Methods for evaluation of early bone healing at titanium implants

KARIN HEDERSTIERNA

Institute of Biomedicine
Department of Medical Biochemistry and Cell Biology
The Sahlgrenska Academy

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Karin Hederstierna
Institute of Biomedicine, Department of Medical Biochemistry and Cell Biology, The Sahlgrenska Academy, University of Göteborg, Box 420, SE 40530 Göteborg, Sweden

ABSTRACT

Titanium has for a long time been the implant material of choice when in need for abilities as strength, biocompatibility and stability. Although numbers of studies have been made, there is need for more research on the early events of implant healing.
The experimental procedures included implantation of titanium discs in rat tibia and explantation after different periods of time. This procedure allowed studies by immunocytochemistry of cell division, apoptosis and cell differentiation into osteoblasts. The results show that few cells that adhere to the surface are involved in the activities measured. The experimental procedure was then changed to include explantation in situ, and direct studies of bone formation.
The aim was then to develop methods for evaluating bone formation around implants. We used common histological methods and imaging time-of-flight secondary mass spectrometry (TOF-SIMS) to evaluate the implant healing. With the Bi_3^+ cluster ion source used, it is possible to detect fragments specific of hydroxyapatite (HA) within an area of 40µm from the implant. These results are the first showing the localization of hydroxyapatite in tissue. The mineral content of the tissue/implant interface area may be argued to be important for the functional performance of the implant.
Bone formation and resorption and the relationship between those two during the first weeks have been of interest in this thesis. After 7 days bone was in close contact with the implant but from this day and evident after 14 days bone resorption was seen. The initially formed bone was resorbed and replaced by mature lamellar bone in a process similar to the organization of a callus in fracture healing. Our findings indicate that healing around implants starts primarily in the periphery growing toward the implant. Early callus formation and resorption are crucial steps in these early phases and possibly the net bone production is influenced by these factors. Findings in the fourth paper indicate that magnesium coatings may decrease bone resorption and increase net bone production.

Keywords: TOF-SIMS, titanium, porosity, Magnesium, hydroxyapatite, implant surface, bone resorption, bone formation,

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CONTENTS

List of papers

This thesis is based on the following papers which will be referred to in the text by their Roman numerals.

I. C. Eriksson, H. Nygren and K. Ohlson
Implantation of hydrophilic and hydrophobic titanium discs in rat tibia: cellular reactions on the surfaces during the first 3 weeks in bone
Biomaterials 25(2004) 4759-4766

II. C. Eriksson, K. Börner, H. Nygren, K. Ohlson, U. Bexell, N. Billerdahl, M. Johansson
Studies by imaging TOF-SIMS of bone mineralization on porous titanium implants after 1 week in bone
Applied Surface Sciences 252(2006) 6757-6760

III. C. Eriksson, K. Ohlson, K. Richter, N. Billerdahl, M. Johansson, H. Nygren
Callus formation and remodelling at titanium implants
Journal of Biomedical Materials Research Part A, 2007 Dec 15;83(4):1062-9

IV. H. Nygren , C. Eriksson, K. Hederstierna, P. Malmberg,
TOF-SIMS analysis of the interface between bone and titanium implants – effect of porosity and magnesium coating
Applied Surface Sciences, in press, doi: 10.1016/j.apsusc.2008.05.143

Abbreviations

AES	Auger Electron Spectrocopy
ALP	Alkaline Phosphatase
BMP-2	Bone Morphogenetic Protein type 2
DABCO	1, 4-diazabicyclo-[2, 2, 2]-octane
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extra cellular Matrix
FDA	Fluorescein Diacetate
FGF	Fibroblast Growth Factor
HA	Hydroxy Apatite
IL	Interleukin
MMP	Matrix Metalloproteinase
PBS	Phosphate Buffered Saline
PDGF	Platelet-derived Growth Factor
PI	Propidium Iodide
SEM	Scanning Electron Microscope
TG	Transglutaminase
TGF	Transforming Growth Factor
TOF-SIMS	Time-of-flight Secondary Ion Mass Spectrometry
VEGF	Vascular Endothelial Growth Factor

Introduction

At the time this study was initiated, there was much discussion about the mechanism of titanium implant healing and about strategies for improved healing, both with regard to healing rate and endurance. A common view was that osteoblasts could spread on the implant surfaces thus mineralizing the tissue/implant interface. Influential researchers like Davies, [1, 2] have coined terms like " bone conducting capacity" and "guiding", which means surface properties optimized to "guide" the bone cells at the surface. This is still a subject of investigation and recently published articles demonstrate the existing thoughts around this matter [3-5]. In these studies *in vitro* technique is frequently used. The bone cells have been extracted and the cell-implant interaction has been isolated.

In previous studies, our group had studied the early phases of implant exposure to blood, looking at selection of blood proteins and blood cells at the implant surface and its dependence on implant properties. The first paper of the present thesis was a continuation of that project and was made in order to detect dividing cells, apoptotic cells and cell differentiation in the granulation tissue to osteoblasts as a function of material properties. The strategy differs from that of most other studies on osteoblast reactions with implant materials where cultured cells are exposed to the material surfaces. The results were quite disappointing in that few cells were seen on the surfaces, few of the cells present showed signs of differentiation and areas devoid of cells were common at time points of healing longer than 4 days. The paper was still published in order to emphasize the need to make studies in vivo and follow cell interaction with the material during healing.

The second paper was initiated in order to detect mineralization of the tissue adhering to implants. Mineralization, the endpoint of osteoblast differentiation, is a natural measure of implant healing and is often evaluated by light microscopy. In our second paper, imaging mass spectrometry was used to detect mineralization of the implants during healing into rat tibia. With the Ga primary ion source, used in this study it is only possible to localize small fragments of hydroxyapatite, like Ca, CaO and CaOH, which can not be claimed to be specific for the mineral. These species were evenly distributed over the surface. However, by normalizing to the distribution and intensity of Ti ions, it was possible to see differences in the content of Ca-containing species of the implants used. The experimental system based on harvesting of implants, used in the two first papers of the thesis will always contain a certain risk for loss of tissue during the explantation. Our results also indicate that such loss increase with time. Therefore, we changed the technique to in situ analysis in the last two papers.

In paper III, light microscopy was used to detect bone formation around implants with time. Bone incisions without implants were used for comparison with implant healing. This simple strategy enabled us to detect early mineralization of the bone marrow cavity, stemming from the endosteum. We also found that the callus formation seen after one week of healing was reorganized and resorbed within the following weeks. There were definite similarities between the callus formation in the bones with incisions only, and the bones with implants. The mineralization of the interface between tissue and implant was again interesting in the in situ model, and in the meantime between the publication of paper II and III, new primary ion beams for imaging ToF-SIMS had been developed, making possible detection of hydroxyapatite in tissues.

The principle of SIMS was first invented in the late 40's and further developed in the early 60's. Reviews on the early development of SIMS can be found in [6, 7].

Time-of-flight (ToF) refers to the type of mass analyses used in most organic SIMS instruments. Imaging SIMS can also be performed using other mass analyses e.g. quadropoles [8], but most results published have been obtained with ToF-SIMS instruments, first described in 1981 [9].

Monoatomic ion sources, like Cs^+, Ga^+ and In^+, have been used in imaging TOF-SIMS, all sharing the properties of well focused beam spots, giving a spatial resolution of 100-200 nm. However, the high energy impact of the primary ions on biological samples results in poor yield of high-mass secondary ions, complicating the identification of the original molecule [10]. The possibility of using monoatomic ion sources in biological applications has been excellently reviewed [8, 11, 12] .

The introduction of ionization promoters in the samples improved the signals of some analytes, especially that of cholesterol [13]. Surface modifications, including matrix-assisted SIMS and metal-assisted SIMS do enhance the yield of secondary ions in SIMS,[14], but also include a preparation step that may introduce new sources of artefacts. Given the successful development of polyatomic primary ion sources, the benefit of using ionization promoters is declining.

The ability of polyatomic ion sources to increase the yield of secondary ions, especially of high-mass ions, was first reported in 1989 [15]. A buckminsterfullerene (C60) - based primary ion beam system has been developed for TOF-SIMS analysis of organic materials. The resulting yield efficiencies were reported to be 30-100 times those observed from gallium sources. The C60 source was also reported to favour the formation of high-mass secondary ions [16]. The C60 ion source can also be used for depth profiling [17] i.e. 3D imaging of

organic layers and cells [18]. The ion beam of the C60 ion source, as reported [16], may be focused to a spot with a diameter of 3 microns, giving a practical image resolution in the range of 3-9 microns i.e. low resolution imaging. In a comparative study, using 8 different ion sources, the possibility of using Bi_n clusters was presented [19]. As a general result the efficiency of the secondary ion yield was found to be improved with the mass of the monoatomic ions. A further increase was found with the use of polyatomic ions. According to this, highest efficiencies were obtained for C60, the lowest for Ga. The results reveal the potential of cluster SIMS to overcome existing limitations and to establish ToF-SIMS for new applications in the fields of biology and medicine [19]. The ion beam of the Bi-cluster ion source may be focused to a spot with a diameter of 300 nm, giving an image resolution of 300-500nm, which is within the range needed to allow analysis at subcellular resolution. During the last two years, our group has established the analysis of hydroxyapatite with ToF-SIMS [20].

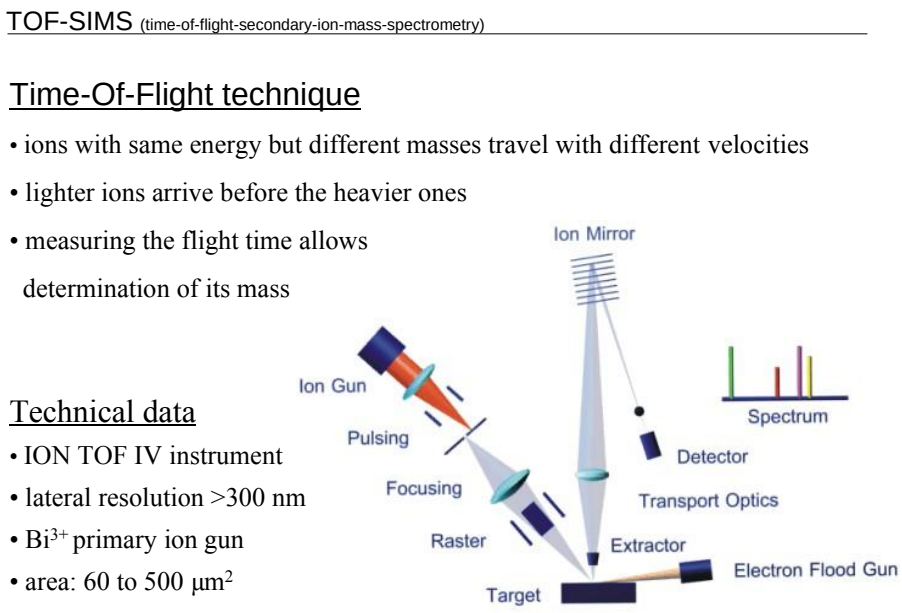


Figure 1. Principle of the TOF-SIMS instrument. By the courtesy of ION-TOF, Germany

The last paper thus describes the analysis of mineralization of the interface between granulation tissue and implant. We now know that the bone is formed from the periphery and approaches the implant. We have developed strategies for quantitative and comparative analysis of tissues and used the technique for studies of the effect of magnesium on bone healing. With modern methods, we are now back to old issues in implant research.

Grove, after a great series of experiment on different animals over a long period of time, macroscopically observed that nickel-plated steel had no irritating effect on bone and that magnesium was rapidly absorbed and acted as a powerful stimulant to bone formation. He also noted that the tissue readily tolerated indifferent aseptic foreign bodies. [21]
1924 Zierold wrote about reactions of bone to various metals and his paper was based on a quite extended experimental series on dogs. The prospect of the study was to determine whether metal per se, when implanted in bone, exerted an influence other than that of any foreign body, and if so, whether that was a property common to many metals or varying with each individual. He used 63 dogs in five different series of experiments and investigated reactions between bone and metal in tibias, ribs, femurs and part of the skull. The anatomical parts chosen because of there developmental and remodelling differences. The metals implanted in femurs were implanted via the joint, trying to evaluate early toxic and stimulant effects in this sensitive medium. Observation time ranged from two to six weeks. As evaluation methods he used x-ray and histological technique, the latter improvised, with focus on the relationship between the various parts of the bone. As a result, the recorded findings merely enumerated the general classification of marrow cell types without attempting a finer division, related to difficulties in preparation. He found that gold, aluminium and stellite (an alloy including tungsten, cobalt and chromium) were well accepted by the tissue[22].
However, it took until 1940 when Bothe et al, for the first time used titanium as an implant material. As experimental animals he used cats, in which femurs he vertically inserted implants. In each bone he inserted 3-4 implants from different metals with the idea that electrolytic action might be exaggerated and to see whether this be of major importance for the healing process. No conclusion that the electrolytic activity influenced the bone-implant healing could be drawn, but he found that titanium was even better than the non-corrosive alloys used before in that titanium was seen to grow in contact with the bone[23]. In the article “Titanium, a metal for surgery”, Leventhal for the first time more seriously investigated titanium as an implant material. This was related to the conclusions that vitallium and stainless steel, even though rather well accepted by the tissue, did not act satisfactory for the fixation of fractures due to unexpected losses and even breakage of screws and plates. Especially the stainless steel plates also were known to need handle with great care because of a tendency to produce eddy currents in the tissue if even the slightest nicked. Until the 50s the titanium was difficult to extract for commercial purposes, but with time extraction of the metal was being carried out more extensively, ironically for use in the war industry. (In fact the titanium used for these experiments were submitted by Remington Arms Co.) The

experiments by Leventhal were conducted on rabbits and rats where the rabbits were used to study soft-tissue reaction through implanting titanium bars into the subcutaneous tissue. The tissues then were examined after 2, 4, 6, 8 and 10 weeks. Not at any time investigated, there were signs of indurations and no reaction to the metal was found. Bone reaction was studied by the insertion of screws into the femora of rats. The animals were sacrificed at 6, 12 and 16 weeks. In no animal there was any infection, indurations or discoloration. At the end of six weeks, the screws were slightly tighter than when originally put in; at twelve weeks, the screws were more difficult to remove; and at the end of sixteen weeks, the screws were so tight that in one specimen the femur was fractured when an attempt was made to remove the screw. Microscopic examinations of the bone structure revealed no reaction to the implants. The trabeculation appeared to be perfectly normal. As a conclusion Leventhal found that titanium would be ideal as implant material thanks to its capability to grow in contact with the bone, its strength and its failure to cause tissue reactions[24]. Today titanium is widely used as implant material, thanks to its good capability of *osseointegration,* which means the capacity to make intimate contact with bone. During the 60s and 70s experiments with different porosities were made and both porous stainless steel and steel coated with porous titanium was introduced. The exploitation of titanium as an implant material at great length started in Gothenburg during the 70s with Branemark and his colleagues' work. The dental implants developed by Branemark are famous and used globally and the technique has become state of the art in how to work with titanium[25-29]. Recently, gene-expression of different adhesive proteins related to bone healing has been studied during the early healing of bone defect and bone-implant interface in animal experiments. The gene-expression of fibronectin, collagen I, bone sialoprotein II and osteopontin in non-implant and bone-implant defects were examined using semi-quantity reverse transcription-polymerase chain reaction. All four proteins peaked at 8 days, indicating that the gene-expression of the four adhesive proteins is different between bone defect and bone-implant interface and that intracellular synthesis of these proteins are accelerated during the early healing stages of the bone-implant interface[30]. Besides titanium different ceramics, plastics, rubber and liquids are used as implants today. An expanding research field concerns the usage of biodegradable polymers, which means implants that gradually break down without leaving marks in the tissue [31-39]. This could lead to a new chapter within biomaterial history.

Bone tissue

Bone is essentially a highly vascular, living, constantly changing mineralized connective tissue. It is remarkable for its hardness, resilience and regenerative capacity, as well as its characteristic growth mechanisms. Like all other connective tissues, bone consists of cells and an intercellular matrix, the great majority of its cells, osteocytes, lying embedded within it. The matrix is composed in part of organic materials, mainly collagen fibres. The rest consists of inorganic salts rich in calcium and phosphate. Together these give bone its unique mechanical properties.

Vascular canals ramify within bone, providing its cells with metabolic support and creating avenues of entry for other cells, including osteoclasts, capable of removing bone, and osteoblasts which deposit it. While these features are found in all bone, their details differ widely with developmental state, site, prevailing mechanical forces and the metabolic state of the body. The collagen framework of bone, permeated with mineral salts, varies from almost randomly orientated coarse bundles (woven bone) when young, to a system of highly ordered, parallel-fibred sheets or lamellae (lamellar bone) in the mature condition. Collagen fibres and mineralized matrix together usually form minute cylinders (osteons) arranged concentrically around blood vessels both in woven and lamellar bone while, in the mature state, the inner and outer surfaces of bones are lined by a few layers of continuous circumferential (outside) and endosteal (inside) lamellae. The outer surface of bone is always lined by a fibrocellular layer, the periosteum, and on the inner surface is a similar, though thinner layer, the endosteum. In these layers lie osteoblasts, osteoclasts and other cells important in the biology of bone. The texture of mature bone also varies between dense (compact) and spongy (cancellous) osseous tissue which has distinctive mechanical and metabolic roles, often related to their positions within bones. Developmentally, bone may form either by the direct transformation of condensed mesenchyme (intramembranous bone) or be receded by a cartilage model which bone later replaces (endochondral bone). However, bones of different origins may show any of the features mentioned above, and can only be distinguished by a study of their genesis.

Macroscopically, living bone is white, with either a dense texture like ivory (compact bone), or a honeycombed texture with large cavities, the bone being reduced to a latticework of bars and plates (trabeculae), in which case it is called *cancellous, trabecular* or *spongy* bone. The compact bone is usually limited to the cortices of mature bones, called cortical bone. It is of

supreme importance in providing strength and differs in thickness and architecture depending on where it is located. Cancellous bone on the other hand, lies chiefly in the interior and particularly, in the case of long bones, within their expanded ends (metaphyses and epiphyses). Cancellous bone gives additional strength to cortices and supports the bone marrow. The proportions between compact and cancellous bone vary greatly. In the shaft of a long bone, a thick cylinder of compact bone presents only a few trabeculae and spicules on its inner surface so that a large central medullary or marrow cavity is enclosed, communicating freely with the intratrabecular spaces of the expanded bone ends. In other bones, especially flat ones such as the ribs, the interior is uniformly cancellous, compact bone forming the surface. These cavities are filled with marrow, either red, haemopoietic, or yellow, adipose, its character varying with age and site[40].

Bone formation and remodelling

Bone is composed of different types of cells embedded in a stiff calcified matrix. The first bone is seen after about 6 week's in-utero and continuously throughout life bone is remodelling, although the rate of remodelling decreases with age. The term remodelling means that bone continuously are degraded and formed, in the adult individual with the goal to let the bone persist in its original form. During childhood the resorption and bone formation is focused so an increased growth can proceed. This is possible due to the epiphyseal plate, an area where new cartilage matrix is laid down, which acts as a model for the bone cells. The principle is that new bone is formed at one site, the epiphyseal plate, and resorption (and formation) takes place at other sites. The result is that the epiphyseal plate is pushed away from the diaphysis, thus causing an elongation of the bone. The bone growth does not continue over life thanks to a programmed elimination of the epiphyseal plate. This is referred to as epiphyseal closure and is stimulated by the hormone rush during the teens which first stimulates an increased growth and later the closing of the epiphyseal plate.
Primitive mesenchymal cells differentiate into osteoblast precursor cells (osteoprogenitor stromal cells, pre-osteoblasts), which give rise to various other bone cells; osteoblasts which lay down bone; osteocytes within bone; bone lining cells on its surface and osteoclasts which erode it [41, 42]. This gives rise to the formation of matrix and later mineralization. The relation between osteoclasts, a cell type of haematopoietic origin, which degrades bone and the osteoblasts which forms new bone is called bone remodelling - or coupling. The coupling process ensures that an equivalent amount of bone is laid down following the previous

resorption phase. The haematopoietic cells proliferate and differentiate into osteoclasts through a mechanism involving cell-to-cell interaction with osteoblast stromal cells [43, 44]. Next the bone surface is prepared by removal of the unmineralized osteoid layer by the lining of osteoblasts. A variety of proteolytic enzymes, for example MMPs (Matrix metalloproteinases), collagenase and gelatinase are produced [45]. This exposes the mineralized matrix which maybe chemotactic to the osteoclast. The osteoblast also directly stimulates osteoclast activity. During the resorption process growth factors are released from the matrix which then activates osteoprogenitor cells. The osteoprogenitor cells mature into osteoblasts and ultimately replace the resorbed bone. The mechanism by which osteoblasts are directed to form bone only in the resorption lacunae may be due to the presence of molecules such as TGF-β and BMPs which are left behind during osteoclastic activity. The resorption process also inhibits the osteoclast function. Bone remodelling is regulated by systemic hormones and by local factors, which affect cells of both the osteoclast and osteoblast lineages and exert their effects on replication of undifferentiated cells, recruitment of cells and differentiated function of cells. The end product is the maintenance of a mineralized bone matrix and the major organic component of this matrix is type I collagen. Perturbations of this cellular activity, resulting in an imbalance between the activities of the two cell types, are the key element in many bone metabolic diseases, disuse atrophy and microgravity-induced ostopenia [46]. The bone also consists of cells from the vascular and nervous system, as well as other components in the periosteum, endosteum and marrow. The osteoprogenitor stromal cells, osteocytes, bone lining cells and osteoblasts are all closely related compared to the osteoclasts that origin from a haematopoietic cell type.

Healing around implants and normal fracture healing

Healing around implants consists, as normal wound healing as well as normal fracture healing, of different phases; inflammation, soft callus formation, hard callus formation and remodelling. Soft and hard callus formations are collectively equivalent to the proliferative phase of wound healing. The main difference between normal fracture healing and implant-associated wound healing is that in the latter situation the implant material may interfere with the healing process, by its selection of surface-adsorbed plasma proteins and the adhesion of cells to the protein-coated surface. Except for that, healing around implants mimic ordinary fracture healing in that there are both endochondral and intramembranous ossification side by side. Wound healing is initiated by bleeding from the surgical wound. When blood contacts

the implant surface, a rapid adsorption of ions and water and then proteins take place [47, 48]. Since the adsorption is so rapid, cells never encounter the actual implant surface [49]. The protein composition is dependent on surface properties of the implant material, which will lead to certain proteins being preferentially adsorbed on the surface [50-53]. The adsorption event is followed by adhesion of platelets and the initiation of the coagulation cascade, which leads to blood clot formation around the implant and vasoconstriction. The surrounding of the implant is hypoxic and hypoxia has been shown to upregulate the BMP-2, an osteogenic cytokine, at the fracture site [54, 55]. Inflammatory cells are then being recruited to the site of injury which increases blood flow and vasopermeability. Within a few days a fluid phase is found around implants. The fluid contains scattered inflammatory cells proteins, including networks of fibrin. With time the fluid diminishes and disappears as the outer tissue border grows towards the implant surface [56-58]. The inflammatory phase overlaps with the phase of soft callus formation and typically lasts for about 4 days. In this process pro-inflammatory cytokines IL-1 and IL-6 show increased levels of expression[59]. The importance of these factors is illustrated by the delay of callus mineralization and maturation when IL-6 has been knocked out[60]. The soft callus formation, which occupies approximately 3 to 4 weeks develops between and around fragments of bone and contains proliferating preosteoblasts, fibroblasts and sometimes chondroblasts, embedded in a matrix, rich in glycoproteins and collagen, into which new blood vessels grow. At the fracture site the soft callus will form whether or not the fractured parts of the bone are in apposition to each other, either because they were not displaced or because they were set by a physician. The same is seen during early implant healing although there is no splinter. The soft callus acts as a model for the new bone and helps stabilizing and binds together the fractured bone. The most important function for the soft callus during implant healing is to act as a model for the hard callus[61]. Expression of growth factors, such as FGF, PDGF, TGF and VEGF suggest that these may be of importance to the bone healing process. VEGF seems to influence all the subprocesses of bone healing which is supported by studies on the expression of VEGF mRNA during bone healing [62]. VEGF-labelled cells were seen from day 1 and both VEGF and osteocalcin-positive cells were seen at day 5[63]. When osteoclasts resorb bone, the result is the creation of a demineralised bone matrix which becomes the recipient surface for new bone formation. The resorption surface of old bone provides a highly topographically complex surface into which new bone matrix will be deposited, and with which the latter can interdigitate and interlock [61]. Gradually, the soft callus is converted into woven bone through both endochondral and intramembranous ossification. In our own study of bone formation after

drilling holes in rat tibia, Callus formation and remodelling at titanium implants [64], it was shown that new bone is first formed at the endosteum after 4 days. This was seen whether or not implants were inserted in the holes. The immature woven bone was seen to expand from the endosteal origin to fill more than half of the bone marrow volume around the implant after 1 week. The same pattern was seen in drilled holes without implants which rendered the conclusion that the implant has little effect on initial bone healing. After the first week, both cellularity and vascularisation increases and this proceeds after 3 to 4 weeks after injury and continues until attainment of firm bony union. The same general process of bone healing has been described in canine mandibula, where the kinetics of the process was found to be slower than in the rat tibia, with a peak in formation of woven bone after 4 weeks of healing, and resorption and maturation within 8 weeks [65]. Osteogenic buds from the new bone invade the callus and begin to deposit new bone within the callus, gradually replacing the original fibrous and cartilaginous callus with the hard, bony callus[61]. Usually the first soft callus is seen to exceed its normal proportions, but it is later normalized due to the continuous resorption. During this period of time osteogenesis is in progress and there is also osteoclastic activity that continues at the fracture site. This phase is called the remodelling phase and can continue for years. The osteoclasts remove excess bone from the exterior of the periosteal collar if it is a fracture that heals, and remodel its endosteal aspects so that the medullary cavity is restored across the site of the fracture [40]. At the implant site a thin bone capsule is left around the implant indicating an effect of the implant on bone remodelling [66]. Much effort has been spent in attempts to improve the bone healing around implants. Incorporation of Mg^{2+} and surface roughness seems to positively affect bone healing [67]. Corroding Mg^{2+} rods induce an increased bone formation during their corrosion [68]. A significantly higher torque removal values and shear strength between bone and implants has also been seen when including F^- into implants [69].

AIMS OF THE THESIS

To study early (1-28 days) bone healing around implants

To develop methods for evaluating bone healing around implants

To investigate the role of bone formation at the interface zone between implant and bone in the evaluation of implant healing.

To investigate the time sequence of bone remodelling at the implant-tissue interface

To develop tissue preparation methods for TOF-SIMS for measuring hydroxyapatite content in the tissue around implants.

Materials and Methods

Surface Preparation

Titanium discs (machined, not blasted;) were obtained from Elos AB (Timmersdala, Sweden). The discs differed in size between 0,7 mm (paper I) – 1, 0 mm (paper II-IV) in thickness and had a diameter of 2,5 mm.

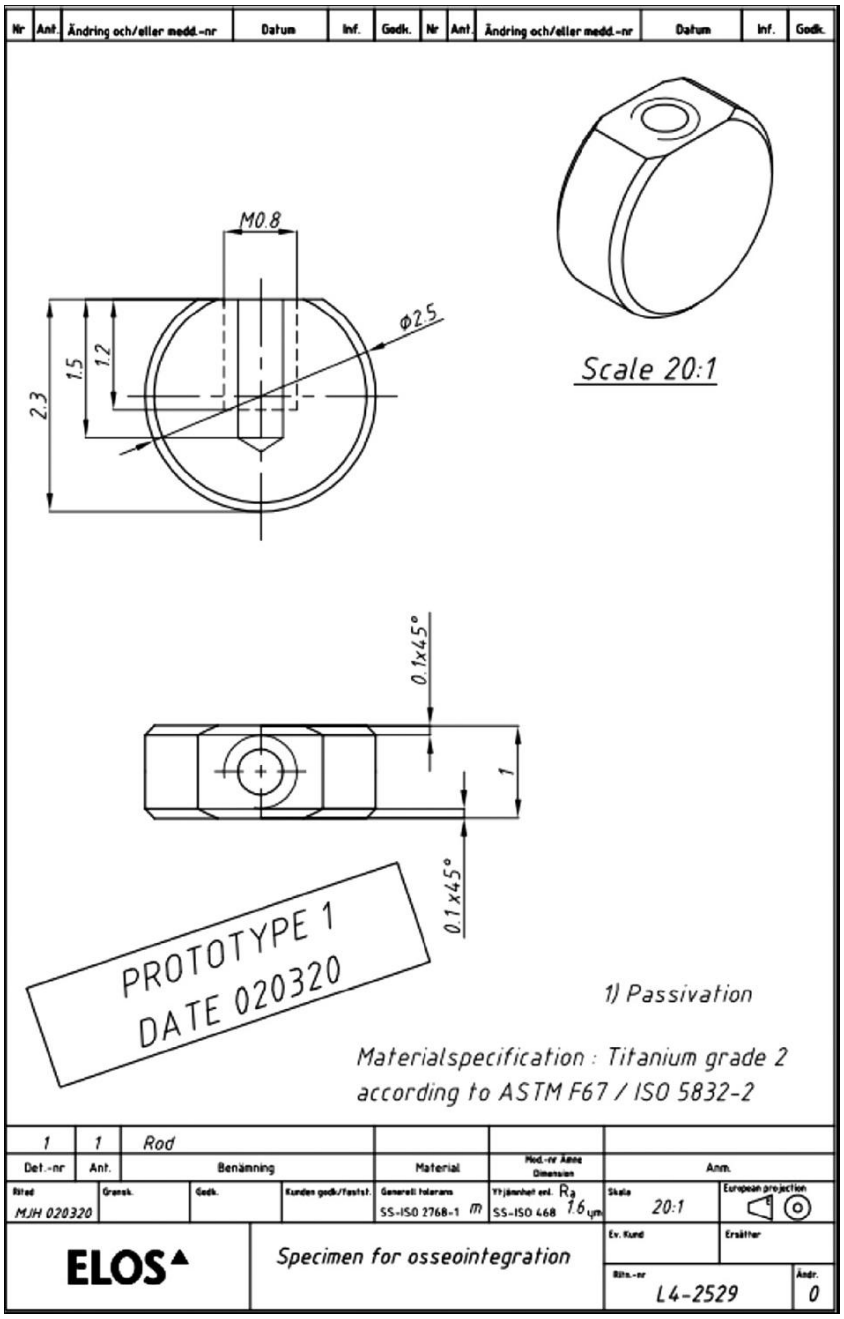


Fig 2. Geometry and dimensions of the titanium discs (machined; grade 2; ISO 5832-2) used in the study. Measurements are given in millimetres. Ra< 0.8 µm according to the manufacturer. 4760 C [64].

The discs were cleaned and in the first paper (I) discs were made hydrophilic by boiling in NH4OH (25%):H2O2 (30%):H2O (1:1:5 v/v) for 5 min followed by thorough rinsing in distilled water. Other discs were made hydrophobic by ultrasonication in 1part H2O and 9 parts 1-buthanol for 30 min, followed by ultrasonication in 99.5% ethanol for 3-10 min. All discs were stored in 70% ethanol until use. In previous papers written by our group the contact angle for hydrophilic and hydrophobic titanium has been estimated to $<11^{O}$, as indicated by measurement of saline drops. No measurement were done at these discs because of their small size, but the contact angle was considered similar as in previous papers, while the cleaning methods used was the same [70].

In paper II-IV the discs were passivated in 4.9 M HNO3 for 20 minutes at 50ºC and washed in alcohol, containing more than 90% 3-butoxy-2-propanol, at 70ºC. The specimens were then rinsed in deionized water and air-dried at room temperature. In paper II-IV the discs were made porous through anodic oxidation. An electrochemical cell containing 30 ml electrolyte, was used. A platinum band formed a ring around the disc and served as cathode while the anode was made of titanium. During the anodic oxidation two to three different acids (H2SO4, H3PO4 and HF) were used in different concentrations and combinations. Voltage, current and process time was also varied. The coating with Magnesium in the fourth paper was made electrochemically and the coated surfaces were also analyzed without coating as comparison. The exact description of the electrolyte composition for each surface can upon request be obtained from Elos Medical AB.

Characterization of surfaces

Paper I: All samples, except the ones stained with PI/FDA, were mounted with 1, 4-diaza-bicyclo-[2,2,2]-octane mixed with glycerol (DABCO) to keep the fluorescence from fading. Viewing and photography were done within 6 h. All specimens were photographed in a fluorescence microscope (Zeiss Axioskop 2 plus) equipped with a CCD camera (SPOT 2, Diagnostic instruments inc., MI, USA). Coverage and number of stained cells or cell nuclei on the discs were analysed.

Paper II: After implant retrieval the discs were fixed in absolute ethanol at -20^{o}. Before measurement the samples were warmed to room temperature, dried and analysed in a TOF-SIMS IV instrument (ION-TOF GmbH, Germany), equipped with a gold liquid metal ion gun.

Measurements were performed at the Swedish National Testing and Research Institute SP (Borås, Sweden). The samples were sputtered at 3 kV in order to remove organic material. Positive mass spectra were recorded and the ion profiles of the surfaces were imaged. A tooth sample, containing 98-99% HA in the enamel, was used as reference. In addition to the porous samples, not anodized titanium samples were also implanted. These served as titanium controls. Bone from the implant site was decalcified, histologically prepared and then investigated microscopically.

Paper III: After the anodic oxidation the specimen's were rinsed in deionized water and air-dried at room temperature. Prior to implantation the surfaces were analyzed by Time-of-flight secondary ion mass spectrometry (TOF-SIMS). Measurements were performed at Tascon (Münster, Germany) using a TOF-SIMS V instrument (ION-TOF, GmbH, Germany) equipped with a Bi_1^+ ion gun. Both positive and negative spectra were recorded. The analyzed area was 100x100 μm2. For purity assessment implants were also analyzed by AES at Materiex AB (Borlänge, Sweden).The porous surfaces were examined and photographed in a Gemini 982 (SEM, Zeiss, Germany) using 3 kV. For image analysis the program ImageJ, a java based program, was used. The SEM images were segmented, and based on histograms threshold levels were determined. Mean pore diameter, number of pores/μm^2, and surface porosity was then calculated.

Paper IV: TOF-SIMS analysis: Time-of-flight secondary ion mass spectrometry (TOF-SIMS) is a technique allowing identification and localization of unknown molecules at sample surfaces [71]. It has several advantages over alternative methods e.g. its sensitivity to all elements, detection of all isotopes, excellent spatial resolution (< 200 nm), and simultaneous imaging of the surface distribution of detected elements and molecules [72]. The method was used both for assessment of purity of the surfaces, and for analysis of hydroxyapatite at implant tissue interfaces.
Measurements were performed at Tascon (Münster, Germany) using a TOF-SIMS V instrument (ION-TOF, GmbH, Germany) equipped with a Bi_1^+ ion gun. Both positive and negative spectra were recorded and the analyzed area was 100x100 μm^2. The TOF-SIMS spectra of the implant surface prior to implantation showed peaks corresponding to Ti, O, Mg and low molecular weight carbon containing compounds within the limits of commercially pure implants.

After implant retrieval measurements were performed under commercial conditions at Tascon GmbH, Münster, Germany. The sections were analysed with a TOF-SIMS mass spectrometer (IONTOF TOF-SIMS V). The instrument was equipped with a Bi_3^+ liquid metal ion gun (Bi_3^+ LMIG). Images were taken in the bunched mode (high mass resolution, < 2 μm lateral resolution). Bi_3^+ primary ions were applied with a target current of 0.2-0.4 pA. The total primary ion doses were 1.9-3.27 x 10^9 ions, thus kept far below the static SIMS limit. Fields of view ranged from 300 x 300 μm to 500 x 500 μm and the pixel density was 256 x 256.

Implantation procedure

All animal work was approved of and conducted according to guidelines stipulated by the Gothenburg animal experiment ethical committee. Male Spraque Dawley rats, 400–500 g (B&K Universal AB, Sollentuna, Sweden), were used. The rats were anaesthetised with Isofluran Baxter (Baxter Medical AB, Kista, Sweden).

After shaving and cleaning the calves with iodine, muscles and bone were exposed by a lateral incision of 2 cm. The gastrocnemius muscle was firmly kept to the side and the periosteum was opened. To insert an implant a hole was drilled (0.7mm (I): 1.0 mm (II-IV) in diameter) in the lateral side of the tibia, using a low-speed drill under generous irrigation with saline. Emanating from the hole, an incision (2.5mm long, 0.7mm (I) or 1.0 mm (II-IV) wide and 2.5mm deep) was made, into which the implant was placed. One or two implants were placed in each tibia. In paper III some incisions were left without implants as controls. The skin was sutured with suturamide 4-0 (Johnson & Johnson Intl. Brussels, Belgium). Postoperatively, the animals were given buprenorphin (Temgesic; Reckitt & Coleman, Hull, Great Britain; 0.05 mg/kg b.wt.) subcutaneously.

The animals were allowed free postoperative movements with food and water ad libitum.

Implant Retrieval and histological preparation

Paper I: The animals were sacrificed after 1, 4, 8, 14 and 21 days, under anaesthesia. A total amount of 89 hydrophilic and 75 hydrophobic implants were used. Usually only one implant were implanted in each tibia, but in some animals two implants per tibia were implanted. The retrieved samples were rinsed in DPBS prior to fixation, and were then used for different analysis. The retrieved discs were fixed and then rehydrated. After rinsing in DPBS, the discs were placed on a cooling plate (Histolab, Stockholm, Sweden) at 0 ^{O}C and incubated with goat anti-osteocalcin antibodies (Biogenesis, England, UK) for 30 min, followed by rinsing in

DPBS. The discs were then incubated with FITC-conjugated donkey anti-goat antibodies (Jackson Immuno Research Laboratories Inc, West Grove, PA, USA), for 30 min. Some of the discs were used to detect cells positive for BMP-2, VEGF and ALP respectively, where specified antibodies were used. Only BMP-2 were tested after 1, 2 and 4 days. 4 hydrophilic and 3 hydrophobic surfaces (day 1), 5 hydrophilic and 5 hydrophobic surfaces (day 2) and 4 hydrophilic and 5 hydrophobic surfaces (day 4). No double-staining with Hoechst 3342 were done until after 4 days. After 8 days BMP-2 was tested on 6 hydrophilic and 7 hydrophobic surfaces. ALP was tested on 6 hydrophilic and 6 hydrophobic surfaces and VEGF was tested on 6 hydrophilic and 6 hydrophobic surfaces. After 14 days BMP-2 was tested on 5 hydrophilic and 4 hydrophobic surfaces. ALP was tested on 5 hydrophilic and 4 hydrophobic surfaces and VEGF was tested on 4 hydrophilic and 4 hydrophobic surfaces. After 14 days osteocalcin was used as a marker tested on 4 hydrophilic surfaces and 4 hydrophobic ones. Finally, after 21 days, BMP-2 was tested on 6 hydrophilic and 6 hydrophobic surfaces. ALP was tested on 4 hydrophilic and 4 hydrophobic surfaces. VEGF was tested on 4 hydrophilic and 4 hydrophobic surfaces and osteocalcin was tested on 5 hydrophilic and 4 hydrophobic ones. The viability of the cells adhered was tested on different discs with a double staining with propidium iodide (PI; Fluka- Biochemica, Buchs, Switzerland) and fluorescein diacetate (FDA; Fluka-Biochemica). The viability was tested after 4 and 8 days. After 4 days 6 hydrophilic and 5 hydrophobic surfaces were tested. After 8 days 5 hydrophilic and 4 hydrophobic surfaces were tested. All discs, except the ones stained with PI/FDA were double stained with Hoechst 33342 (Sigma Chemical Co, St Louis, MO, USA) for 3 min to visualize cell nuclei and were investigated face-on the surface.

Paper II:

The discs were implanted in rat tibia for 1 week. The animals were then sacrificed and the discs were fixed in absolute ethanol at -20 ^{O}C. 6 different porosities were made through the oxidation process although only 3 were analyzed more thoroughly and presented in the article. From some of the implants (n), more than one cut was made. These are presented within parenthesis. C (control) n=4(5), A (B11): n=4(7) and B (G1): n=2(4).

Paper III:

The same implants as in paper II were used (Control, B11, G1) plus another surface: G4. From some of the implants (n), more than one cut was made, which are presented within parentheses. C (control): n=4(5), B11: n=4(7), G1: n=2(4) and G4: n=4(4). At 4, 7 and 14 days the animals were sacrificed under anaesthesia. The part of bone where the insertion had

been made was retrieved and fixed in 1% paraformaldehyde in PBS for 3 days. The samples were then decalcified for 2 weeks with EDTA. The decalcifying medium was changed every third day. After decalcification the samples were rinsed in water for 15 minutes. Implants were not removed until after the decalcification and rinsing steps to avoid disturbing the interface between implant and tissue. The samples were dehydrated in graded series of ethanol, placed in xylene, and imbedded in Histowax imbedding medium (Histolab Products AB, Göteborg, Sweden) at 60ºC. The imbedded samples were cut in sections and mounted on Superfrost Plus glass slides (Menzel-Gläser, Germany) and stained with Mallory's trichrome according to Ladewig at Histocenter AB, Göteborg, Sweden.

Paper IV:

At 2 and 4 weeks the animals were sacrificed under anaesthesia. Implants and surrounding tissue were retrieved and fixed in absolute alcohol at –20^{o}C. The specimens were imbedded in resin and cut with an Isomed^{TM} Low Speed Saw (Buehler, Illinois, USA), equipped with a diamond wafering blade. From each specimen 2 cross sections were produced. The first section was 100 µm thick and was used for TOF-SIMS measurements. The next, adjacent section was 75 µm thick and was stained with basic Fuchsin (Fluka, Switzerland). Two sections were thus made from each specimen and the stained section was later used for orientation of the unstained section in the TOF-SIMS measurements, thereby ensuring that relevant areas were measured. The staining technique used was a modified version of a method by Krompecher. In short, the sections were stained with increasing strength of alcohol (50-95-100%) varied with decreasing amounts of Fuchsin (8-2-1,5g). The stained sections were placed on glass slides and mounted with DPX mountant (BDH Laboratory Supplies, England). Sections were then viewed and photographed in a Zeiss Axioskop 2 plus microscope, equipped with a CCD camera (SPOT 2, Diagnostic instruments inc., MI, USA). The amount of hydroxyapatite in the interface between implant and tissue was measured as follows:

1. ***Areas were selected by light microscopy***

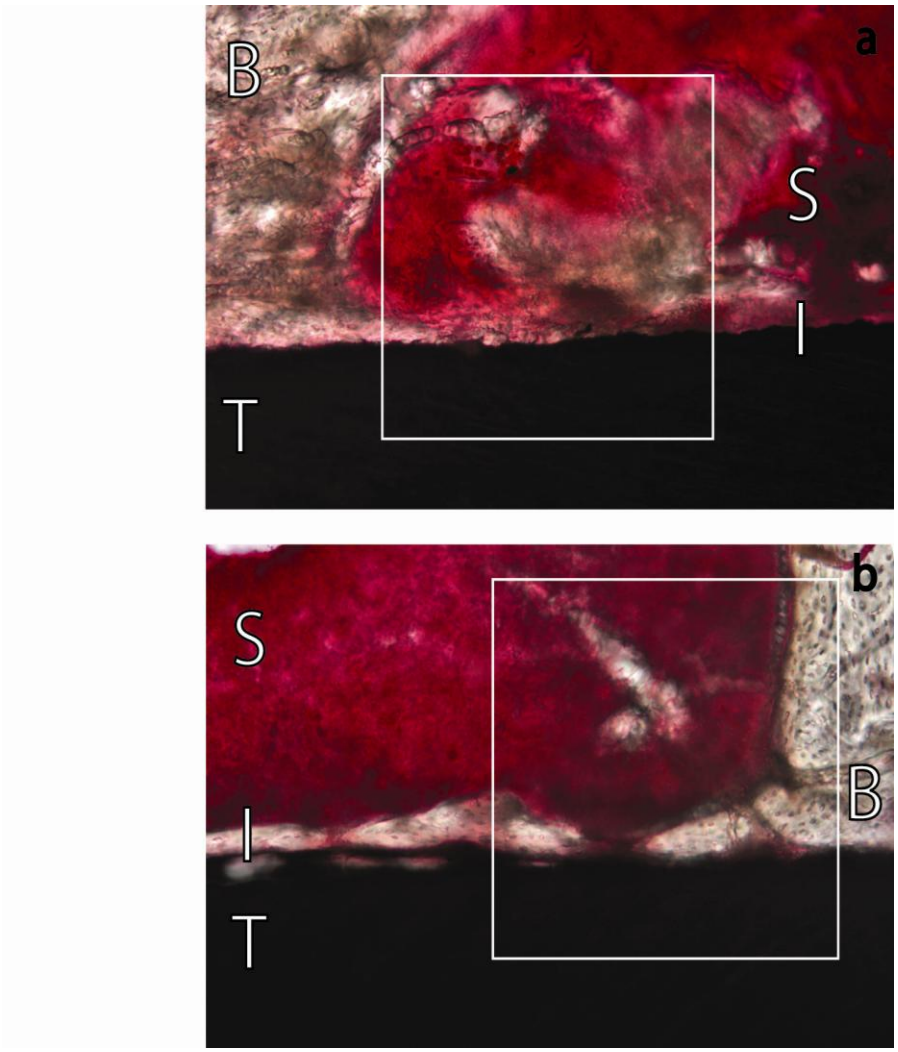


Figure 3. Light micrograph of bone section showing the titanium implant, the interface zone and soft tissue. The square shows the area selected for TOF-SIMS analysis.

2. TOF-SIMS analysis

ROI – principle

- The selected area is defined in the instrument
- An imaged is created in the bunched mode
- ROI is determined in the image
- Image of HA-fragment

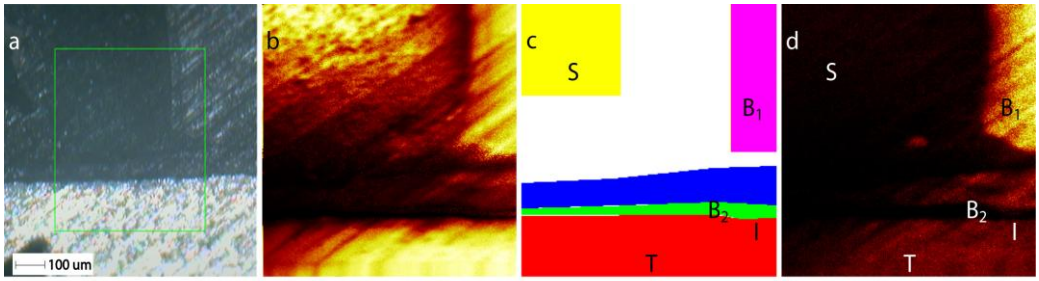


Figure 4. The ROI analysis of the content of hydroxyapatite.

Statistics

Paper I: Results were expressed as mean ±standard error of mean (SEM). Two values were compared with each other (either at two different time points for the same surface or the same time point for two different surfaces). Students paired t-test was used as statistical method and the value for significance was set to $p<0:05$.

Paper II: One way ANOVA with post hoc test was used as statistical method and the value for significance was set to $p<0.05$.

Paper III: Results were expressed as mean ±standard error of mean (SEM). One way ANOVA with post hoc test was used as statistical method and the value for significance was set to $p<0.05$.

Results and discussion

The major aim of this thesis was to elaborate methods for evaluation of implant healing and to further investigate early implant healing at the implant site and at the interface. The focus has been on early implant healing, in a time span between 1 and 28 days, and on investigating how different porosities and surface treatments affect the implant healing process. There has also been a focus on finding criteria's for healing between bone and implants.
Tradition when studying implant healing has been to analyze implant healing after months to years. From a clinical point of view this is easy to understand since the importance of biocompatibility and strength of the osseointegration becomes evident first after a longer period of time. The knowledge about early implant healing has been limited, as a result of that most studies have focused merely on healing after 6-8 weeks and later. However it is of great interest to study the early interactions between bones and implant surface. This could possibly predict the course of implant healing over time and give information about mechanism of healing that could be used to improve the healing process.

In the first paper early implant healing on hydrophilic and hydrophobic surfaces were investigated and the aim was to see whether the hydrophilic surface differed from the hydrophobic one, when comparing early cellular events after implantation. It has earlier been shown that blood-surface interactions are influenced by surface energy, and that thrombin generation and cell activation are more pronounced on hydrophilic surfaces than on hydrophobic ones in vitro [73, 74]. As in the paper written by Suda et al [44] and Meikle et al [45], the sample surfaces were removed and tissue adhering to the samples was studied face-on. This means that the tissue is not necessarily in close contact with the surface, but must adhere with a certain force to withstand retrieval. Immunofluorescence techniques were used to detect signs of bone formation. Cell viability, ALP activity, presence of osteocalcin and cells positive for BMP-2 and VEGF were investigated. BMP-2, ALP, VEGF and osteocalcin are all well known markers used to study bone formation [75-78]. BMP-2 was more prominent on the hydrophilic discs compared with the hydrophobic discs after 1 week. The angiogenesis was considered similar on both surfaces in that VEGF was detected after 8 days in the same amount on both surfaces. Also ALP was detected equally on both surfaces after 8 days. Osteocalcin positive cells were found from 2 weeks. Both viable and non-viable cells were found and only initially there was a visible difference between the hydrophilic and

hydrophobic surfaces; 4% and 56% viable cells respectively. After 3 weeks the surfaces elicited similar responses, indicating that surface energy is more important during the initial rather than subsequent healing of implants in bone. The implants were studied face-on, i.e. after extraction we investigated the implant surface, compared with sectioning. In this study we came to the conclusion that surface energy is of minor interest and that the early healing is more influenced by other properties, for example surface structure. In the study it was possible to see not only the interface but also cells on the implant surface indicating that healing at least partly could be effected by the implant surface. This was a quite interesting finding although our intention to seek for osteoblasts as indicators of bone growth did not fully succeed in that they were rare. However our finding is supported in a paper from another research group, where the aim was to investigate initial and early tissue reactions to modified and conventional (sand-blasted, large grit and acid-etched) titanium implants [79]. The implants in this study were placed in the mandibles of dogs and retrieved after 1, 4, 7 and 14 days respectively. The research group used non-decalcified tissue sections using conventional histology and immunohistochemistry with monoclonal antibodies to TG and osteocalcin. After 4 days the first signs of osteocalcin were seen and staining for TG indicated that angiogenesis was correlated to new bone formation after 7 days. After 14 days the surfaces seemed to be surrounded by mature, parallel-fibred woven bone [79].

Titanium can be modified in order to improve healing and to speed up healing time. Examples of modifications are increasing roughness and oxide thickness of the metal as well as introducing pores [80-82]. In the second paper we investigated early events at surfaces modified by anodic oxidation creating different porous layers on the surfaces. Questions asked in this study was whether the implant surface is mineralized prior to the surrounding bone or the other way around, and how implant porosity influence mineralization and bone formation. In this study we used the same implantation technique as in the first paper. After implant retrieval, bone formation was investigated face-on by measuring HA with TOF-SIMS. TOF-SIMS as a method for evaluating surface structures is well established [83]. Recently, TOF-SIMS has also been used for characterization of biological structures, so besides cellular distributions of inorganic compounds, e.g. calcium, magnesium and boron, organic substances and biomolecules can be detected, e.g. phosphatidylcholine and cholesterol [13, 84-91]. With help from TOF-SIMS, we could detect formation of adhering bone around all the implants after 7 days by measuring hydroxyapatite. Although the differences between porous and non-porous surfaces were not significant, hydroxyapatite was

found on all surfaces and it was possible to see differences between the surfaces. It has earlier been stated that porosity influence cellular events, a fact that became obvious during our investigation. However, since the most porous surface only had the third highest HA coating, factors other than porosity might influence the mineralization process. One such factor could be pore size. Another explanation could be influence from the different acids used for anodization and etching, like sulphur, phosphor and fluoride, which perhaps could become incorporated in the growing oxide and thereby, influence the mineralization process. An interesting thing seen was that bone formation following implantation was observed already after 4 days, though no mineralization could be detected at this time point. A possible conclusion from this study is that bone forms in the tissue around the implant before the implant surface is mineralized. These findings are supportive to our findings stated in the first paper about rare findings of osteoblasts in direct contact with the implant, in that it seems clearer that the osseointegration starts in the periphery gradually enclosing the implant. Although our conclusions in the first paper considered bone growth directly on the implant surface, it seems more suggestible that the osteoblasts found were parts of the formation of callus rather than indicative of real bone growth at the implant surface.

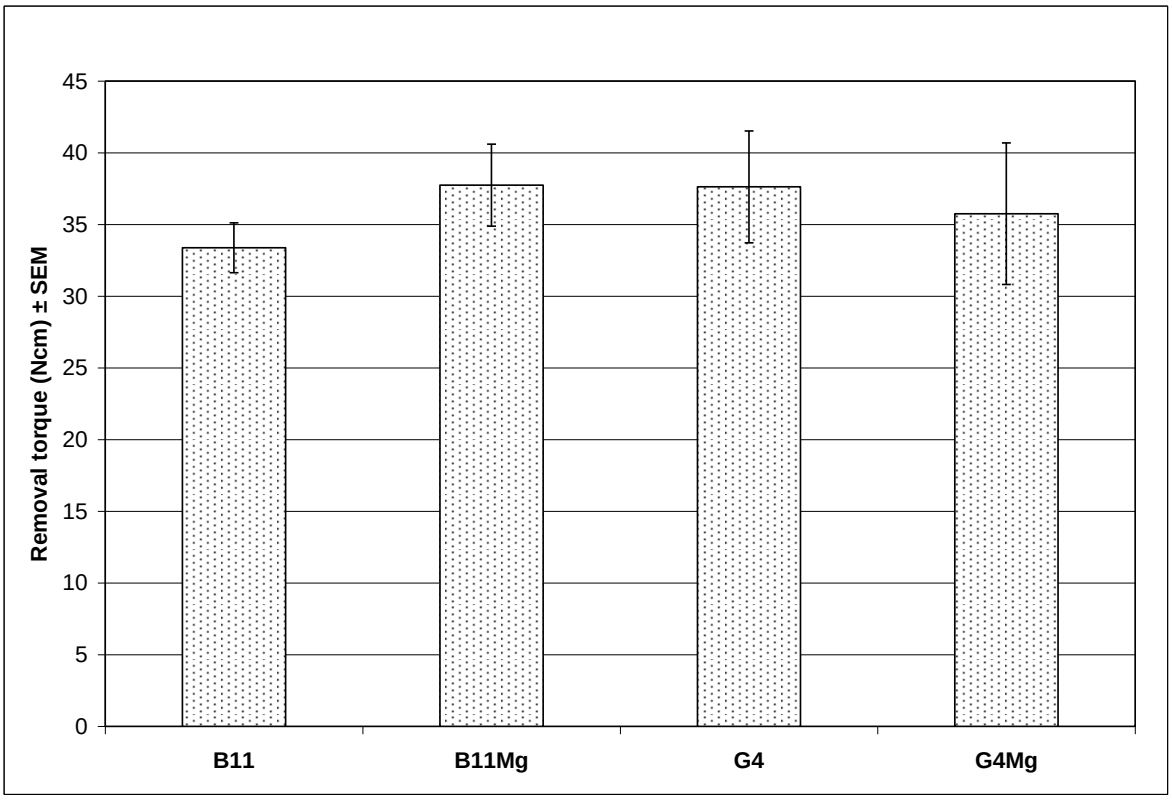
As a continuation of this the focus in the third paper was on bone formation, resorption and the relationship between these two. The same technique as in the previous papers was used regarding preparation of the surfaces and implantation but the sample were fixed in situ, decalcified and sectioned. The sectioning gives an opportunity to see the relation between bone and the titanium implant. Also before the implantation we used TOF-SIMS to characterize the surfaces. We could then see that during the oxidation process compounds are built into the oxide as for example sulphur, fluoride and phosphor. This was not known before and supported our theory in the second paper, that other property than porosity might influence the healing process although the porosity plays a crucial role. Small islands of bone were seen after 4 days both during normal wound and implant healing. After 7 days the bone was in close contact with the implant, including the marrow cavity. However, resorption of bone in the marrow could be seen from day 7 and was evident after 14 days, which has been reported before [63, 92]. After 14 days the initially formed bone was resorbed and replaced by mature lamellar bone, mostly in close contact with the implant. These findings, compared with those done by Takeshita et al. indicates that resorption plays a much greater role than earlier considered. The findings by Takeshita showed that after the early healing period bone contact, bone thickness and area of bone surrounding the implants increased significantly [93]. Although significant, the increases were small. This indicates that most of the bone

thickness is established early in the healing process and gives us a clue about the importance of bone resorption and what could be achieved if the resorption is possible to influence. A similar response was seen by Lindhe et al [65]. Our findings, together with the results from Takeshita and Lindhe is important in that it suggests that it is not the initial bone formation that is crucial but how much of the newly formed bone that remains after the initial resorption. Probably this process is possible to influence and the focus when studying implant healing should therefore be changed from measuring amounts of bone after a certain time span to study and influence the dynamic procedure that is bone healing. Saying that, it is interesting that in our study, the general healing process, when comparing normal wound (fracture) healing and early implant healing did not differ and our results indicate that porosity did not affect the healing process during the first 7 days.
Another difference in this study compared with the others, is the use of decalcification as a method to be able to get thin slides. The decalcification was executed prior to removal of the implants to avoid disturbing the interface between implant and tissue. The common procedure before has been to cut thicker slides without prior decalcification [94] or to look face-on the implants. A possible problem with this execution is that the retrieval of implants risk destroying or disrupting structures at the implant surface, the interface and connective tissue respectively.

In the final paper focus was on the mineralization process at the implant-tissue interface. In paper III we investigated the early bone formation and remodelling. After two weeks we found a thin line of bone surrounding the implant although there had been large amounts of callus before. With this callus degeneration in mind, we wanted to investigate the amounts of high mass fragments of HA with TOF-SIMS technique. The amount of HA fragments would indicate mineralization capacity i.e. osseointegration for the chosen surface. The surfaces investigated were complemented with two surfaces coated with magnesium. Magnesium is known to strongly induce bone growth, but is itself rapidly absorbed during this process. This has restricted the use of magnesium as an implant for fixation [23, 95]. In this paper the thought was to combine the bone inducing properties of magnesium with the strong, non-absorbable metal titanium to see whether this could improve healing and fixation. Implantation time varied from 2 to 4 weeks and the implants were after retrieval cross-sectioned with a low-speed saw. One section from the implant was stained with basic Fuchsin and the other part was analyzed with imaging TOF-SIMS using a Bi_3^+ cluster ion source. The TOF-SIMS technique has been used for inorganic materials for a longer period of time, but

there have been problems with biological material because of the force within the ion source. It has simply destroyed cells that were about to be investigated. As mentioned before the TOF-SIMS today is capable of imaging also biological compounds and it has then also been possible to see HA as signs of mineralization, although the ion sources have suffered from poor secondary molecular ion production efficiency, which only have made it possible to see small fragments. In this study we used Fuchsin-staining to find proper areas to use for TOF-SIMS analysis. The amount of HA within an area of 40 μm from the implant was measured. The areas were also analyzed histologically. We found a correlation between histology and TOF-SIMS regarding healing. After 4 weeks it was possible to see well defined areas between bone, soft tissue (bone marrow) and interface. After 2 weeks it was more difficult trying to separate bone from soft tissue, since bone resorption in the marrow was not completed and therefore mineralization spots were still present. This was observable with both Fuchsin images and TOF-SIMS images. We could detect high mass HA fragments in areas defined as bone. No HA fragments were found in areas defined as soft tissue, but HA fragments were found both after 4 and 2 weeks in the interface. This was an unexpected finding, and to our knowledge this has not been reported before. Previously calcium deposits have been found in the ground substance of the interface but no high mass HA fragments [96-98]. The possibility to evaluate early bone healing has previously been limited to the amount of callus seen after a certain time span. No possibility to evaluate the mineral content, amount and stability has been given. Measuring HA fragments after different time spans indicates both callus turnover and is an indicator of whether the implants chosen is valuable as osseo integrative or not. The HA fragments indicates how well the initially formed callus remain as a solid mineral containing bone. Our opinion is that this is of importance and could improve further studies of implant healing. It can also enhance further evaluation of different implant modifications.
We also noticed a region adjacent to the implant, which turned up black in the TOF-SIMS image, indicating that no secondary ions were generated from this area. This region was located between the metal and the interface tissue. The same phenomenon has been reported before [97, 99, 100]. A probable cause of this is the histological preparation, where the dehydration can cause shrinkage of the tissue. It is also possible that cutting the sample causes some dislocation between implant and tissue. This region was considered to be an artefact and not part of the interface tissue. In the first two papers we looked face-on the implants, compared with the last two which we cross-sectioned. With the first technique it is much more difficult to anticipate properties of the investigated surface compared with the latter. This might in part explain our misjudgement in the first study.

It has earlier been state of the art to use removal torque tests to see whether the implant healing has succeeded [69, 101-104]. In this thesis we have used TOF-SIMS as a method for studying hydroxyapatite. Unpublished results, although not significant, indicates that there is some correlation between those two test methods and that TOF-SIMS as a method could become of value for evaluating healing around implants. Our findings indicate that implant healing has great similarities with ordinary fracture healing, i.e. callus formation and remodelling. This mean that implant healing, implant properties and cellular reactions to the surface properties should be studied *in vivo* to get a true picture of the process. With these findings a whole new spectra of literature opens up. If, as we suspect the early implant healing acts as a modified fracture, more of the clinical orthopaedic literature becomes relevant and our findings might be useful for evaluation of early healing around implants. Clinically, studies with focus on bone formation and resorption and how to influence those two are of great interest. There is, for example, a number of studies focusing on titanium coated with bisphosphonate which show improved biocompatibility [105-108]. Experimental studies on postoperative infusion with zoledronic acid after hip-fractures once yearly have been found to decrease mortality and decrease the amount of new fractures [109, 110] The knowledge about the importance of resorption and how to influence the resorption with for example zoledronic acid will be a subject for further investigation and hopefully the findings in this thesis will improve the possibilities to evaluate the results with higher specificity than before.



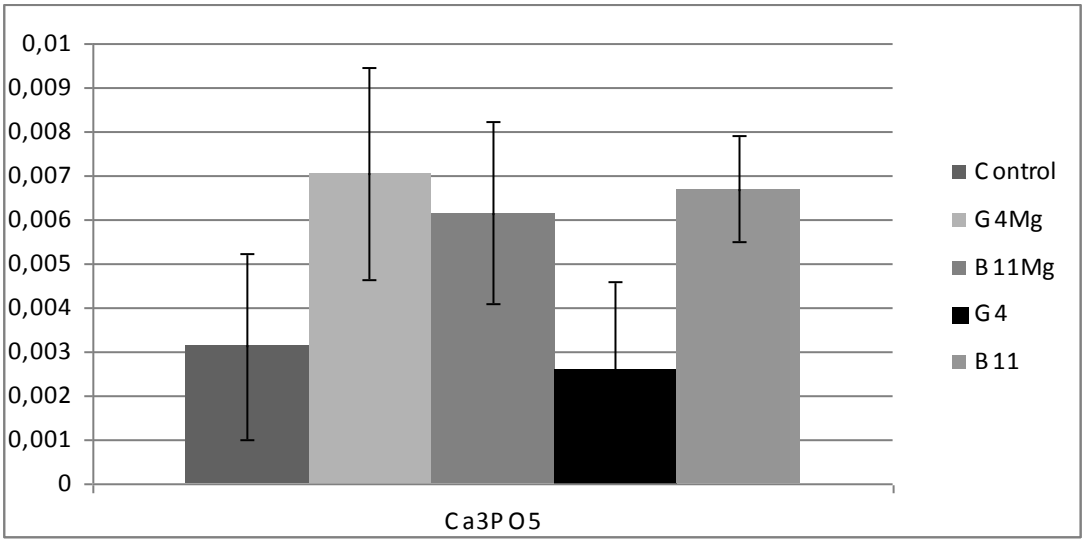


Fig 5. A comparison between tables with detectable, although not significant, similarities. The first table shows removal torque tests after 6 weeks of implantation in rabbit bone. The second table shows relative corrected intensity with 95% confidence intervals from m/z 231, Ca_3PO_5, after 4 weeks of implantation in rat bone.

Conclusions

Healing around implants starts primarily in the periphery growing toward the implant. Early callus formation and resorption are crucial steps in these early phases and the net bone production is possibly influenced by these factors.

Porosity may influence the osseointegration and stimulate bone growth in that the net bone production may be influenced. We found a correlation between pore size and implant healing but the implant healing might also be influenced by compounds built in during the oxidation process.

Porosity together with magnesium coating was seen to positively influence bone growth. Our findings in paper IV may indicate that Magnesium coated implants could decrease resorption and increase the net bone production. This might be of great value in further studies of early implant healing.

Imaging TOF-SIMS with a Bi_3^+ cluster ion source is a suitable tool for investigating high mass Hydroxyapatite fragments.

High mass HA fragments were found in the interface, something that has not been reported before and could act as an important indicator in further studying of biocompatibility.

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