# CHARACTERIZATION OF BIOMATERIAL-BONE INTERFACES WITH TRANSMISSION ELECTRON MICROSCOPY

# CHARACTERIZATION OF BIOMATERIAL-BONE INTERFACES WITH TRANSMISSION ELECTRON MICROSCOPY

By

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## Abstract

Understanding the interfacial reactions to synthetic bone regenerative scaffolds in vivo is fundamental for improving osseointegration and osteogenesis. Using transmission electron microscopy, it is possible to study the biological response of hydroxyapatite (HA) and zirconia (ZrO<sub>2</sub>) scaffolds at the nanometer scale. Using this technique, the bone-bonding abilities of HA and ZrO<sub>2</sub> scaffolds produced by free form fabrication were evaluated in the human maxilla at 3 and 7-months. A novel focused ion beam (FIB) sample preparation technique enabled the production of thin lamellae for study by scanning transmission electron microscopy (STEM). Interface regions were investigated using high-angle annular dark-field (HAADF) imaging, energy dispersive x-ray spectroscopy (EDXS) analysis and Z-contrast electron tomography. The absence of an interfacial apatite layer in the ZrO<sub>2</sub> samples suggests the formation of a direct contact with bone, while HA bonds through an apatite layer that shows indications of resorption with increasing implantation time. Interfacial apatite layers of 80 and 50nm thickness were noted in the 3 and 7-month HA samples, respectively and bone growth was discovered in micropores up to 10µm into the samples. Viewing this structure in three dimensions enabled us to observe the nanometer differences in orientation of hydroxyapatite crystals in the collagen matrix of the bone and crystals precipitated on the implant surface. This study demonstrates the potential of hydroxyapatite and zirconia scaffolds for use as bone regenerative materials.

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## List of Abbreviations

2D	Two-Dimensional
3D	Three-Dimensional
EDXS	Energy Dispersive X-Ray Spectroscopy
FEG SEM	Field Emission Gun Scanning Electron Microscope
FIB	Focused Ion Beam
НА	Hydroxyapatite
HAADF	High-Angle Annular Dark-Field
SBF	Simulated Body Fluid
STEM	Scanning Transmission Electron Microscopy
TEM	Transmission Electron Microscopy
XRD	X-Ray Diffraction
β-TCP	Beta Tri-calcium Phosphate

## **Glossary of Terms**

- Biocompatibility The ability of a material to elicit the appropriate host response.
- Biomaterial Any material, natural or synthetic, that comprises whole or part of a living structure or biomedical device which performs, augments, or replaces a natural function.
- Chemotaxis The characteristic movement or orientation of an organism or cell along a chemical concentration gradient either toward or away from a chemical stimulus.
- *In vitro* In a non-living environment.
- *In vivo* In a living environment.
- Osseointegration Direct contact between living bone and implant surface.
- Osteoconductive The conduction of bone growth along a surface or into pores, channels or pipes.
- Osteogenesis The formation and development of bone.
- Osteolysis Dissolution or degeneration of bone tissue resulting from disease.

## **1** Introduction

## 1.1 Bone

The support structure of the human body, the skeleton, is composed of bone. On the macroscale, two main forms of bone exist; cortical and trabecular. Cortical bone, also known as compact bone, forms the dense outer shell of bone's hierarchical structure, while trabecular bone forms the interior. Mature bone forms a well-organized lamellar structure, referred to as Haversian channels. It is important to note the vascularization of bone and its dependence on a blood supply for growth and survival. The location of cortical versus trabecular bone and the arrangement of Haversian channels are demonstrated in Figure 1.



Figure 1. (A) Schematic long bone structure identifying regions of cortical and trabecular bone. (B) The structure of cortical bone including 1. Bone lamallae. 2. Periosteum. 3. Osteocytes. 4. Volkmann channels. 5. Blood vessels. 6. Cement line separating Haversian channels. 7.Endosteum. 8. Marrow cavity. 9. Haversian systems. (Palmquist, 2008)

Bone is a composite material of inorganic and organic constituents. The organic component accounts for approximately 35% of bone mass and is made up of 90% collagen Type I (Vallet-Regí and González-Calbet, 2004). The remaining 10% of bone's ... organic component consists of proteoglycans and various other proteins. The inorganic 65% of bone is composed of ion-substituted forms of the mineral hydroxyapatite (HA), Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub>. The most common ion-substituted form of HA found in bone is carbonated-hydroxyapatite, however strontium, magnesium and fluorine substituted forms have also been documented (Vallet-Regí and González-Calbet, 2004, Dorozhkin and Epple, 2002).

On the nanometer scale, a relationship between the organic and inorganic bone constituents exists. Organic Type I collagen forms fibrils approximately 300nm in length and 15nm in diameter (Katz and Li, 1973). These fibrils are aligned parallel to one another such that they form gap and overlap zones (Hodge and Petruska, 1963). The inorganic hydroxyapatite mineral is a hexagonal crystal that forms plates along the direction of the c-axis, up to 100nm in length. This mineral is arranged within the gap zones of collagen such that its c-axis is parallel to the long axis of the collagen fibrils (Weiner and Traub, 1986). The arrangement of collagen and hydroxyapatite is shown in Figure 2.



Figure 2. Schematic of the nanoscale organization of hydroxyapatite and collagen to form the structure of bone. (Lakes, 1993)

Bone is a living structure that continually undergoes a remodeling process to repair its damaged tissue, improve bone quality and respond to mechanical stresses (Lanyon, 1993). Two main cell types participate in this remodeling process; osteoclasts and osteoblasts. Osteoclasts secure themselves to the surface of bone and resorb it, creating tunnels in the process. Osteoblasts are attracted to these cavities and lay down new bone in them. On becoming inactive, osteoblasts embed themselves in the bone structure and are referred to as osteocytes. Bone's structure is ever changing due to this remodeling process.

## **1.2 Bone Graft Substitutes**

Patients may suffer from reduced bone volume for any number of reasons including trauma, disease or degeneration. This volume reduction may occur in long bone regions such as the bones of our limbs, or quite frequently in cranial or maxillofacial locations. To repair this loss of bone, treatment options include bone replacement, augmentation or regeneration, and ideally a combination of all three. A number of factors must be considered to promote bone healing in the design and production of bone graft substitutes, among these the materials selection and geometrical considerations are of most importance.

#### 1.2.1 Bone Healing & Osseointegration

Of the properties associated with biomaterials, biocompatibility is perhaps one of the most important factors to consider. Biocompatibility was traditionally defined as the ability of a material to elicit the appropriate host response (Williams, 1987). In the case of implants intended for contact with hard tissue, such as bone, the appropriate host response involves osseointegration; the direct contact between living bone and implant surface (Brånemark et al., 1977). Originally, when this term was defined this contact was on the light microscopic resolution level. However, electron microscopy enables evaluation of materials at a much higher resolution and allows determination of direct contact on an ultrastructural level.

Trauma associated with implant placement causes a chain reaction of events in the body including an inflammatory response, and if favorable bone formation. The response of the body is dependent on many factors, but is strongly influenced by the type of material in contact with bone and surrounding fluids. Bone formation begins with the rapid deposition of a third class of bone, the unorganized structure known as woven bone. Through the remodeling process this bone is eventually organized into a lamellar structure and gains mechanical strength (Doblarè et al., 2004). Implant materials that promote the rapid formation of bone in their vicinity are sought after for bone regenerative applications.

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#### **1.2.2 Materials Selection**

While a number of biological and synthetic alternatives for bone augmentation and regeneration exist, they do not come without their share of disadvantages and imperfections (LeGeros, 2002). For many years, the gold standard in bone graft substitutes has been autografts, from the patient themselves, or allografts, from a human donor patient (LeGeros, 2002). Serious potential drawbacks include for example, limited supply, donor site infection and increased morbidity rates with use of autografts (Arrington et al., 1996, Younger and Chapman, 1989). Additionally, the use of allografts poses potential risks such as transference of disease and incompatibility (LeGeros, 2008). An increase in the number of xenografts, transplants from other species, has been noted with applications such as bovine bone granules used extensively (Richardson et al., 1999). Despite numerous protocols in place to protect patients, disease transference or implant rejection remain high risk factors. There is a distinct need for improvement of synthetic materials for bone graft substitutes and a deeper understanding of their interfacial interactions with the human body.

Materials selection and interfacial stability are intimately related concerns for improving the biocompatibility of bone graft substitutes. Biomaterials have been categorized depending on their interfacial behaviors in the body. Hench has described the different classes of biomaterials; bio-inert, bioactive, and resorbable (Hench, 1991). The musculo-skeletal system can respond to biomaterials in one of four ways; (1) if the material is toxic, it causes cell death in surrounding tissues, (2) if the material is nontoxic and inert, it results in the formation of a encapsulating fibrous tissue, (3) if the material is non-toxic and bioactive, it forms an interfacial bond with tissue, and (4) if the material is non-toxic and dissolvable, it is replaced by tissue (Hench, 1991). In the case of bone regenerative materials, the desirable interactions are the formation of an interfacial bond between implant material and bone, and ideally, resorption of the implant material over time (Hench, 1998). The rapid formation of an interfacial bond between implant and bone is essential for reducing micro-movements, which contribute to inflammation and the formation of a fibrous encapsulating layer. In some cases, these small micro-movements at the interface lead to osteolysis and aseptic loosening of the implant (Sundfeldt et al., 2006). Choosing a material that readily and rapidly forms an interfacial bond with bone reduces implant movement, and therefore decreases the risk of aseptic loosening. Another important factor to consider is the implant placement. Physical mechanical interlocking between implant and bone, achieved by proper placement and size of implant can also reduce risks of implant loosening (Plenk, 1998).

Ceramic, polymer and polymer-ceramic composites are accepted choices for nonload bearing bone regenerative scaffolds, as they can be similar to bone in composition, structure or mechanical properties. A wide range of calcium phosphate materials are approved for use in humans including hydroxyapatite, tricalcium phosphate, octacalcium phosphate, Apatite-Wollastonite® glass-ceramic and Bioglass® (LeGeros, 2008, Hench, 1998).

#### 1.2.3 Scaffold Design

In addition to materials selection, complete osseointegration of scaffolds depends on a number of factors including implant design. A unique definition of biocompatibility has evolved from the original definition given by Williams in 1982 specifically for scaffold materials.

"The biocompatibility of a scaffold or matrix for a tissue engineering product refers to the ability to perform as a substrate that will support the appropriate cellular activity, including the facilitation of molecular and mechanical signaling systems, in order to optimize tissue regeneration, without eliciting any undesirable local or systemic responses in the eventual host." (Williams, 2008)

Therefore, proper scaffold geometry is an important factor for ensuring mechanical support and encouraging tissue regeneration. Pore morphology, and interconnectivity are known to greatly influence bone ingrowth in bone graft substitutes. It has been cited that interconnected macroporosity >100 $\mu$ m is necessary for vascularisation associated with osteogenesis (Jones et al., 2009a, Uchida et al., 1985, Shimazaki and Mooney, 1985).

In addition to macroporosity, microporosity plays an important role in improving biocompatibility (Jones et al., 2009a). Microporous surfaces have been shown to enhance bone attachment and growth (Malmström et al., 2007).

While conventional casting techniques constrain implant shape and design, recent developments in computer assisted design (CAD) and rapid prototyping methods such as free form fabrication provide a feasible solution. Free form fabrication, which employs a 3D inkjet printing principle, is an effective method to control pore architecture (size, shape, interconnectivity) and tailor scaffolds for specific clinical applications.

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## **1.3 Bone-Implant Interfacial Interactions**

Biocompatibility is of the utmost importance when considering a material for use *in vivo*. As mentioned, the biological response to materials is of such importance it has been used to formulate the classification system for all biomaterials (Hench, 1991). Materials are placed into the following categories; inert, bioactive or bioresorbable.

Inert biomaterials, also considered first generation biomaterials, illicit no or little biological response *in vivo*. This class of materials includes many used in load bearing orthopaedic applications such as zirconia, alumina, titanium and stainless steel. In some cases, these materials may form a fibrous capsule around them, isolating them from interacting with the body. This type of interaction is the least desirable, as it prevents the body from fully integrating with the implant material (Hench, 1991).

Bioactive materials, on the other hand, are a much better alternative to inert materials from a biological perspective. Common bioactive materials include calcium phosphates, calcium aluminates, Bioglass® and glass-ceramic Apatite-Wollastonite® (Hench, 1991, LeGeros, 2002). The unique characteristic of these materials is their ability to form a chemical bond with bone through a biologically active apatite layer that forms on their surface *in vivo* (Ducheyne and Qiu, 1999). However, as many bioactive materials consist of brittle or soft ceramic or polymeric materials, these materials are generally restricted to non-load bearing applications and quite frequently, are used as coatings to improve biocompatibility of inert materials.

The most desirable of all biomaterials are those belonging to the bioresorbable class. These third generation biomaterials focus on regeneration of soft and hard tissue, rather M.A.SC. THESIS • K. GRANDFIELD • MCMASTER • MATERIALS SCIENCE & ENGINEERING 2010

than just replacement and augmentation. These materials are resorbed by the body and eventually, replaced completely by natural tissue. In this way, the material provides temporary relief and encourages regeneration while the body heals. Bioresorbable materials include  $\beta$ -tricalcium phosphate, calcium carbonate and polylactic-polygycolic acid (Hench, 1998). The challenge with these materials remains tailoring their resorbable rate to match the body's natural regeneration rates. As this factor varies greatly from patient to patient, and implant site-to-site, perfect tailoring of resorbable materials can be difficult.

Of the possible bone graft substitute materials, hydroxyapatite and zirconia based scaffolds have achieved much attention and clinical use. These materials are further discussed.

#### 1.3.1 Zirconia-Bone Behavior

Zirconia ( $ZrO_2$ ), while not a bioactive material, has considerable widespread use in the biomaterials field. Its chemical inertness, combined with its strength has made it ideal for dental and load bearing applications. Stable anchorage of zirconia implants requires a direct contact between implant and bone, and has been demonstrated in the rabbit model (Sennerby et al., 2005, Malmström, 2007).

## 1.3.2 Hydroxyapatite-Bone Behavior

Calcium phosphate ceramics have vast applications in the biomedical field. Of these calcium phosphates, hydroxyapatite (HA)  $(Ca_{10}(PO_4)_6(OH)_2)$  is of particular interest due to its similar composition to the mineral component of bone. It is well known that HA is a

bioactive material, in that it precipitates an apatite layer on its surface *in vivo*, enabling it to form a chemical bond with bone (Hench, 1998). Such bone bonding capabilities are of particular interest in the bone regeneration field. The proposed mechanism for bone bonding is a dissolution–reprecipitation process to form a biologically active apatite layer on the HA surface (Ducheyne and Qiu, 1999). A most versatile biomaterial, non-load bearing applications for HA range from coatings to cements, and to scaffolds. Improving the design, performance and longevity of bone regenerative scaffold materials depends strongly on a thorough understanding of their interfacial interactions *in vivo*.

The behavior of HA *in vitro*, on the other hand, is quite well understood. When placed in a simulated body fluid (SBF) solution, with ion composition similar to that of blood plasma (Table 1) given by Kokubo's bioactivity test, HA precipitates carbonate-substituted HA on its surface (Kokubo and Takadama, 2006). Kokubo's bioactivity test in SBF has been shown to be useful in predicting the behavior of materials *in vivo*. According to the test, a material that forms an apatite layer on its surface *in vitro* is likely to form a similar surface-active layer *in vivo* (Kokubo and Takadama, 2006).

	Ion concentration (mM)							
	Na <sup>+</sup>	K <sup>+</sup>	Mg <sup>2+</sup>	Ca <sup>2+</sup>	Cl	HCO <sub>3</sub>	HPO4 <sup>2-</sup>	SO4 <sup>2-</sup>
Human Blood Plasma	142.0	5.0	1.5	2.5	103.0	27.0	1.0	0.5
Corrected SBF	142.0	5.0	1.5	2.5	147.8	4.2	1.0	0.5

Table 1. Simulated Body Fluid concentrations as given by Kokubo. (Kokubo and Takadama, 2006)

The mechanisms that govern apatite formation *in vitro* have been explained thoroughly by Kim et al. and are depicted in Figure 3. The surface of HA has a slightly

negative charge, owing to the oxygen ions. This charge creates a gradient for positive calcium ions from the SBF to deposit on the surface of hydroxyapatite. With an accumulation of calcium ions, the surface charge becomes slightly positive, and with this change phosphate ions from solution are attracted to the surface. The phosphate ions are incorporated onto the surface and an amorphous calcium phosphate layer results. With increasing soaking time, this amorphous layer reorganizes into a crystalline form (Kim et al., 2005).



Figure 3. Schematic outlining the formation of bone-like apatite on the hydroxyapatite surface in SBF. (Kim et al., 2005)

The mechanisms governing apatite formation become extremely more complicated *in vivo*. Unlike *in vitro*, ions are not the only factors present to influence apatite formation. A number of proteins, cells, and biological factors are present in blood plasma and contribute to the formation of apatite. Ducheyne et al. (1999) have outlined the possible processes occurring on the surface of a hydroxyapatite sample *in vivo*. Shown in Figure 4, eleven possible interactions occur simultaneously and in no preferred order; (1) dissolution from the ceramic; (2) precipitation from solution onto the ceramic; (3) ion exchange and structural rearrangement at the ceramic–tissue interface; (4) interdiffusion

from the surface boundary layer into the ceramic; (5) solution-mediated effects on cellular activity; (6) deposition of either the mineral phase (a), or the organic phase (b), without integration into the ceramic surface; (7) deposition with integration into the ceramic; (8) chemotaxis to the ceramic surface; (9) cell attachment and proliferation; (10) cell differentiation; and (11) extracellular matrix formation. (Ducheyne and Qiu, 1999)



Figure 4. Processes occurring on the hydroxyapatite surface in vivo. (Ducheyne and Qiu, 1999)

Apatite and bone formation *in vivo* can be simplified by considering a dissolutionreprecipitation mechanism that governs the system. *In vivo*, the dissolution of HA occurs creating a supersaturation of calcium and phosphate ions in the near vicinity. Simultaneously, proteins adhere to the surface while an apatite layer reprecipitates out of the supersaturated solution. The proteins that are a critical part of the system have receptors that attract osteoblasts, the bone building cells, from the surrounding fluid. The osteoblasts then play a key role in the formation of new bone around hydroxyapatite.

## **1.4 Bone-Implant Interfacial Analysis with Electron Microscopy**

#### 1.4.1 Scanning Electron Microscopy

The gold standard in determining percentage of bone-implant contact and bone-ingrowth area is backscattered scanning electron microscopy (Kokubo, 2008). Obtaining an accurate depiction of bone formation around and along implants is crucial for determining their success *in vivo*. Light microscopy techniques require extensive sample preparation such as staining and sectioning, and still lack the resolution needed to obtain accurate measurements. SEM requires little sample preparation and does not require staining with heavy elements.

The scanning electron microscope is a valuable characterization tool for boneimplant interfacial analysis. Of the variety of electron-matter interactions that occur in the SEM, the detection of backscattered electrons is the most useful.

Backscattered electrons are highly Z-dependent and therefore create Z-contrast images. This is crucial for studying hydroxyapatite in contact with bone, since their chemical similarities make them difficult to distinguish otherwise. In addition to its application for bone contact measurements, backscattered electrons are useful for choosing sites of good bone-implant contact for further investigation with TEM.

#### **1.4.2 TEM Sample Preparation Techniques**

Particularly for the structural and interfacial characterization of bone growth around such implants, preparation of biomaterial-bone interfaces is a major challenge (Giannuzzi and Stevie, 2005). Due to the differences in mechanical strength of the adjacent materials, M.A.SC. THESIS • K. GRANDFIELD • MCMASTER • MATERIALS SCIENCE & ENGINEERING 2010

conventional TEM preparation by ultramicrotomy results in a variety of artifacts (Giannuzzi et al., 2007). Problems related to delamination of interfaces, plastic deformation of soft materials, fracture of brittle materials, and limitations in specimen thinness have been reported to constrict the acquisition of elemental and high resolution data (Giannuzzi and Stevie, 1999). Focused ion beam (FIB) sample preparation eliminates the artifacts associated with ultramicrotomy. Using a dual beam instrument equipped with an SEM column and in-situ lift-out probe, site-specific samples can be made (Engqvist et al., 2006). A schematic outlines the main features of the FIB in Figure 5. Interface integrity is maintained with FIB and therefore has been used for all sample preparation in this study.



Figure 5. Schematic of the main components of a dual-beam FIB. (A) Sample stage. (B) Sample. (C) Electron beam column. (D) Tungsten micromanipulator. (E) Ion beam column. (F) Gas injector System. (G) Detector.

#### 1.4.3 Transmission Electron Microscopy

#### 1.4.3.1 High-Angle Annular Dark Field STEM

Imaging specimens containing bone is most successfully performed using high-angle annular dark-field scanning transmission electron microscopy (HAADF STEM). This technique has a number of advantages when analyzing beam sensitive materials such as bone. A high-angle annular detector, which as the name suggests, is shaped in an annulus, collects electrons scattered to high angles. These electrons are predominantly incoherently scattered and sensitive to the atomic number Z of elements under the electron beam, without contribution from diffraction contrast (Williams and Carter, 1996). Therefore, the simultaneous acquisition of morphological and compositional information is accomplished with HAADF STEM. As such, STEM is extremely useful for the study of biointerfaces where only slight chemical changes occur, such as at the interface between hydroxyapatite and bone.

While the attainable resolution is theoretically lower with STEM versus TEM imaging, STEM presents other benefits. For example, STEM imaging is less dependent on specimen thickness so slightly thicker specimens still produce good quality images (Williams and Carter, 1996). The scanning beam is also an advantage to imaging in STEM mode versus TEM. The rastering beam effectively reduces beam damage by reducing the exposure of the sample to electrons.

Bone has an extremely unique morphology in the TEM due to the arrangement of collagen. As mentioned previously, collagen forms gap and overlap zones, which create what is referred to as collagen banding visible in the TEM. The gap and overlap zones

create a banding of light and dark contrast with periodicity of 67nm (Hodge and Petruska, 1963). This unique characteristic of bone enables us to determine the orientation of collagen, since collagen fibres are arranged perpendicular to the banding pattern. Furthermore, since hydroxyapatite is arranged parallel to the collagen fibres, the presence of collagen banding in an electron micrograph also enables us to confirm the direction of hydroxyapatite mineral. Figure 6 demonstrates the origin of the banding.



Figure 6. Schematic illustrating the origin of collagen banding in electron micrographs. The cylinders on the right of the diagram represent the natural arrangement of collagen fibrils.

#### 1.4.3.2 Analytical Electron Microscopy

A variety of analytical electron microscopy techniques are possible due to the interaction of electrons with matter (Botton, 2007). Perhaps one of the most widespread techniques, Energy Dispersive Electron Spectroscopy (EDXS), is extremely useful for probing the chemical structure of bone-implant interfacial regions.



Figure 7. Signals generated from electron-matter interactions with a thin sample. (Botton, 2007)

When electrons interact with matter, one possible chain of events is the production of characteristic x-ray signals. These x-rays are detected and the signals are quantified to give chemical information about the sample. Collecting quantitative information from interface regions in EDXS may present a challenge. Sample geometry and alignment relative to the detector affect the absorption of x-rays in adjacent materials (Botton, 2007, Williams and Carter, 1996). To reduce effects associated with absorption, the sample interface should be aligned parallel to the detector (Botton, 2007).

#### 1.4.3.3. Electron Tomography

Biointerfaces have long been imaged with light, x-rays and electrons. Most of these techniques however, only provide low-resolution or two-dimensional information. With the advances in modern day transmission electron microscopy, new hardware and increased software computational speeds enable high-resolution visualization and analysis of three-dimensional structures (Midgley and Weyland, 2003, Midgley and Dunin-Borkowski, 2009).

A transition from two to three-dimensional analysis is becoming standard practice. The advent of laboratory micro-computed x-ray tomography equipment has resulted in the routine 3D analysis of biomaterials themselves. Indeed, the ability to visualize and quantify scaffold geometries is crucial for improving their design, although without the use of a synchrotron x-ray source, a resolution of only a few micrometres is attainable (Jones et al., 2009b, Weiss et al., 2003). With the exception of recent developments in focused ion beam slice and view reconstructions (Giannuzzi et al., 2007), the 3D analysis of biomaterial-bone interfaces by other techniques is quite limited. The drawback of x-ray and dual beam techniques, such as FIB, is quite simple; their low-resolution limits their use in understanding bone bonding at the nanometer or ultrastructural level.

Shifting to electron tomography marks a new threshold for understanding biointerfaces in 3D. Z-contrast electron tomography is a valuable tool for visualizing three-dimensional structures through the collection of a series of 2D projections. Utilizing scanning transmission electron microscopy to collect images over a large angular range presents a number of advantages over conventional TEM-based tomography. The advantages to using STEM, mentioned previously, give rise to the usefulness of STEM Z-contrast tomography for the study of biointerfaces where slight chemical changes occur.

One of the limitations associated with electron tomography is caused by the physical geometry of the TEM system. Although specially designed tomography holders are employed, a limit to the tilt-range exists. The angular range over which projections cannot be collected is referred to as the 'missing wedge' (Figure 8) and results in

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incomplete information of the sample in both real and Fourier space (Baumeister et al., 1999). Reducing the missing wedge increases the theoretical resolution of the resulting tomogram.

Another problem that results in conjunction with the missing wedge is the uneven sampling in Fourier space. The Fourier space is incompletely sampled at high frequencies, resulting in the need to apply a weighting function to the 2D images to produce the correct 3D reconstruction. Applying a weighting function to produce the 3D structure is one reconstruction algorithm referred to as weighted back projection. Often several defined iterations are carried out during a reconstruction.



Figure 8. The limited tilt-range leads to a missing wedge (red), and under sampled high frequencies in Fourier space.(FEICompany, 2003)

The theoretical resolution of electron tomography far exceeds that of x-ray computed tomography, which has resolution limits of only a few micrometres (Weiss et al., 2003). While precise resolution limits have not been determined, the approximate resolution can be estimated from

 $d = \pi D/N$  (Crowther et al., 1970)

where N is the number of projections acquired, and D is the diameter of the volume to be reconstructed which for an extended slab geometry is approximated by

$$D = T/cos(\alpha_{max})$$
 (Radermacher, 2006)

where T is the thickness of the specimen and  $\alpha_{max}$  is the maximum tilt-angle.

Using these equations and assuming all images acquired in the tilt-series are perfectly in focus, the theoretical resolution for a sample approximately 70nm thick and over a tilt range of 146° is less than 5nm, a significant improvement over x-ray computed tomography. In practice, resolution better than this can be obtained with HAADF STEM and careful alignment of the images.

## 2 Research Objectives

The general aim of this research was to evaluate the biological response of human bone to ceramic scaffolds at the nanometer scale using advanced electron microscopy techniques.

In detail, the aims included:

- To compare the effect of material chemistry on human bone response by investigating the differences between hydroxyapatite and zirconia scaffolds interfacing with bone.

- To determine the evolution of the implant-bone interface with increasing implantation time.

- To develop a unique set of electron microscopy methods suitable for the evaluation of implant-bone interfaces on the nanometer scale in two and three dimensions.

## **3** Materials & Methods

## **3.1 Scaffold Fabrication**

Porous hydroxyapatite and zirconia scaffolds ( $\phi = 3 \text{ mm}$  and l = 4 mm) were created by free form fabrication. Square-shaped interconnected channels (approximately 350 $\mu$ m x 350 $\mu$ m) define the macroporous structure that was created using a CAD tool (Solid Works, Concord, MA, USA) shown in Figure 9.



Figure 9. CAD drawing of the scaffold ( $\phi = 3 \text{ mm}$  and l = 4 mm) showing macroporous square-shaped channels ( $350 \mu \text{m} \ge 350 \mu \text{m}$ ).

The mould structure was formed from thermoplastic building material (Proto build, Sanders, USA) and supported by a wax-based surround (Proto support, Sanders, USA). Free form fabrication equipment (Model Maker II<sup>®</sup>, Sanders, USA) using an inkjet printing method, with layer thickness of 50µm, was used to create the moulds. Ceramic suspensions of HA (Plasma Biotal, Tideswell, Buxton, UK) and ZrO<sub>2</sub> (Tosoh Corporation, Tokyo, Japan) prepared by ball milling were loaded into the moulds 48vol% M.A.SC. THESIS • K. GRANDFIELD • MCMASTER • MATERIALS SCIENCE & ENGINEERING 2010

and 50vol%, respectively. The addition of a binder (LDM7651S, Clariant, Sweden) enabled the variation of interconnected microporosity and improved green strength of the HA scaffold. Slip casting on a plate of plaster was used to remove excess water. The cast materials were heated at a rate of 1°C/min up to 600°C to burn away the mould and additives. Further heating of 5°C/min up to 1200°C for HA and 1500 °C for ZrO<sub>2</sub>, holding of the temperature for 2h and then decreasing by 5°C/min produced the sintered scaffolds.

Fabricated HA scaffolds contain 22.3vol% microporosity (22.1vol% open and 0.2vol% closed) and zirconia scaffolds contain 0.7vol% closed porosity, measured by Archimedes's principle. The square shaped interconnected pore channels contribute to 40vol% macroporosity.

The phases present in the zirconia and hydroxyapatite scaffolds were analyzed by xray diffraction (XRD), using a Gunier-Hägg camera and CuK $\alpha$  radiation. Grain size of the scaffold material was determined using transmission electron microscopy.

## **3.2 Surgical Procedures**

Patients recommended for implant treatment in the premolar region of the maxilla and between the ages of 20 to 75 were included in this study. Exclusion criteria consists of a clinical history of smoking (>5 times per day), immunosuppressive agents, recent cardiovascular disease, cardiovascular/renovascular drugs, hormonal disease, radiotherapy in the head/neck region, and infection. Ethical approval for this study was obtained from the ethical research committee at Linköping University, Linköping, Sweden (Dnr. M35-05) and further approval for electron microscopy studies from the Faculty of Health Sciences Research Ethics Board at McMaster University, Hamilton, Ontario, Canada (09-238-T).

Twelve patients (six men and six women, 48-72 years old) received the implants in the maxilla. Anesthesia (10-12ml, Xylocain Dental Adrenalin<sup>®</sup> 2%, 12.5µg/ml, Dentsply, Skarpnäck, Sweden) was administered locally. Twist drills with a diameter of 3mm were used to prepare holes 4mm deep under profuse irrigation with saline (NaCl 9mg/mg, ACO, Upplands Väsby, Sweden). Scaffolds were press fit into the holes, rinsed with saline, and muco-periosteal flaps sutured with Vicryl<sup>®</sup> 5-0 (Johnson & Johnson, Sollentuna, Sweden). Postoperatively, patients received analgesics (Diclofenac T ratiopharm 50mg, ratiopharm AB, Helsingborg, Sweden, three times daily for 1-2 days). Antibiotics (phenoxymethylpenicillin 4g daily or clindamycin 600mg daily) were prescribed for seven days. Patients were advised to rinse with a 0.1% chlorhexidine digluconate solution (Hexident, Ipex, Solna, Sweden) daily for 2 weeks postoperatively.

Specimens were retrieved with surrounding bone tissue using a trephine drill (5mm inner diameter). Retrieved specimens were fixed by immersion in glutaraldehyde (2.5% in 0.05M sodium cacodylate buffer, pH 7.4) and dehydrated in ethanol. Undecalcified specimens were embedded in plastic resin (LR White, the London Resin Co. Ltd., Hampshire, UK). Sawing divided specimens in half longitudinally (Exakt cutting and grinding equipment, Exakt Apparatebau, Norderstdt, Germany) (Donath and Breuner, 1982). Surfaces were polished and sputter coated with 10nm of gold for SEM analysis. While 12 patients initially took part in the investigation, only 4 blocs were allotted for SEM and TEM analysis, while the remainders were intended for another study.

## **3.3 Focused Ion Beam Microscopy**

Samples for transmission electron microscopic study were prepared by a novel focused ion beam technique and *in-situ* lift out method (Jarmar et al., 2008). A Zeiss NVision 40 dual-beam FIB equipped with a 30kV gallium ion column, FEG SEM, carbon gas injector system (GIS), and Kleindiek probe drive system was used. Areas of apparent bone-implant contact were selected for preparation. A layer of carbon, approximately 1µm thick, was deposited in a rectangle 30x2µm to protect the underlying surface from ion-implantation damage. Trapezoidal shaped trenches were milled on either side of the carbon deposit to a depth of approximately 11µm using a beam current of 30nA. The resulting lamella was cut free underneath and on one side using a beam current of 6.5nA. The tungsten lift-out probe was attached to the top of the lamella by carbon deposition. The final side of the lamella was cut free and the sample lifted out insitu. A TEM grid was inserted into the FIB chamber, and the lamella was attached to the side of the grid using C deposition. Finally, the sample was thinned to electron transparency using beam currents from 1.5nA down to 40pA.

## **3.4 Scanning Electron Microscopy**

Preliminary scanning electron microscopy investigation was performed with a JEOL 7000F FEG SEM operated at an acceleration voltage of 15kV. Backscattered electron SEM analysis enabled the identification and quantification of bone-scaffold contact.

## **3.5 Transmission Electron Microscopy**

Transmission electron microscopy was performed on a FEI Titan 80-300 transmission electron microscope (FEI Company, Eindhoven, The Netherlands) equipped with a Schottky field-emission gun and a CEOS hexapole-based aberration corrector for the image forming lens. The microscope was operated at an acceleration voltage of 300kV.

## 3.5.1 High-Angle Annular Dark-Field STEM

Images were obtained using a high-angle annular dark-field detector to enhance Zcontrast compared to bright field imaging (Williams and Carter, 1996). The microscope is fitted with a Model 3000 in-column high-angle annular dark-field detector (Fischione Instruments, Pennsylvania, USA) for imaging in STEM.

#### 3.5.2 Energy Dispersive X-Ray Spectroscopy

Elemental maps and line profiles were collected using an Oxford EDXS detector and Inca software in STEM-mode. Acquisition times varied between 20min to 1h.

#### 3.5.3 Electron Tomography

The Advanced Tomography Holder Model 2020 (Fischione Instruments, Pennsylvania, USA), specially designed for tomographic series collection, was used with the sample interface aligned parallel to the tilt axis. Automated focusing, image shift, and acquisition of a single-axis tomographic tilt-series were achieved using the Inspect 3D (FEI Company, Eindhoven, The Netherlands) software. A linear tilt scheme was used with image acquisition increments of  $2^{\circ}$  up to tilt angles of  $\pm 60^{\circ}$ , and  $1^{\circ}$  for further angles up to  $\pm 75^{\circ}$ . Images were recorded between  $-74^{\circ}$  and  $+71^{\circ}$  on the HAADF detector.

The <u>3D</u> reconstructions were computed using a simultaneous iterative reconstruction technique with 20 iterations in Inspect3D (FEI Company, Eindhoven, The Netherlands). Models for 3D visualization were created in Amira Resolve RT FEI edition 4.1.2 (Visage Imaging) by guided segmentation.

## 4 Results

## 4.1 Scaffold Composition

XRD analysis, shown in a previous study, confirmed the presence of mainly tetragonal zirconia, with small amounts transformed to monoclinic when the material was crushed during sample preparation (Malmström et al., 2009). The hydroxyapatite scaffolds contained minor amounts of  $\beta$ -TCP. Grain size was measured in TEM (Figure 10) to be approximately 1.2µm and 390nm for hydroxyapatite and zirconia scaffolds, respectively. Drastic changes in grain size after implantation were not observed.



Figure 10. TEM micrographs of the a) hydroxyapatite and b) zirconia scaffolds prior to implantation.

## **4.1 SEM Evaluation**

All surgical sites healed uneventfully. Bone overgrowth was noted in a few samples. Light microscopy was performed in detail in a previous study (Malmström et al., 2009). Morphometry indicated a significantly higher bone area and bone-to-scaffold contact for HA scaffolds compared to ZrO<sub>2</sub>, and no presence of an intervening fibrous structure (Malmström et al., 2009). Similar conclusions were drawn using backscattered scanning electron microscopy.



Figure 11. Low magnification backscattered electron micrographs showing intervening bone around a) hydroxyapatite and b) zirconia scaffolds.



Figure 12. High magnification backscattered electron micrographs showing intimate bone contact with a) hydroxyapatite and b) zirconia scaffolds. Rectangular boxes denote the location FIB samples were prepared from.

### 4.1.1 Hydroxyapatite-Bone Interface

The hydroxyapatite scaffold-bone interface is shown in Figure 11a). The intervening bone structure can be seen surrounding and in direct contact with the implant. SEM was useful for noting bone growth into the macroporous channels of the scaffold, a strong indication of the osteoconductive property of hydroxyapatite.

Figure 12a) displays a higher magnification backscattered SEM micrograph of a region showing intimate bone-implant contact. A slight difference in contrast is visible between some micropores in the bulk of the sample and near the surface. This result suggests that bone growth has permeated into the microporosities, which will be confirmed with the use of TEM. The rectangle on the micrograph indicates the region from which focused ion beam preparation of a TEM sample was carried out.

#### 4.1.2 Zirconia-Bone Interface

The zirconia-bone interface shows some similarities to the hydroxyapatite-bone interface. A small amount of bone growth surrounding the implant is shown in Figure 11b). However, unlike hydroxyapatite, in the case of zirconia no bone growth was noted in the macroporous channels of the scaffold.

Figure 12b) indicates a region of good implant-bone contact at a higher magnification. In this micrograph, the rectangular outline again represents the region selected for FIB sample preparation.

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## 4.2 Sample Preparation by Focused Ion Beam

Samples for TEM analysis were prepared from the selected regions shown above, as well as similar sites on other samples not shown. The procedure outlined previously, was followed and resulted in successful TEM sample preparation in almost all cases. An example of the successful preparation of a hydroxyapatite bone sample is shown in Figure 13. The final sample is visibly electron transparent under the SEM in the FIB unit, and is of an approximate thickness of 70nm.



Figure 13. FIB preparation method for TEM samples. From top left to right: deposition of a protective C layer, ion milling of trenches, in situ sample lift out with micromanipulator and final thinned lamella on TEM grid.

Table 2 indicates the samples successfully prepared using the FIB technique. Preparing a TEM sample from the zirconia-bone interface at the 3-month time period was not possible due to insufficient bone contact and bonding. Upon milling, breakage at the interface and incomplete bone contact were revealed. In addition, all samples were prepared from the exterior of the implant-bone interface. Although bone growth was present in the macroporous channels of hydroxyapatite scaffolds, direct and intimate contact necessary to prepare a FIB sample was not maintained during implant extraction and fixation.

	Native	Material	3-m Impla	onth ntation	7-month Implantation	
	HA	$ZrO_2$	HA	$ZrO_2$	HA	ZrO <sub>2</sub>
FIB successful?	30-3		3>>	**	3++	3+

Table 2. Samples successfully prepared with FIB method.

## **4.3 TEM Evaluation**

### 4.3.1 Zirconia-Bone Interface

HAADF STEM images were collected from the bone-zirconia interface after 7months implantation in the human maxilla. An example of the interface is shown in Figure 14.



Figure 14. HAADF micrograph of the zirconia-bone interface.

The instability of the interface resulted in the production of a rather thick FIB sample, as such; collagen banding in the bone structure is only visible in a small region of the bone. In the absence of an interfacial apatite layer, as expected with zirconia, bone forms a direct contact with the scaffold. This result is demonstrated by performing an EDXS line-scan across the  $ZrO_2$ -bone interface. The scan, shown in Figure 15, yields a dramatic drop in Ca and increase in Zr content at the interface, confirming the absence of an interfacial apatite layer.



Figure 15. a) HAADF STEM image of the cortical bone-ZrO<sub>2</sub> scaffold interface implanted for 7-months, and b) the corresponding EDXS line-scan profile. The lack of a concentration gradient over this interface indicates the absence of an interfacial apatite layer and confirms the direct bond of ZrO<sub>2</sub> to bone.

Lack of intimate bone-implant contact prevented the preparation of a zirconia sample at 3-months.

## 4.3.2 Hydroxyapatite-Bone Interface

#### 4.3.2.1 Apatite Layer Evolution with Time

In hydroxyapatite scaffolds implanted for 3-months, images reveal the *in vivo* formation of an interfacial apatite layer that exhibits intimate contact with bone along the interface region, shown in Figure 16. According to the proposed *in vivo* mechanisms of hydroxyapatite dissolution-reprecipitation and a previous study in the rabbit model, it is expected that the interfacial apatite layer spans the entire surface.



Figure 16. HAADF STEM image of the cortical bone-HA scaffold interface implanted for 3-months. An interfacial apatite layer, 80nm in thickness, is indicated by the arrow. Collagen banding is observed perpendicular to the interface.

A similar apatite layer is noted in some regions in the 7-month sample. However, indications of apatite resorption and replacement with bone are denoted in Figure 17.



Figure 17. HADDF STEM image of the cortical bone-HA scaffold interface implanted for 7-months. Region a. denotes an interfacial apatite layer 50nm in thickness. Regions b. mark the interface without an interfacial layer. Collagen banding with 67nm periodicity is clear in the bulk bone and at the interface regions absent of interfacial apatite. Region c. denotes the arrangement of collagen banding parallel to the arrow. Evidence of effective bone ingrowth and implant fixation is further demonstrated by the detection of bone growth into micropores, seen in Figure 18 and confirmed by FIB cut and view (not shown), up to 10µm from the surface in both HA samples. Figure 18 is also an excellent example of collagen banding in bone; the banding is visible perpendicular to the scaffold surface.



Figure 18. HAADF STEM image of the HA scaffold implanted for 7-months. Bone growth into the micropores is observed and collagen banding is visible perpendicular to the scaffold surface

A closer look at the bone in micropores, Figure 19, indicates a similar structure to that of bone at the exterior of the implant. The interconnected nature of the micropores enables this bone growth to reach into the scaffold.



Figure 19. HAADF STEM image of a micropore in the hydroxyapatite scaffold implanted for 7-months. Bone growth is noted inside the micropore.

## 4.3.2.2 Elemental Analysis of Interfacial Apatite

Energy dispersive x-ray spectroscopy line-scans across the HA scaffold-bone interface at 3 and 7-months reveals a composition gradient, confirming the existence of an interfacial apatite layer richer in Ca and P closer to the scaffold surface. The increase in C content away from the interface indicates both apatite formation of a carbonate-substituted form and the presence of collagen moving into bone. Compositional analysis on growth in micropores yields similar results to bone in bulk.

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Figure 20. HAADF STEM image of the cortical bone-HA scaffold interface implanted for 7-months, a), and accompanying EDXS line scan profile, b), showing a gradual decrease in Ca and P, and increase in C concentrations across the interface, confirming the presence of an interfacial apatite layer.

## 4.3.2.3 Three-Dimensional Structure of the Interface

To demonstrate the power of Z-contrast electron tomography, a reconstruction of the interface between a hydroxyapatite scaffold implanted for 7-months and human bone was carried out. A single-axis tomographic tilt-series was collected over the bone-scaffold interface and using back projection, with a simultaneous iterative reconstruction technique, a three-dimensional reconstruction of the section was created.

The complete tilt-series represents images obtained over an angular range of 146°. A selection of these images is shown in Figure 21, while the complete tilt-series can be viewed on the attached CD.



Figure 21. Images from the tilt-series obtained at approximately a) 0, b) -20, c) -40, and d) -60 degree tilt. The arrow indicated the collagen banding visible perpendicular to the surface, a- bone, b- interfacial layer and c-HA scaffold.

The tilt-series illustrates both the characteristic collagen banding of bone (Hodge and Petruska, 1963), with 67nm periodicity perpendicular to the scaffold surface, and the dense interfacial apatite layer formed *in vivo*. The reconstructed volume represents a section of the tilt series measuring 320nm x 260nm x 70nm, and is shown from different angles in Figure 22. The complete reconstruction showing both volumetric slicing and surface rendering is also available on the attached CD.



Figure 22. Volumetric reconstructions of the HA scaffold-bone interface from two views, (a) and (b). The reconstructed volume measures 320nm x 260nm x 70nm, with the bone and the interfacial region represented by purple, on the left, while the hydroxyapatite scaffold is yellow, on the right. Notice the clear difference in orientation of crystals closest to the interface and further into the bone.

The reconstructed 3D volume clearly reveals the distinct orientation of HA crystallites in the fibrous bone structure and in the dense interfacial layer. The HA crystals in the bone are aligned parallel to the scaffold surface, while the HA crystals of the precipitated apatite layer appear to be strongly oriented perpendicular to the scaffold surface. This distinct feature could not be deduced directly from the individual images; only with the aid of tomography and the 3D reconstruction is this characteristic visible. A schematic diagram summarizing the orientations of HA crystals is depicted in Figure 23.

![](_page_51_Figure_2.jpeg)

Figure 23. An illustration of the human bone-scaffold interface indicating the differences in hydroxyapatite crystal orientation visible with electron tomography. Crystals in the bone are oriented with their long axis (c-axis) parallel to the scaffold surface, while crystals in the interfacial apatite layer have their c-axis oriented perpendicular to the scaffold surface.

## 5 Discussion

## **5.1 Sample Preparation**

Use of the focused ion beam for sample preparation was critical for site-selective preparation and analysis of the scaffold-tissue interface. Precautions to avoid ion beam damage were taken such as sequential reduction in ion beam current. FIB-induced damage is not suspected as structures resemble those in the literature prepared by FIB and other methods where the collagen banding is also observed (Jantou et al., 2009, Giannuzzi et al., 2007). Challenges associated with preferential milling of bone versus hydroxyapatite and zirconia presented themselves. In most cases, extra milling was required on HA and  $ZrO_2$  to fully remove it. Separation of bone from the scaffold interface, whether due to specimen retrieval or processing, prevented high-resolution analysis of scaffold-bone interfaces for the  $ZrO_2$  sample at 3-months and inside the square-shaped macroporous channels for all samples.

### **5.2 Role of Materials Selection on Bone Response**

The scaffolds were analyzed with a variety of electron microscopy techniques to determine their bone-bonding performance *in vivo*. HA samples at both 3 and 7-months exhibit an interfacial apatite layer. The proposed mechanism for apatite formation on hydroxyapatite is a dissolution-reprecipitation sequence (Ducheyne and Qiu, 1999). Apatite layers as thick as 1000nm have been reported and precipitation of crystallites

observed in as little as 3h post-implantation (Porter et al., 2002, Bruijn et al., 1995). In this investigation, layers of 80 and 50nm thickness are reported. The absence of formation of a dense apatite layer for zirconia samples may be due to the inert chemical nature of the material. Zirconia, known to be a bio-inert material, has limited interaction in the body (Akagawa et al., 1993). Results for both materials are in agreement with surface response observed around the scaffolds implanted in rabbit tibia in a previous study (Malmström, 2007).

The addition of microporosity to scaffold materials has been reported to enhance the bioactivity of bone substitutes (Malmström et al., 2007, Hing et al., 2005). These hydroxyapatite scaffolds in particular displayed increased bone ingrowth and bone contact with a microporous structure versus those fabricated without microporosity (Malmström et al., 2007). It is clear that the pores show an affinity for bone growth. Detection of bone in micropores demonstrates the open network of pores in the scaffolds and migration of bone forming cells into the structure, which is essential for improving bioactive fixation of the implant. It is interesting to note the absence of an interfacial apatite layer and collagen-banding structure in HA detected in the micropores. Instead, the HA exhibits a fibril structure, which was also seen in the rabbit model (Malmström, 2007). Bone growth located at the exterior implant interface exhibits the standard 67nm banding periodicity typical of cortical bone (Hodge and Petruska, 1963). The origin of the lack of collagen banding in micropores is unknown. It can be speculated that the bone in micropores is woven bone, which formed rapidly and without time for organization in the lamellar structure seen in more mature bone.

While the hydroxyapatite scaffolds showed indications of osteoconductivity at all stages of the electron microscopic study; such as bone growth along the implant surface, and into both macro and microporosities, the zirconia scaffolds presented differently. Although TEM indicated an intimate contact between bone and the zirconia scaffold, the lack of bone contact overall prevented a more thorough investigation on the nanometer scale. In addition, the zirconia scaffolds did not induce bone growth into the macroporous channels.

Maintaining a constant scaffold size and pore geometry enabled the direct comparison of bone behavior in the vicinity of hydroxyapatite versus zirconia scaffolds for bone regeneration. While neither scaffold resulted in a negative reaction such a fibrous encapsulation, the hydroxyapatite scaffold exhibited characteristics of osteoconductivity.

## **5.3 Evolution of Implant-Bone Interface with Time**

Ideally, scaffolds for bone regeneration should be comprised of resorbable materials, those that naturally dissolve and are replaced by bone growth (Hench, 1998). Evidence of the initiation of resorption is seen in hydroxyapatite samples implanted for 7-months. The average thickness of the interfacial apatite layer decreased from the 3 to 7-month sample. This result indicates the resorption of the apatite layer and its replacement by natural bone tissue with increasing implantation time. Other indications of resorption are the rough interface and lack of interfacial layer in areas along the interface, which are key features of enhanced biocompatibility and integration. Furthermore, the apparent resorption of apatite layer and direct growth of bone into the scaffold appears most

prominent in regions adjacent to grain boundaries, as demonstrated in Figure 17. This may be caused by the increased dissolution rate of HA at grain boundaries.

## 5.4 Three-Dimensional Analysis of Implant-Bone Interface

The formation of an interfacial apatite layer on hydroxyapatite is known to be governed by a dissolution reprecipitation mechanism resulting in carbonated HA growth. However, there are controversial opinions on the arrangement of apatite particles during formation. Analytical techniques such as electron and x-ray diffraction, as well as imaging with high-resolution TEM have suggested both preferred and non-preferred orientation of crystallites on the HA surface (Daculsi et al., 1990, Daculsi et al., 1989, Fujita et al., 2003, Jarcho, 1981). Previous TEM investigations have exhibited crystal growth perpendicular to the surface of the original material (Daculsi et al., 1990). Other observations involving atomic resolution imaging have even suggested the possibility of epitaxial growth of apatite on HA (Fujita et al., 2003). It certainly appears from our reconstructions that a preferential orientation of precipitated hydroxyapatite particles exists perpendicular to the scaffold surface.

The origin of the orientation differences between HA crystallites present in bone and those formed in the interfacial apatite layer is not well understood. It can be speculated that the disparity in orientation results from differences in cellular versus solutionmediated hydroxyapatite formation (Ducheyne and Qiu, 1999). Due to its small size, diffraction over the interfacial apatite layer to confirm its orientation in relation to the scaffold is not possible. However, the reconstruction gives us a lot of information not attainable if considering only the 2D projections formed in HAADF STEM images. M.A.SC. THESIS • K. GRANDFIELD • MCMASTER • MATERIALS SCIENCE & ENGINEERING 2010

Information regarding the morphology and arrangement of HA crystallites at the interface between hydroxyapatite and bone was extracted from both the tilt-series and tomographic reconstruction. In bone, the HA crystals are aligned such that their long axis is parallel to collagen fibers. Tomograms from calcified tendons have contributed to understanding this arrangement of collagen and HA mineral (Landis et al., 1993). In this study, the arrangement corresponds to the c-axis of HA aligned parallel to the scaffold surface. This result, demonstrated clearly in the tomogram, is consistent with the orientation of the collagen-banding present in the tilt-series. The banding, shown perpendicular to the scaffold in the tilt-series, confirms arrangement of collagen, and therefore HA in bone, parallel to the scaffold surface.

## 6 Summary, Conclusions & Future Work

The performance of bone regenerative scaffolds depends strongly on interfacial interactions. Synthetic hydroxyapatite and zirconia scaffolds, produced with interconnected macro and microporosity by free-form fabrication, were evaluated in the human maxilla. Sample preparation using the focused ion beam technique enabled the investigation of the nanometer scaled region at the scaffold-tissue interface. Scanning transmission electron microscopy revealed the development of an interfacial apatite layer on HA scaffolds in vivo, confirming the formation of bioactive fixation. A concentration gradient exists across this interfacial layer, suggestive of its development by a dissolution-reprecipitation mechanism. Indications of resorption were noted with increasing implantation time. In addition, extensive bone growth into microporosities indicates the great potential of hydroxyapatite scaffolds as a bone regenerative material. Zirconia scaffolds, on the other hand, showed a direct contact to bone in the absence of an interfacial apatite layer. Since scaffold geometry and macropore size morphology and volume were identical, the differences in *in vivo* response can be attributed to the material chemistry and microporosity.

The ability to visualize biointerfaces in three-dimensions has vast implications for the field of life sciences. Shifting from two to three-dimensional imaging has, for example, allowed us to better understand the relationship between hydroxyapatite scaffolds and bone. Indeed Z-contrast electron tomography as a characterization technique is applicable to all forms of biointerfaces. This work has presented the tomogram from a ceramic-bone interface, however STEM tomography can easily be extended to the study of other biointerfaces involving metals, polymers and natural materials. Acquiring 3D structural information promotes tailored implant design, better functionality and ultimately improved patient quality of life. The feasibility of using STEM tomography for the three-dimensional structural analysis of biointerfaces at the nanometer scale has been demonstrated.

While Z-contrast electron tomography provides additional interfacial information not visible in images, it does raise more questions about the behavior of scaffolds *in vivo*. Tomography confirmed the preferred orientation of hydroxyapatite crystals in the interfacial apatite layer, but it does not answer the question of why or how these crystals form with a preferred orientation. Further investigations on a greater number and variety of hydroxyapatite materials *in vivo* may be an interesting study.

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