2] Without ridge preservation, studies have found that the residual ridge can lose roughly 50% of horizontal bone width, mostly within the first 3 months.[2,3] Current approaches to ridge preservation can be technically challenging and/or cost prohibitive[3]. For example, autologous bone can be harvested from a donor site, but comes at increased patient morbidity and discomfort. Other biomaterials available on the market, which include allografts, xenografts, and alloplasts, can be applied in deficient ridges in lieu of autologous bone. For example, OsteoGen Plug (Implandent) is marketed as a bioactive and resorbable calcium apatite crystal bone graft that is physicochemically & crystallographically like human bone, according to the manufacturer. Bio-Oss Collagen (Geistlich Pharma) is 90% anorganic bovine bone matrix combined with 10% porcine collagen marketed for ridge preservation and bone augmentation.

Though allografts and xenografts have been around for a long time and provide predictable outcomes, they are relatively expensive and require stringent processing, storage, and tracking protocols. Furthermore, some patients may be adamantly opposed to getting bone grafts from cadaver or animal sources. As such, an alternative material that is biocompatible, biodegradable, and cost effective with user friendly handling properties is highly desirable.[7,16]

Since the 1970s, polycaprolactone (PCL) has been used extensively in the biomedical field, including as a drug delivery device and as an adhesion barrier. Polycaprolactone is a synthetic polymer that can be electrospun into nanofibers. It is inexpensive, biocompatible, and biodegradable, and is FDA approved for use in humans. Electrospun nanofibers have the potential to mimic the extracellular matrix, and the electrospinning process allows for manipulation of its composition to obtain the desired properties and functions. This study focused on PCL's potential application as a nanofibrous scaffold. Collagen is added to further mimic the extracellular matrix and enhance cellular adhesion.[16] The PCL and collagen scaffold can then be mineralized with hydroxyapatite (HA) to enhance mechanical strength and osteoconductivity, which allow for ease of integration with native bone.[4,5] However, the optimal %

amount of collagen and HA is yet to be determined.[24]

Numerous studies have investigated the effect of collagen and mineralization on synthetic scaffolds. In a previous *in vitro* study, Jones et al. (2021) showed that OsteoGen had significantly greater osteoblast proliferation compared to Bio-Oss Collagen due to its highly porous structure, with a high collagen to mineral content. On the other hand, Bio-Oss Collagen had also been clinically proven to induce bone fill.[26] A high mineral content enables a socket grafting material to hold its shape and withstand mechanical forces until bone turnover is completed. Thus, investigating the mechanical properties and porosity of synthetic bone graft materials such as PCL based scaffolds is necessary to develop a viable alternative that has similar mechanical properties while allowing for cellular proliferation.

The aim of this study was to compare the porosity and indentation modulus and hardness properties of three electrospun PCL scaffolds (e.g., PCL only, PCL-collagen, and PCL-collagen-hydroxyapatite) with two commercially available products used in alveolar ridge preservation (OsteoGen and Bio-Oss Collagen). The hypothesis was that there is no significant difference in these properties among the three materials.

MATERIALS AND METHODS

The 3D PCL scaffolds tested in this study were fabricated using the processes outlined in the following sections.

Electrospinning

PCL was dissolved in hexafluoro-2-propanol (HFP) at 8% w/v and stirred at room temperature until the polymer was completely dissolved. The polymer solution was loaded into a 10 mL Luer-Lock syringe and fed through an 18-gauge line to the tip of an 18-gauge blunt tip needle. The syringe was loaded into a syringe pump and set to 2.0 mL/hr. The power supply was set to 10 kV and humidity was maintained between 16-26% during the electrospinning process. The spherical copper mesh collector was placed 15 cm from the needle tip to the front plane of the collector and the electrospinning process was carried out until an even distribution of fibers had been spun across the entire grid. The scaffold was collected, and a 4 mm biopsy punch was used to collect multiple 4 x 1 mm (diameter x height) samples from the same electrospun scaffold.

Collagen Attachment

Aminolysis

Scaffold samples that had been cut by biopsy punches (4 x 1 mm) were used for this experiment. A method previously reported by Zhu et al. (2002) for the optimization of aminolyzing PCL scaffolds was utilized by preparing a 10% w/v solution of hexamethylenediamine (HMDA) dissolved in 2-propanol. Scaffolds were placed in a non-treated 96-well plate and 200 uL of HMDA solution was added to each well. Scaffolds were allowed to incubate at 37°C for 1 hour. Then, each scaffold was individually placed in 100 mL of distilled water and washed on a shaker at room temperature for 24 hours. Finally, scaffolds were removed from the water wash and dried for 24 hours in a vacuum desiccator.

Glutaraldehyde (GA) Method

This method was accomplished by preparing a 1 wt% glutaraldehyde solution and immersing each aminolyzed scaffold in 200 uL of the GA solution.[14] Scaffolds were removed from the solution and individually placed in 100 mL of distilled water and washed on a shaker for 24 hours. Once removed from the water wash scaffolds were immersed in a 6 mg/mL collagen solution (Sigma) that had been previously diluted and pH adjusted to 3.4 for 24 hours at 2-4°C. Once removed from the collagen solution, scaffolds were dunked 3 times in a 1% acetic acid solution to remove excess collagen that did not chemically attach. Finally, scaffolds were rinsed individually in 100 mL of distilled water for 24 hours at 2-4°C and then dried inside a desiccant chamber for 24 hours at 2-4°C.

Mineralization

Passive Mineralization in Simulated Body Fluid (SBF) (Table 1)

Simulated body fluid was prepared according to Kokubo et al. (1990) by dissolving the constituents in the order they are listed, seen in table 1. Because of the super-saturation of apatite in the fluid, precipitation can happen without careful preparation. The solution was kept at 36.5°C throughout the preparation process, plastic beakers were used to prevent precipitation onto glass, and if the solution became cloudy at any point during preparation (indicating precipitation), the experiment was abandoned and began again. Once preparation of SBF was complete, aminolyzed, collagen attached scaffolds were soaked in 50 mL of SBF for 1, 5, and 7 days at 37°C. At the end of each time point, scaffolds were collected and placed in a desiccant chamber to dry and stored at 2-4°C for further testing.

Groups

Three samples per group were tested. The material control was non-functionalized and unmineralized base PCL scaffolds [4x1 mm (diameter x height)]. The bone control was J-Bone® (9x15x18 mm corticocancellous blocks, Impladent). The two PCL test groups were PCL with collagen only (PCL-CLG) and PCL with collagen and hydroxyapatite (PCL-C-HA). OsteoGen (10x20 mm plugs, Impladent) and Bio-Oss Collagen (5x5x7 mm blocks, Geistlich Pharma) were tested unmodified from the manufacturer.

Porosity Analysis

Micro-CT was used to measure scaffold porosity and internal structures. Three samples from each group were scanned using the SkyScan 1172 micro-computed tomography (Bruker, Belgium). Prior to image acquisition, a flat field correction was performed. Filtration of the polychromatic x-ray beam was done with a 0.5 aluminum filter. Each specimen was scanned 180 degrees with a 0.4-degree rotational increment per frame using a source voltage and current of 44 kV and 222 μA, respectively. At each rotational degree, five scans were captured and averaged to give one projected frame. These projected scans were then reconstructed into a three-dimensional volume with a voxel size of 5.98 μm and 2000 x 1048 pixels per slice, using a customized Feldkamp algorithm (NRecon v-1.7.4.6, Bruker, Billerica, MA). Image segmentation and analysis were performed using a proprietary software (CTAn v-1.18.9.0+, Bruker, Billerica, MA). Percentage porosities per sample were characterized.

Hardness testing and indentation modulus

Nanoindentation using the iNano® Nanoindenter (iNano, Nanomechanics, Oak Ridge, TN) was done to determine the hardness and indentation modulus of the materials. Samples were prepared by embedding them in epoxy resin, then polished to a smoothness of 0.05 μm. Because Bio-Oss Collagen and J-Bone have "spongy" and "cortical" regions, these samples were further divided into two areas for indentation testing – "spongy" and "cortical". All indentations were performed after the thermal drift rate reached below 0.05 nm/s threshold. The maximum indentation load was 2 mN, and Poisson's ratio for all specimens was 0.3. An array of indents (100 indents) was imprinted on the specimen surface. Each consecutive indent was spaced 2 μm apart from each other to avoid any interference of residual stresses from adjacent imprints. Force–displacement curves for the indents were used to evaluate the elastic moduli. For each indent, elastic modulus was calculated using the standard methods of Oliver and Pharr (1992). The elastic modulus, E (GPa), per group was computed with the following expression,

$$E = (1 - v^2)\left(\frac{1}{E_r} - \frac{1 - {v_{tip}}^2}{E_{tip}}\right)^{-1}$$

where v and E_r (GPa) were the Poisson's ratio (v = 0.3) and reduced modulus, and v_{tip} and E_{tip} (GPa) were the Poisson's ratio (0.07) and elastic modulus (1141 GPa) of the Berkovich indenter, respectively. The nanoindentation hardness was obtained from the indentation load divided by the projected contact area, A (nm^2): Hardness = P/A, where P (mN) was the maximum contact force exerted by the indenter onto the sample.

Statistical Analysis

A mean and standard deviation was determined per group for modulus, hardness, and porosity. Data were analyzed with a one-way ANOVA and Tukey's post hoc test to evaluate the effect of material type on the mechanical property and porosity ($\alpha = 0.05$) using statistical software (JMP v15.2.0 SAS Institute, Cary, NC).

RESULTS

Porosity (Figures 1, 4)

The porosity of different scaffolds was measured and compared. OstenGen had the highest mean porosity (97.16%), while Bio-Oss Collagen had the lowest (52.22%). No significant difference was found between PCL, PCL-CLG, and PCL-C-HA scaffolds in terms of porosity. Similarly, PCL, PCL-CLG, and Bio-Oss Collagen scaffolds had no significant difference in porosity. However, all PCL based scaffolds (PCL = 56.85%, PCL-CLG = 57.59%, PCL-C-HA = 61.51%) had significantly lower porosity than OsteoGen and J-Bone (88.19%) ($p = 0.001$). Moreover, PCL-C-HA scaffolds had significantly higher porosity than Bio-Oss Collagen ($p = 0.0055$), but significantly lower than OstenGen and J-Bone ($p = 0.001$).

Hardness and indentation modulus (Figures 2, 3)

The hardness of PCL alone (0.21 GPa), PCL-CLG (0.18 GPa), and PCL-C-HA (0.24 GPa) are significantly less than cortical areas of Bio-Oss Collagen (0.45 GPa) and J-Bone (0.45 GPa) ($p = 0.001$). There was no statistically significant difference in hardness between any of the PCL scaffolds and OsteoGen or "spongy" Bio-Oss Collagen. Also, there was no statistically significant difference in

hardness between PCL-C-HA and “spongy” J-Bone (0.42 GPa). The modulus results mirrored the findings of the hardness test. There was no statistically significant difference in modulus between any PCL scaffold (PCL = 3.14 GPa, PCL-CLG = 2.82 GPa, PCL-C-HA = 3.73 GPa) and OsteoGen (4.33 GPa) or “spongy” Bio-Oss Collagen (4.65 GPa). The modulus of “cortical” Bio-Oss Collagen (12.91 GPa) was not significantly different from J-Bone (“spongy” = 14.37 GPa, “cortical” = 14.93 GPa).

DISCUSSION

PCL is a synthetic polymer that has been widely used for tissue engineering applications because of its biocompatibility, biodegradability, and mechanical strength.[8] It can be fabricated into porous scaffolds that mimic the extracellular matrix and support cell attachment and proliferation.[22] PCL scaffolds can also be functionalized with collagen and hydroxyapatite, which are natural components of bone tissue, to enhance their physical and bioactive properties.[4,5,6,9,15,19] In this study, the null hypothesis that electrospun PCL scaffolds functionalized with collagen and hydroxyapatite have similar hardness and indentation modulus and porosity to two commercially available bone graft materials (OsteoGen and Bio-Oss Collagen) was rejected.

The micro-CT images of the test samples in this study are shown in Fig 4. The PCL scaffolds, with collagen or with collagen and HA, appeared uniform in structural architecture, with no discernable differences. Bio-Oss Collagen had the largest particles compared to OsteoGen and all PCL scaffolds due to the bovine bone mineral component, which subsequently made it the least porous. The internal structure of OsteoGen appeared the most porous, and it had the highest porosity of all the materials. Addition of collagen and HA to base PCL increased porosity slightly but were not statistically significant. The porosity of PCL based scaffolds were like Bio-Oss Collagen, but significantly less than OsteoGen. The hardness of PCL scaffolds functionalized with collagen and mineralized with HA were like OsteoGen and "spongy" aspect of Bio-Oss Collagen. However, this "spongy" aspect is likely from the porcine collagen component in Bio-Oss Collagen. The indentation modulus of J-Bone (14.37-14.93 GPa) in this study was consistent with Huja et al. (2007), who reported that the alveolar process had an indentation modulus of 8.47 to 16.54 GPa in a dog model, with the mandible exceeding the maxilla.

These results suggest that PCL scaffolds have comparable mechanical properties to some commercially available bone graft materials. The addition of HA microparticles did not significantly improve the hardness or modulus of PCL scaffolds but may have other benefits such as improved cell adhesion and differentiation. While the optimal ratio between collagen and HA in composite scaffolds remained unclear, Prosecká et al. reported a ratio of 50 wt % HA in 0.5 wt % collagen solution as ideal for new bone formation and cell proliferation in vitro. However, this study only tested the mechanical properties of PCL scaffolds *in vitro*. The ratio of collagen and HA and the *in vivo* response of cells and

tissues to the fabricated PCL scaffolds from this study needs to be further evaluated.

PCL by itself has an extremely slow rate of degradation – 6 months up to 4 years[16]. PCL degrades *in vivo* by non-enzymatic hydrolysis to small molecular weight particles, then intracellular degradation via phagosomes. However, the rate of hydrolysis can be altered by copolymerization with other substrates such as glycolides or lactides. This aspect warrants further investigation to determine whether PCL based scaffolds can be a suitable bone graft substitute.

CONCLUSION

Electrospun PCL scaffolds may have similar mechanical properties to OsteoGen, but not to Bio-Oss Collagen, which has higher mineral content and hardness. However, the high porosity of PCL scaffolds may enhance cell growth and bone regeneration compared to Bio-Oss Collagen.

ACKNOWLEDGMENTS

The author gratefully acknowledges Dr. Minju David Yi for his assistance in the data collection for this project.

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FIGURES AND TABLES

Order, amounts, weighing containers, purities and formula weights of reagents for preparing 1000 ml of SBF

Order	Reagent	Amount	Container	Purity (%)	Formula weight
1	NaCl	8.035 g	Weighing paper	99.5	58.4430
2	$NaHCO_3$	0.355 g	Weighing paper	99.5	84.0068
3	KCl	0.225 g	Weighing bottle	99.5	74.5515
4	$K_2HPO_4 \cdot 3H_2O$	0.231 g	Weighing bottle	99.0	228.2220
5	$MgCl_2 \cdot 6H_2O$	0.311 g	Weighing bottle	98.0	203.3034
6	1.0M-HCl	39 ml	Graduated cylinder	—	—
7	$CaCl_2$	0.292 g	Weighing bottle	95.0	110.9848
8	Na_2SO_4	0.072 g	Weighing bottle	99.0	142.0428
9	Tris	6.118 g	Weighing paper	99.0	121.1356
10	1.0M-HCl	0–5 ml	Syringe	—	—

Table 1. Simulated body fluid reagents and order of addition. Kokubo et al.[12,13]

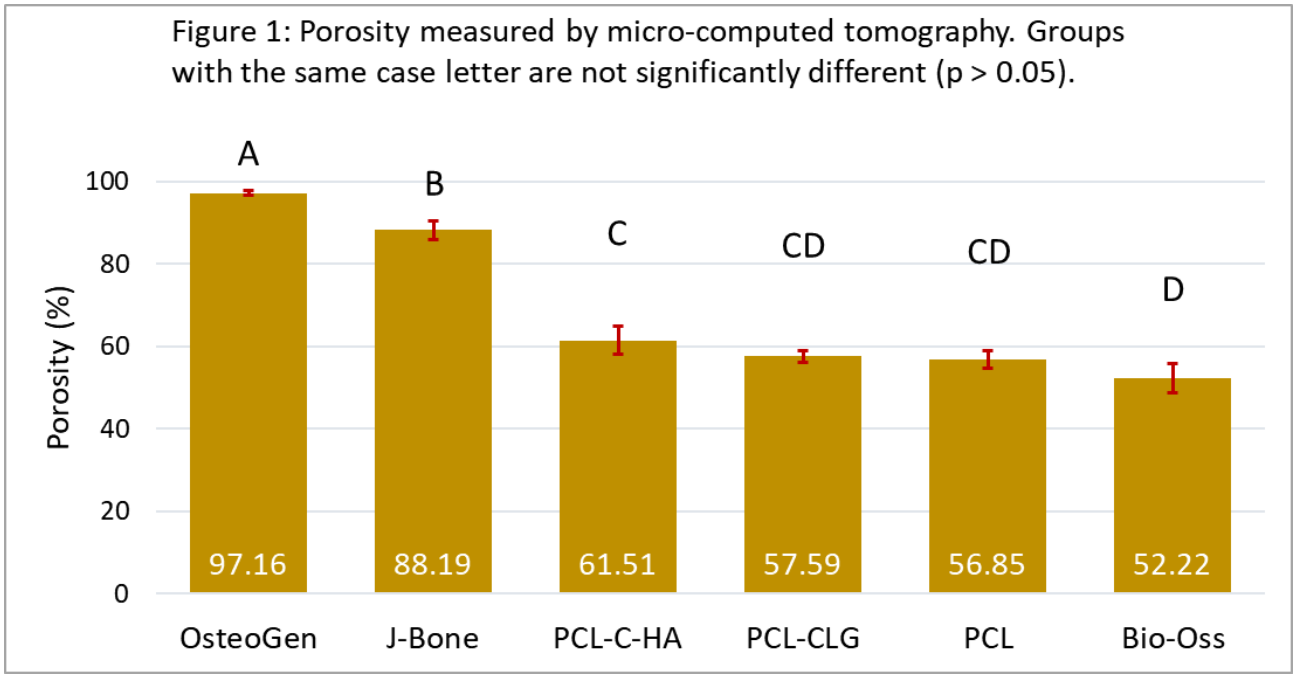


Figure 2: Hardness measured by nanoindentation. Groups with the same case letter are not significantly different ($p > 0.05$).

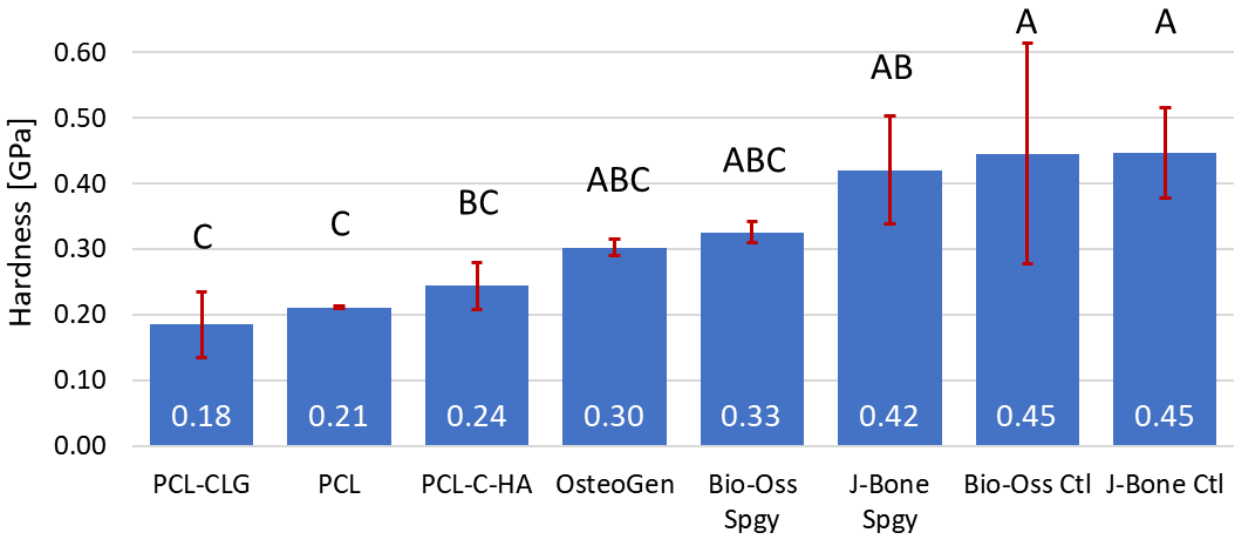
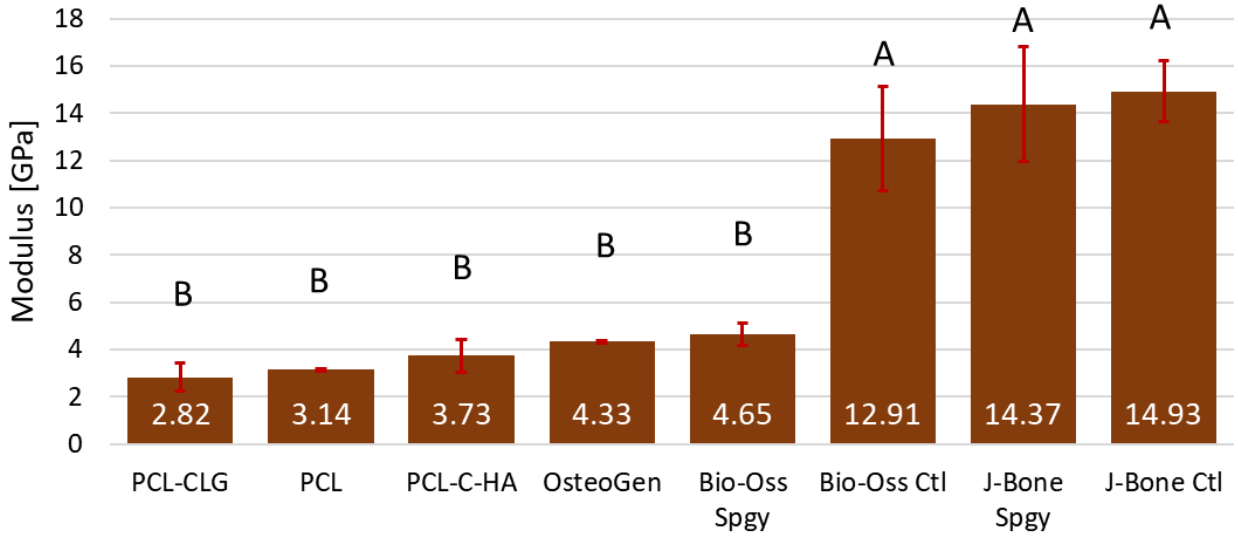


Figure 3: Modulus measured by nanoindentation. Groups with the same case letter are not significantly different ($p > 0.05$).



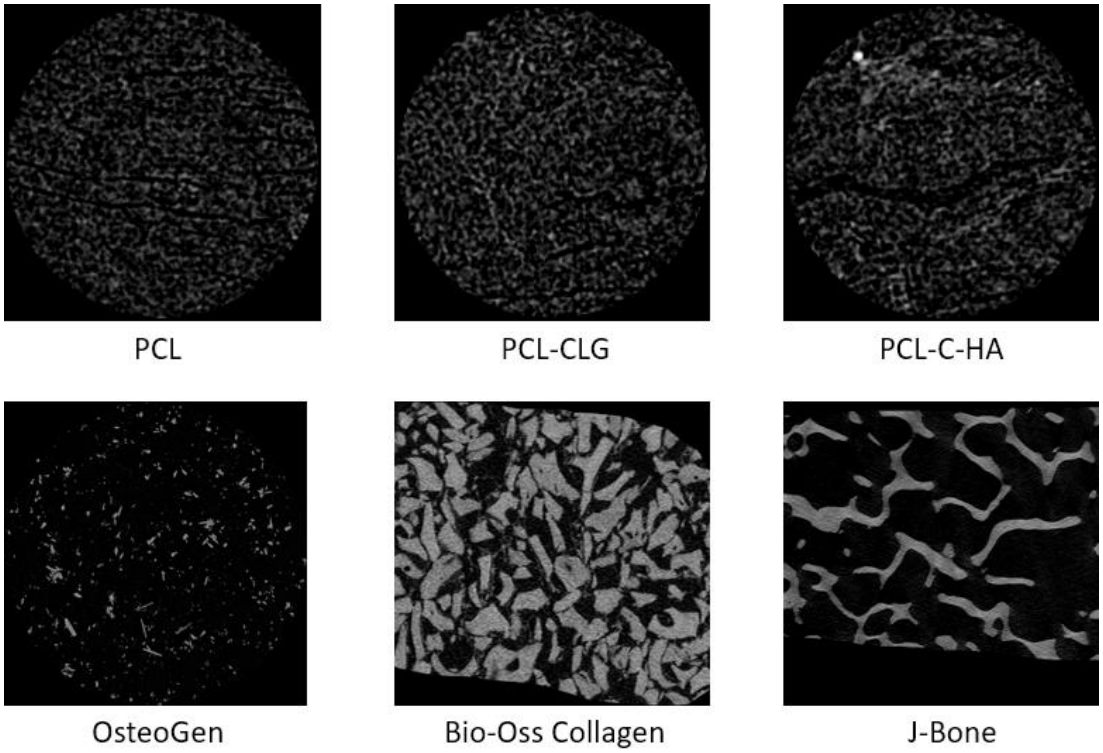


Figure 4. Micro-CT images of scanned samples.