On Possibly Bioactive
CP Titanium Implant Surfaces

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To Thea, Anders and my parents Bodil and Hasse Hansson with love.

This PhD thesis represents number 34 in a series of investigations on implants, hard tissue and the locomotor apparatus originating from the Department of Biomaterials/Handicap Research, Institute for Clinical Sciences at Sahlgrenska Academy, Göteborg University, Sweden.

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Abstract

Background Osseointegrated titanium implants are routinely used in clinical dentistry. Although the overall clinical results are good, there are situations when an improved implant healing is desirable, for instance in compromised bone or in order to decrease healing time. Six factors are proposed to affect titanium implant osseointegration, where one is surface quality. Attempts to optimize surface quality of titanium implants with respect to topography and biochemistry and to prepare possibly bioactive surfaces demonstrate promising results, yet there is a need for further investigations.

Aims The aim of the thesis was to investigate the significance of surface orientation for bone tissue response *in vivo* and, furthermore, to investigate possibly bioactive titanium implant surfaces *in vitro* and *in vivo.*

Materials and Methods The thesis is based on five experimental studies, where 12 differently modified CP titanium implant surfaces were investigated.

Topography and chemistry were characterized by Laser Scanning Profilometry, Optical Interferometry, Scanning Electron Microscope and X-ray Photoelectron Spectroscopy, respectively.

In vivo bone responses were evaluated histomorphometrically and mechanically in a rabbit model (Study I, II).

In vitro cell response were investigated in human primary monocyte (Study III, IV) and osteoblast (Study V) cell culture models, while calcium phosphate nucleation (CaP) capacity of the surfaces were investigated in simulated body fluids (SBF) (Study V).

Results In Study I titanium implants prepared with isotropic and anisotropic surfaces with similar roughness demonstrated similar bone response *in vivo* after 3 months of implantation.

In Study II the non-bioactive (anodized) and possibly bioactive (alkali-heat treated) titanium implants with and without covalently immobilized protein coatings (blood plasma) demonstrated similar bone response *in vivo* after 1 month of implantation.

In Study III the non-bioactive (anodized) and possibly bioactive (anodized/Mg) titanium surfaces demonstrated increased inflammatory cell attachment, yet a similar early inflammatory cell response *in vitro* compared to the turned and blasted control surfaces.

In Study IV the protein coatings influenced the early inflammatory response *in vitro*; however, cells on immobilized catalase surfaces, not fibrinogen, demonstrated the strongest inflammatory response.

In Study V the possibly bioactive surfaces (alkali-heat treated, anodized/Mg, fluoride and nano HA coated), gave rise to an earlier CaP formation than the blasted control surfaces. Furthermore, the SBF treated (72 hours) alkali-heat treated fluoride and anodized/Mg surfaces demonstrated similar or decreased bone cell response, while the SBF treated blasted and nano HA surfaces increased bone cell response compared to the blasted controls.

Conclusions Within the limits of the studies of the present thesis, surface orientation had no effect on bone response *in vivo*. Furthermore, possibly bioactive surfaces did not significantly increase bone response *in vivo*, while possibly bioactive/oxide modified and, in particular, bioactive/covalently immobilized proteins influenced early inflammatory cell response *in vitro*.

Key words: titanium implants, surface modification, bioactivity, bone tissue, cell culture

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Contents

Introduction

The Need for Further Research on Titanium Implants

Oral implants have been used on a regular basis for nearly forty years. Today approximately three to four million implants are placed in patients all over the world, annually.

The overall clinical success rate is > 90%, and implants in the mandibular front region have success rates in the range of 95-100%. Despite these impressive figures, there is still a need for continued R&D of implant surfaces.

Firstly, there is incomplete information on difficult clinical situations, i.e. implant sites with poor bone quality and quantity, e.g. irradiated or transplanted bone. Furthermore, the increase in length of life poses new challenges with patients having old and pathologic bone.

Secondly, there is incomplete information on acceleration of implant integration in normal bone; original protocols prescribed three months of healing in lower jaws and six months in upper jaws before implant loading. Accelerated implant integration would mean functional and social benefits for the patient since they can return sooner to ordinary life. However, the introduction of immediate loading protocols, implants placed in extraction sockets and shortened implants have raised the demands on the standards of implants.

Thirdly, there is incomplete data on the mechanisms responsible for bone healing around titanium implants and proper knowledge is the base for further development.

Albrektsson and co-workers presented six factors that influenced implant integration in bone[1]. These factors concerned the biocompatibility, implant design, surface quality, general and local status of the patient and surgical and prosthodontic techniques.

This thesis will focus on optimizing surface quality of titanium implants with respect to topography and biochemistry.

The performance of modified implants was tested *in vivo* while explanatory models were tested *in vitro*.

Bone Healing around Titanium Implants

Overview

At implant insertion, the trauma causes an acute inflammation characterized by increased blood flow and increased vascular permeability.

The homeostatic system that consists of several interrelated protein systems, such as blood coagulation, fibrinolytic system, immune system, complement system and kinin system is activated and it modulates the inflammatory response and the inflammatory cell recruitment. *In vitro* studies have demonstrated that proteins and platelets adhere to titanium surfaces within nanoseconds and seconds respectively[2, 3] and furthermore, that polymorphnuclear cells (PMN) are the dominating inflammatory cells at titanium surfaces from 8 minutes to 32 hours[4] after placement.

In vivo studies have demonstrated that the interface areas (threads) initially are filled with old bone, debris from preparation and blood cells[5].

After a few days monocytes appear and differentiate into macrophages that phagocytes granulation tissue and debris created by drilling. The blood clot is gradually dissolved due to fibrinolytic systems and, in parallel, mesenchymal precursor cells differentiate into endothelial, fibroblast and bone cells that start to produce vessels and new connective tissue.

Osteoid has been demonstrated on endosteal bone surfaces within a week and mineralized immature woven bone has been observed in contact with the implant two weeks after placement[5]. It has been discussed whether the bone generation is induced by distal osteogenesis (endosteal bone)[5] or by contact osteogenis (implant surface)[6]. Four to six weeks later, bone grow along the entire periostal part of the implant and the woven bone is gradually replaced by mature lamellar bone[5, 7]. The most active phase of the remodeling takes about 6-18 weeks, corresponding to 4-12 months in humans[8]. Although bone seems to be in intimate contact with the implant at light microscopic level of resolution, ultra structural studies performed in TEM observations indicate the presence of an intermediate, un-mineralized amorphous zone of 20-400 nm[9].

Factors Relevant for Present Thesis

Blood Plasma Proteins

Blood plasma is the liquid component of blood, in which the blood cells are suspended. Plasma is the largest single component of blood, making up about 55% of the total blood volume. Blood plasma contains several proteins, mainly fibrinogen, albumin, and globulins.

Except for taking part in the coagulation process, converting to fibrin and mediate binding of platelets to foreign surfaces, fibrinogen is additionally considered to be a prime inflammatory cell attractant and activator[10].

Albumin acts as a carrier for different molecules and regulates the tissue colloidal osmotic pressure. Furthermore, preadsorbed albumin has been demonstrated to passivate platelets and decrease inflammatory response[11].

Material surface adsorbed IgG activates the classical pathway of complement activation[12] while IgA triggers the alternative pathway[13].

Different models of describing the mechanisms of protein adsorption to solid surfaces have been suggested.

However, proteins generally adsorb, undergo conformational changes, bind irreversibly to the surface or desorbs in favour of another protein[14]. Initially the protein adsorption is fast but, gradually the surface becomes saturated, and the process slows down. The time dependent exchange of blood proteins at solid surfaces (talantalum) has been described by Vroman and co-workers[15]. In brief, high molecular weight proteins are present at low concentrations to replace abundant low molecular proteins.

In vitro studies on titanium surfaces show that fibrinogen is frequently the dominant component of the proteins after short time contact but after further exposure it may be replaced by other plasma components such as HMWK and high-density lipoproteins[2]. Soft tissue proteins *in vivo* are additionally found to vary around the titanium implant surface in a time dependent manner[16].

Macrophages

Monocytes are part of the human body's immune system and originate from hematopoietic stem cell precursors called monoblasts in bone marrow.
Monocytes circulate in the blood stream for about one to three days and then typically move into tissues throughout the body. They consist of between 3 to 8 percent of the leukocytes in the blood.
At implant installation, the monocytes adhere and migrate through the endothelium and further through the interstitial tissue towards the chemo attractants released from the site of injury. The monocytes recruited to the place of surgery and implant insertion, undergo maturation to macrophages and remain at the implant surface. The macrophages at the implant sites have different phenotypes and express different surface antigens depending on time after implantation[17, 18]. The adherent macrophages are considered key mediators of implant associated inflammation and foreign body response. Thomsen and Gretzer[19] describe several characteristic of macrophage functions identified as critical events in the material host interaction such as the ability to:

- produce proteins, where especially cytokines and growth factors (IL-1, IL-6, IL-10, TNF-α, TGF, FGF and PDGF) modulate inflammatory response.
- phagocytosis and release of lysosomal enzymes, where the size ratio biomaterial / attached cell will decide whether enfulgment and phagocytosis or “frustrated phagocytos” may occur.
- fuse and form FBGC that is unique to the macrophage phenotype, where the presence of FBGC at the surface of implanted biomaterials is the hallmark of continuous low inflammatory grade to biomaterials that cannot be phagocytosed or digested.

Titanium surfaces in a soft tissue model *in vivo* have demonstrated to cause an increased recruitment of monocytes compared to sham operation sites, although with a transient course[20].

Cytokines

Cytokines are small hormone-like factors produced by different cell types.

Dinarello and co-workers[21] describe several biological effects that regulate immunological and inflammatory host responses by serving as intercellular messenger (mobility, differentiation, growth metabolism apoptosis etc). The effects of cytokines are mediated by their binding to specific cell-surface receptors and the subsequent initiation of various intracellular signalling cascades that produce a wide variety of effects on the functioning of the cell. This may include the up regulation and/or down regulation of several genes and their transcription factors, which result in production of other cytokines, or increase in the number of surface receptors for other molecules, or suppress their own effect by feedback inhibition. The cytokines mainly carry out their signalling function via a paracrine or autocrine route, although some cytokines are found in the general circulation under pathological conditions. Several cytokines share similar functions (redundancy) and they are capable of acting on many different cell types (pleotropic).

Production may be initiated inflammatory mediators such as LPS, IL-1, TNF-α, TGF-β etc. Some cytokines promote inflammation while others suppress inflammation.

Tumor Necrosis Factor-α

TNF-α is primarily a pro-inflammatory cytokine. It received its name from one of its early-defined functions of killing certain kinds of tumor cells.

TNF-α are produced by inflammatory cells, however also by adipocytes, keratinocytes, osteoblasts, epithelial cells and adrenal cells. It is present as membrane type II protein or, in extracellular soluble form, in the blood stream and biological fluids. TNF-α is pyrogen and causes fever, furthermore it is involved in shock syndrome, tissue injury, capillary leakage syndrome, hypoxia, pulmonary oedema and multiple organ failure and is also associated with a number of chronic processes[21].

Tumor necrosis factor (TNF) plays an important role in the pathogenesis of inflammatory bone loss through stimulation of osteoclastic bone resorption and inhibition of osteoblastic bone formation[22].

An early enhanced secretion of TNF-α characterized the titanium surface in a soft tissue model compared to sham operated sites *in vivo*[23].

Interleukin-10

IL-10 is primarily an anti-inflammatory mediator originally known as the cytokine synthesis inhibiting factor (CSIF). It is mainly produced by monocytes, T and B lymfocytes. The major activities include inhibition of cytokine production (pro-inflammatory cytokines) by macrophages and inhibition of the accessory functions of macrophages during T cell activation. IL-10 additionally down regulates the reactive oxygen species (ROS) production, neutrophile chemotaxis and degranulation. However, IL-10 also acts stimulatory towards certain T cells, mast cells and B cells[24]. IL-10 have demonstrated a role in preventing of inflammatory bone loss through inhibition of differentiation of early osteoclastic progenitor cells[25].

In soft tissue model there were no difference in IL-10 concentrations around titanium implants *in vivo* compared to sham operated sites [26].

Catalase

During phagocytosis the leukocytes experience a respiratory burst characterized by increased oxygen consumption[27]. The result is reactive oxygen species ROS (singlet oxygen, hydrogen peroxide, super oxide radicals, and hydroxyl radicals) involved in intra and extra cellular killing[28] . ROS are unstable molecules, since they have an unpaired electron in their outer shell. They easily react with other molecules by either reducing them or oxidizing them, and ROS may thereby cause damage to the surrounding tissue[29]. Most cells contain at least three antioxidant enzyme systems; Superoxide dismutase (SOD), catalase and glutathione peroxidase, where catalase degrades hydrogen peroxide to water and oxygen and finishes the de-toxiofication reaction started by SOD[30].

Studies have demonstrated a low spontaneous hydrogen peroxide production around titanium *in vitro*[31] and *in vivo*[32] in soft tissue models, a peroxide production that increased with stimulation.

Osteoblasts

According to Buchwalter and co-workers[33, 34] osteoblasts are bone forming cells proposed to originate from two cell lines; mesenchymal stem cells (bone canals, endosteum, periosteum and bone marrow) and a hematopoetic stem cell line and additional vascular pericytes.

The cells produce and secrete an extra cellular matrix osteoid that later will be mineralized. The process is stepwise and starts with the production of Type I collagen, one of the early events associated with osteoblast differentiation.
Synthesis of collagen I follows sequential expression of the non collagenous proteins; alkaline phophatase, osteopontin, osteonectin, bone sialoprotein and osteocalcin markers of differentiation and prerequisites for mineralization.
Theories regarding mineralization can be classified along two lines.

- by formation of mineral crystals in extra cellular matrix vesicles being the result of disintegration of osteoblasts, where such vesicles (25-250 nm) have only been seen where there is no previously formed bone present[35].
- by small nuclei of mineralization on the collagenous matrix in the absence of vesicles. It has been suggested that crystals are formed on templates of organic material, so called heterogeneous nucleation or secondary nucleation at the surface of previous crystals[36].

During the mineralization, the osteoblasts will be trapped in the lacune to become osteocytes, while others end up as bone lining cells that are flat and elongated, located on endosteal or trabecular surfaces.

Transforming Growth Factor β

TGF-β is a member of the TGF-β super family, >20 related proteins including bone morphogenetic proteins. The name originates from its ability to transform non-neoplastic fibroblasts in the presence of epidermal growth factors *in vitro*. It is mainly produced by osteoblasts, platelets, and macrophages. TGF-β has been demonstrated to regulate differentiation and morphogenesis in embryogenesis, in general to stimulate mesenchymal cells and inhibit ectodermal cells. TGF-β is considered to participate in bone formation and has an important role in coupling the formation of bone with the resorption of bone by inhibiting the formation of osteoclasts and increase of osteoblastic activity[22]. The most pronounced effect on osteoblasts represents the stimulation of the extra cellular matrix (ECM) synthesis including collagen I and III, proteoglucans and fibronectin deposition[37].

Titanium Surface Modifications

Overview

In the 1960s a new system for permanent anchorage of artificial teeth was discovered when the Brånemark group studied bone marrow cells in bone chambers.
The concept of "osseointegration" was initially defined as "a material in intimate contact with living bone without intervening fibrous tissue"[38-40].
The Brånemark system was for a long time the gold standard based mainly on good clinical records[41]. However, in parallel implant parameters were evaluated for predicting good osseointegration and in the 1980s Albrektsson proposed six parameters being important for the implant performance; material compatibility, implant design and surface quality, status of implant bed, surgical trauma at installation and prosthetic loading[1].
There are several methods by which the titanium surface quality can be modified[42]; physical (turning, blasting), chemical (acid etching, alkali), electrochemical (electropolishing anodizing), deposition (plasma-spraying, sol-gel) and biochemical (SBF, proteins) methods. The different techniques will result in a surface quality with different topographical, chemical, physical and mechanical properties.
Since osseointegration depends on biomechanical bonding i.e. ingrowth of bone into small irregularities of the implant, the topography and especially the roughness of the implants has been an area of interest and has been the subject of numerous research efforts.
Guidelines of how to perform and present the measurements of surface topography in a standardized way have been suggested by Wennerberg and Albrektsson[43].
Furthermore, based on experimental evidence from the mid 1990s a surface roughness of about 1.5 µm S_a (average deviation in height from a mean plane) has been defined as optimal for osseointegration[44]. This is rougher than the original, turned Brånemark implant that demonstrated a surface roughness of about 0.5 µm.
Titanium surface roughness has also demonstrated to affect protein absorption[3], inflammatory cell[45-50] and bone cell[51-64] responses *in vitro.* Furthermore, there have been indications that surface orientation may be of importance[65, 66] for implant bone integration, however, not evaluated in a scientifically controlled manner.

Except for the concomitant change in chemical composition when changing the surface topography, attempts have been made to intentionally modify chemical composition to add a biochemical bonding to the biomechanical bonding.

The theoretical benefit of a chemical bond would be earlier attachment, since it is hypothesized to occur more rapidly than bony ingrowth.

Materials that have the ability to bond to living tissue are defined as “bioactive” and the first possibly bioactive material Bio-glass was described in the 1970s by Hench and co-workers[67]. Furthermore, Jarcho and co-workers were the first to present indications of an possible direct bone bonding to hydroxyapatite (HA)[68].

The mechanism proposed was ion exchange resulting in an apatite layer requested not only by the bone cells but also because proteins that serve as growth factors preferentially adsorb to this layer. The “bioactive” properties of these materials were based on morphological observations of the tissue coalescence by TEM and apatite formation *in vitro* and *in vivo.* However, it must be pointed out that bioactivity or chemical bonding are difficult to prove and that the presented evidence is of an indirect nature. Poor mechanical properties of these materials make them unsuitable for load-bearing, clinical applications. Therefore, experiments were made to coat titanium surfaces with calcium phosphates by the plasma spraying technique. The surfaces indeed showed rapid tissue response initially, but in later stages biodegradation and delaminating of the thick coating was frequently observed[69]. Additionally, the line-of sight problem made the technique unsuitable to use for the coating of complex shapes.

To avoid these problems, alternative techniques were suggested to make CP titanium possibly bioactive; ultra thin coatings of calcium phosphates in sol-gels, etching with fluoride containing acids, alkali-heat treatment and anodization. Another possible approach to enhance the bone response is to immobilize organic bio-molecules to the surface. These five surface modifications will be reviewed in the following section.

State of the Art Bioactive Titanium Surfaces

Fluoride Etched CP Titanium Surfaces

Etching of titanium surfaces with different acids to modify surface roughness has been extensively studied during the last decades[70]. The idea of using fluoride containing acids in low concentrations for the purpose of incorporating fluoride ions on titanium implants in small amount was presented by the Ellingsen group[71].

The action of the fluoride ion has mostly been evaluated in the area of caries research, where the beneficial effect because of its high attraction for calcium and phosphates is of great clinical importance, when the ion is brought in contact with the enamel. Fluoride has also specific attraction for skeletal tissues, e.g. trabecular bone density can be increased by the presence of fluoride ions during remodeling[72].

The proposed effects of the fluoride ion are increased proliferation of bone cells by increasing intracellular levels of the ion, increased differentiation of mesenchymal cells into bone cells and the possible stimulation endogenous growths factor production[73]. Titanium implants with incorporated fluoride ions have been evaluated *in vitro*[74-78]*, in vivo*[71, 74, 79] and clinically. Furthermore, there is a commercially dental implant system (OsseoSpeed), and in addition an orthopedic hip implant available with some clinical documentation[80].

In vitro studies of fluoride modified surfaces have demonstrated both increased[74] and decreased[76] proliferation and increased differentiation[74-77].

In vivo studies has demonstrated increased bone response by means of increased bone implant contact[74, 79] and increased stability[79, 81].

In the present thesis, the fluoride surfaces are compared to other possibly bioactive titanium surfaces *in vitro*.

References - Fluoride Etched CP Titanium Surfaces

In Vitro

Eriksson et al -01[78] compared smooth (polished) and rough (HF etched) surfaces with thick (annealed 700ºC) and thin (HNO_3) oxide. The surfaces were characterized by SEM, Optical Profilometry and AES. After exposure to whole blood for 8 minutes to 32 hours, immunofluorescence and chemiluminescence techniques were used for evaluation of cell adhesion, expression of adhesion receptors and the stimulated respiratory burst, respectively. PMN cells were the dominating cell on all surfaces followed by monocytes. While cells on rough surfaces demonstrated increased expression of adhesion receptors, earlier maximum respiratory burst occurred on the smooth surfaces. It was concluded that surface topography had greater impact on most cellular reactions, while oxide thickness often had a dampening effect.

Cooper at al -06[74] compared grit-blasted (25 and 75 µm) titanium implants with and without fluoride ions (various fluoride concentrations). Cell attachment, proliferation and osteoblastic gene expression were measured by SEM, Tritiated thymidine incorporation and RT-PCR, respectively. There were no differences in human mesenchymal stem cell (hMSCs Osiris) attachment between the differently modified surfaces but cells on the fluoride ion modified implants demonstrated an increased proliferation and differentiation (BSP, BMP-2) compared to grit-blasted implants.

Masaki et al -05[75] compared grit-blasted titanium implants with and without fluoride ions and grit-blasted etched surfaces (OsseoSpeed, TiOBlast, SLA-1 and SLA-2). Cell morphology, attachment, and osteoblastic gene expression were measured by SEM, Coulter counter (electrical conduction) and RT-PCR, respectively. There were no differences in mesenchymal pre-osteoblastic cell (HEPM 1486, ATCC) attachment, while cell morphology differed between the differently modified surfaces. Furthermore, cells demonstrated increased ALP gene expression on the SLA-2 surface, while cells on TiOBlast and OsseoSpeed demonstrated increased expression of Cbfa1/RUNX-2. It was concluded that implant surface properties might contribute to the regulation of osteoblastic differentiation by influencing the level of bone-related genes and transcription factors.

Isa et al -06[76] compared blasted titanium implants with and without fluoride ions. Cell proliferation, alkaline phosphatase specific activity and gene expression were evaluated by Coulter counter, Spectrophotometry and RT-PCR, respectively. The number of cells human embryonic palatal mesenchymal (HEPM) were decreased on the fluoride surface compared to the blasted control. The gene expression was similar, except for Cbfa1, a key regulator for osteogenisis that was up regulated after 1 week on the fluoridated surface.

Stanford et al -06[77] compared blasted titanium implants with and without fluoride ions. Platelet attachment and activation were evaluated by immunofluorescence technique, while human palatal mesenchymal (HEPM 1486, ATCC) morphology and gene expression were evaluated by SEM and RT-PCR, respectively. The number of attached platelets was decreased, while activation was increased on the fluoride surface compared to the blasted control. The gene expression was similar for the surfaces, except for Cbfa1 and bone sialoprotein that were increased on the fluoride modified surfaces.

In Vivo

Ellingsen et al -95[81] compared turned titanium implants with and without fluoride ions (various fluoride concentrations NaF). The surfaces were characterized before installation and after push out test by SEM. It was demonstrated that fluoride modified surfaces had increased push out values in rabbit ulna after 4 and 8 weeks compared to untreated implant surfaces. Furthermore, on the fluoride modified surfaces fractures occurred in bone, while for the turned surface it occurred in the bone-implant interface.

Ellingsen et al -04[79] compared blasted titanium implants with and without fluoride ions (HF). The surfaces were characterized by Optical Profilometry. It was demonstrated that fluoride modified surfaces had an increased amount of bone-implant contact in a rabbit model after 1 and 3 months compared to untreated implants. Additionally, the fluoride modified surfaces demonstrated increased RTQ and shear strengths between bone and implant after 3 months. It was concluded that fluoridated implants achieved greater bone integration after short healing time compared to blasted controls.

Cooper at al -06[74] compared blasted surfaces with and without fluoride ions (HF). The surfaces were characterized by SEM. The results demonstrated improved bone formation by means of bone-implant contact in a rat tibia model for the fluoridated surface compared to the blasted surface after 3 weeks.

Clinic

OsseoSpeed (Astra Tech, Gothenburg, Sweden) is a commercially available dental implant system that has been clinically evaluated in approximately 5-10 articles since their launch in 2004. The longest follow up period is 1 year[82]. The surface has mainly been used in poor bone and in early loading situations where it in general has demonstrated good results.

Alkali-Heat Treated CP Titanium Surfaces

The Kokubo group introduced the alkali-heat treated surface by the middle of the 1990s[83]. NaOH treatment results in a sodium titanate hydrogel, and the subsequent heat treatment at 600 degrees result in an amorphous sodium titanate surface layer[84, 85]. The possibly bioactivity of the surfaces are based on its ability to give rise to apatite formation in simulated body fluids (SBF) and has been thoroughly investigated[83-91]. The apatite formation process on the surfaces has been carefully described[87, 88] and is attributed to Ti-OH groups exchanging sodium ions from the material and hydronium ions from the solution. Thereafter, adsorption of calcium ions from the fluid takes place to form calcium titanate. This calcium titanate surface then causes adsorption of phosphate as well as calcium ions to apatite nucleation layers. Once this layer is formed bone like apatite growth follows spontaneously.

Furthermore, studies have demonstrated an increased differentiation of bone cells compared to untreated controls *in vitro*[92, 93].

In vivo studies have shown increased bone response by means of bone-implant contact, detachment load and tensile failure load compared to untreated surfaces[94-98]. However, the bonding strength seems to be time dependent with an initial high bonding strength and no further increase or difference compared to controls at later time points[95]. If the surface were pre-immersed in SBF, the apatite layer on the surface significantly increases the bone response resulting in increased failure loads[97, 98].

Increased bone response *in vivo* by means of enhanced bonding strength has additionally been demonstrated after sodium removal in hot water immersion or, as reported lately, by immersion in HCl[99].

If the bulk is a porous titanium material, the surface has been shown to induce ectopic bone formation *in vivo* in dog soft tissue model[100, 101].

This surface has so far not been applied to dental implants. However, clinical trials of seventy hip arthroplasty patients have been successfully concluded.

In the present thesis the alkali-heat treated surfaces are compared to other possibly bioactive surfaces and further the surface is coated with covalently immobilized protein for hypothesized enhanced performance *in vivo*.

References - Alkali-Heat Treated CP Titanium Surfaces

General

Kim et al -97[84] evaluated bonding strength of the apatite layer formed in SBF on alkali treated implant surfaces with and without subsequent heat treatment (500, 600, 700, 800ºC) and compared it to bonding strengths of apatite formed on Bioglass 45S5-type glass, glass-ceramic AW and dense sintered HA. The results showed the highest bonding strengths of the apatite layer to the alkali treated titanium surfaces that were maximized after a subsequent heat treatment in 500-600ºC. It was concluded that bioactive titanium metal was useful as bone substitutes, even under load-bearing conditions.

Kim et al-99[85] compared the structure of alkali-heat treated titanium surfaces (5M NaOH 60ºC 24h) prepared with various hydrothermal treatment (600 or 800ºC). Furthermore, the bonding strengths of the apatite layer formed on the various surfaces after soaking in SBF. The surfaces were characterized by SEM, AES, Raman spectroscopy, TF-XRD, XPS and ICP. At 600ºC an amorphous sodium titanate layer with a smooth graded surface was formed, while at 800ºC a crystalline rutile sodium titanate with an intervening thick oxide was formed. The apatite layer prepared in 600ºC demonstrated the tightest bond to the surface.

In Vitro

Kim et al -96[83] evaluated apatite formation in SBF (1-4w) on titanium and titanium alloy surfaces subjected to alkali (NaOH or KOH) and heat treatment (5º C/min to 400-800º C). The surfaces were characterized by SEM-EDX, TF-XRD, ICP and pH- metry. Apatite was formed on the SBF treated titanium and titanium alloy surfaces, though, not on cobalt chromium and stainless steal surfaces.

Kim et al -00[83] subjected alkali-heat treated (5M NaOH 60ºC 24h+ 600ºC 1h) macroporous titanium (plasma-spraying method) to SBF. The surfaces were characterized by SEM-EDX and TF-XRD. The induction period for apatite formation was 3 days, which is comparable to bioactive glass-ceramics A/W. It was concluded that alkali-heat treatment is an effective method for preparation, irrespective of the surface macro-texture.

Wang et al -01[91] compared heat-, H_2O_2-, and NaOH treated titanium surfaces. The surfaces were characterized by SEM, FTIR and XRD. Dense oxide layer, titania gel and sodium titanate gel was formed on the surfaces, respectively. Some of the specimens were pre-immersed in distilled water up to 5 days before SBF. The discs were arranged with (contact surface) and without (open surface) contact with the bottom of the container. It was concluded that bioactivity of titania gel originated from the favorable structure of the gel itself because it formed apatite on open surface and after water immersion, while the sodium titanate was dependent of ion release and therefore was unable to produce apatite on open surfaces and after water immersion (decreased ion concentration). Subsequent heat treatment decreased the apatite forming ability of the treated surfaces, but not the untreated titanium surfaces.

Nishio et al -00[93] compared titanium, alkali-heat treated titanium (5M NaOH 60ºC 24h + 600ºC 1h) and alkali-heat treated titanium subjected to SBF for 2 weeks. The surfaces were characterized by SEM, TF-XRD and XPS.
Cell number (Primary rat bone marrow cells), differentiation and gene expression (OC, OP, ON COL) were evaluated by DNA content, ALP activity and Northern blot, respectively. Results demonstrated that cell differentiation increased on the apatite prepared surfaces, while cell number was similar for the differently modified surfaces. It was concluded that apatite formed on the surfaces favored osteoblast differentiation and that alkali-heat treatment favored apatite formation.

Takadama et al -01[87] carefully described the apatite forming process on alkali-heat treated titanium surfaces by TF-XRD, ICP, pH-metry and XPS. It was stated that "Bioactive titanium metal with a surface sodium titanate layer forms a bone-like apatite layer on its surface in the SBF by the following process; The Na^+ ions were released from the surface sodium titanate via the exchange with H_3O^+ ions in the SBF to form Ti-OH groups. These Ti-OH groups induce the apatite nucleation indirectly, by forming a calcium titanate. The initial formation of the calcium titanate may be attributable to the electrostatic reaction of the negatively charged Ti-OH groups and the positively charged calcium ions in the SBF.

Takadama et al -01[88] further described the structure of apatite formation on alkali-heat treated titanium (5M NaOH 60ºC 24h + 600ºC 1h) subjected to SBF by TEM-EDX, ICP and pH-metry. The Ca/P ratios of the apatite were 1.4, 1.62 and 1.67 after 36, 48 and 72 hours in SBF, respectively.

Muramatsu et al -03[102] compared thrombus resistance of alkali-heat treated titanium (5M NaOH 60ºC 24h + 600ºC 1h), alkali-water treated titanium (distilled water 40ºC 48h) and alkali-heat treated titanium subjected to SBF.
The surfaces were characterized by AFM, XRD and contact angle measurement. Platelet attachment and protein adsorption were evaluated and it was concluded that SBF treated alkali-heat treated titanium behaved thrombus resistant probably because heparin was preferentially adsorbed to its surface.

Uchida et al -03[90] compared apatite forming ability of Ti-OH with different structural arrangements in SBF after 14 days by SEM, TF-XRD and ICP. Gels with anatase and rutile structures induced more apatite on their surfaces compared to amorphous surfaces. It was concluded that crystalline planar arrangement in anatase structure was superior to rutile structure for apatite formation.

Lu et al -04[86] subjected an alkali-heat treated titanium (10M NaOH 60ºC 24h + 600ºC 1h) surface to SBF for 1 month. The apatite formed was characterized by Profilometry, SEM, TEM-EDS and TF-XRD. The study showed that octacalcium phosphate (OCP), not apatite, was formed on the surface after immersion in SBF.

Chosa et al -04[92] compared TCP, titanium and SBF treated (8 days) alkali-heat treated titanium (5M NaOH 60ºC 24h + 600ºC 1h). The surfaces were characterized by SEM, TF-XRD, FTIR and XPS.
Cell (Human osteoblast SaOS-2) differentiation-related gene expression (ALP, COL, OPN, BSP, OSC) was evaluated by RT-PCR after 1, 2, 3 and 4 weeks. The results indicated that the treated implants accelerated middle (OPN, BSP) and late (OSC) stage differentiation, while early differentiation was down-regulated (ALP, COL).

Takemoto et al -05[89] compared macroporous titanium (plasma-spraying method) with and without alkali-heat treatment (5M NaOH 60ºC 24h + 600ºC 1h). The surfaces were characterized by micro-CT/3D reconstruction and SEM. Mechanical tests by means of compression strengths, four-point binding strengths and compressive fatigue strengths were performed of the surface. In vitro bioactivity was evaluated in SBF for 3-7 days and in vivo histomorphometric evaluation was performed after 2, 4, 8 and 16 weeks in rabbit femur. Apatite formation in vitro was apparent after 3 days on the alkali-heat treated surfaces, while no apatite could be detected after 7 days on the control surfaces. Bone-implant contact and bone-area in growth were significantly higher on alkali-heat treated implants at all evaluation times. In addition, the surface had mechanical properties sufficient for clinical use in load bearing conditions.

Maitz et al -05[103] compared bioactivity of titanium following sodium plasma immersion, ion implantation and deposition (alkali) in SBF for 7 days. The surfaces were characterized by AES. In a parallel experiment, cell (rat bone marrow cells) viability, proliferation and differentiation was evaluated by LDH test, Alamar blue test and ALP activity, respectively. It was concluded that ion implantation and deposition could well substitute alkali treatment.

In Vivo

Yan et al -97[98] compared titanium, alkali-heat treated titanium (5M NaOH 60ºC 24h + 600ºC 1h) and SBF treated (4 weeks) alkali-heat treated titanium implants. Tensile testing demonstrated that both treated surfaces showed significantly increased failure loads after 4, 8 and 16 weeks in the rabbit tibia compared to the control. Furthermore, both treated surfaces demonstrated direct bone contact with no intervening soft tissue capsule in a histological evaluation after 4 weeks, whereas untreated implants formed direct contact with bone only at 16 weeks.

Yan et al -97[97] compared titanium and SBF (4weeks) treated alkali-heat treated (10M NaOH 60ºC 24h + 600ºC 1h) titanium implants. The surfaces were characterized by SEM-EPMA and TF-XRD. Tensile testing demonstrated that the treated surfaces showed significantly increased failure loads after 6, 10 and 25 weeks in the rabbit tibia compared to the control. Histologic examination demonstrated that the treated surfaces demonstrated more immediate bone contact compared to the control titanium surface at all evaluation times.

Nishiguchi et al -99[96] compared titanium, alkali-treated titanium and alkali-heat treated titanium implants (5M NaOH 60ºC 24h + 600ºC 1h). The surfaces were characterized by SEM. Mechanical and histomorphometrical evaluations were performed after 8 and 16 weeks in the rabbit tibia. The alkali-heat treated surfaces demonstrated direct bone-implant contact after 8 weeks, while alkali treated implants demonstrated an intervening fibrous capsule. Additionally, the alkali-heat treated surfaces demonstrated significantly increased failure load after 8 and 16 weeks. It was concluded that heat treatment is essential for preparing a bioactive surface, even though the alkali surface had previously demonstrated apatite formation in SBF, since implants with gel surfaces are unstable and difficult to preserve and install.

Nishiguchi et al -01[104] compared macroporous titanium (plasma-spraying method), macroporous titanium coated with AW-glass ceramic and alkali-heat treated macroporous titanium (5M NaOH 60ºC 24h + 600ºC 1h).
Mechanical and histomorphometrical evaluations were performed after 4 and 12 weeks in dog femur. Bone-implant contact was significantly increased on alkali-heat treated implants at 4 and 12 weeks. Push out test revealed increased shear strengths for the alkali-heat treated surfaces compared to the other surfaces after 4 weeks. It was concluded that alkali-heat treated implants provided earlier stable fixation than control implants.

Nishiguchi et al -01[95] compared titanium and titanium alloy implants with and without alkali-heat treatment (5M NaOH 60ºC 24h + 600ºC 1h).
Histomorphometric evaluations and push out tests were performed after 4 and 12 weeks in dog femur. Alkali-heat treated implants showed direct bone-implant contact; while alkali treated, implants demonstrated an intervening fibrous capsule. After 4 weeks, the heat-treated surfaces demonstrated increased push out shear strengths compared to untreated surfaces. However, after 12 weeks the untreated implants demonstrated a catch up compared to the treated implants.

Nishiguchi et al -03[94] compared titanium and alkali-heat treated implants (5M NaOH 60ºC 24h + 600ºC 1h). Mechanical and histomorphometrical evaluations were performed after 3, 6 and 12 weeks in the rabbit femur. Alkali-

heat treated implants demonstrated increased bone-implant contact and increased bonding strengths (pull out test) compared to untreated surfaces at all evaluation times.

Fujibayashi et al -01[99] evaluated the effectiveness of sodium removal from alkali-heat treated titanium surfaces, where CP titanium were used as controls. The in vivo detaching failure load was evaluated after 4, 8, 16 and 24 weeks in rabbit tibia. Thereafter, the surfaces were evaluated by SEM. It was concluded that sodium removal accelerated bone bonding because of the anatase structure. However, the adhesive strengths decreased for the sodium free surfaces.

Fujibayashi et al -04[100] compared ectopic bone formation of porous (plasma-spraying) and mesh titanium surfaces with and without alkali-heat treatment (sodium removed). Evaluations were performed in dog muscle after 3 and 12 months. In a parallel experiment the surfaces were immersed in SBF for 7 days. The surfaces were evaluated by SEM and micro-CT/3D reconstruction. The porous alkali-heat treated surfaces demonstrated osteoinductive ability after 12 months.

Takemoto et al -06[101] compared ectopic bone formation of alkali-heat treated porous titanium, alkali-heat treated (sodium removed by hot water) porous, and alkali-heat-treated (sodium removed by HCl and hot water) titanium surfaces. The surfaces were characterized by SEM-EDX and TF-XRD and evaluated in dog muscle after 3, 6 and 12 months. In a parallel experiment, the surfaces were immersed in SBF for 1, 3 and 7 days. The porous sodium free alkali-heat treated surfaces demonstrated osteo inductive ability after 3 months, while apatite formation could be seen on all surfaces after 1 day.

Clinic

So far, there are no commercially alkali-heat treated dental implant systems available.

Anodized CP Titanium Surfaces

Electrochemical modification of titanium surfaces related to implant research has been performed since the 1970s.

The process called anodic spark discharge (ASD) was proposed by Kurze and co-workers, but was further described by Ishizawa and co-workers[105-107].

Anodized titanium surfaces have been extensively investigated *in vitro*[100, 108-110], *in vivo*[105, 106, 111-134] and additionally there are commercially available implant systems[135]. However, since the oxide properties can be controlled by anodic forming voltage, current density, different electrolytes, electrolyte concentrations and temperature, agitation speed etc., the resulting surfaces present heterogeneous characteristics by means of surface chemistry, oxide thickness, morphology, surface roughness, pore configurations (pore size, porosity, pore density and crystal structure)[136, 137].

In vitro studies have demonstrated various results with either increased[100, 108] or decreased[109, 110] bone cell attachment and increased[110] or decreased[109] differentiation compared to control surfaces.

In general with some exceptions[112, 114, 121, 124], the anodized surfaces demonstrate increased bone response compared to control titanium surfaces *in vivo*[111, 117, 122, 129, 130, 132, 138]. This is attributed to the changes of topography, but also the oxide thickness, pore configurations and crystal structure of the oxide layer, where an oxide thickness of > 600 nm has demonstrated to be favorable[129, 130, 132].

However, when incorporating certain ions such as calcium[128] and magnesium[125-127, 133, 139], the increased bone response has been attributed to chemistry and a potential biochemical bond. Potentially indications of biochemical bonding (bioactivity) has been proposed on the basis of ultrastructural analysis of interfacial fracture (SEM), ion movement/exchange at the interfacial tissue (EDS) and speed and strength of implant integration to bone (RTQ)[125, 126, 139]. Furthermore, calcium incorporated anodized surfaces have demonstrated apatite formation in simulated body fluids[140, 141].

In the present thesis, a non-bioactive anodized surface is compared to possibly bioactive surfaces *in vitro* and *vivo* and additionally the surfaces are coated with covalently immobilized protein for hypothesized enhanced performance *in vivo*.

Furthermore, a potentially bioactive magnesium incorporated anodized surface was evaluated *in vitro* for the first time.

References – Anodized CP Titanium Surfaces

General

Ishizawa et al -95[107] compared anodized titanium surfaces prepared with different anodic voltage 150-400 V (50mA/cm^2), electrolytes and concentrations. Spark discharge occurred at 200V. The surfaces were characterized by SEM, EDX and XRD. Calcium acetate monohydrate and ß-glycerophosphate turned out to be suitable electrolytes, since the resulting Ca/P had a ratio equivalent to HA. HA crystals were precipitated by an additional heat treatment.

Hall and Lausmaa -00[142] introduced an anodized surface that later resulted in the commercially available TiUnite. The surfaces were characterized by Optical Interferometry, SEM, AES and XRD. The surface had a roughness of 1,2 µm (Ra), an oxide thickness of 1-2 µm at the cervical part and 7-10 µm at the apical part, a pore size in the range of 1-2 µm. The surface contained 15% Ti, 55% O, 20% C, 5% P, 1% S and 1% Si. Furthermore, it was demonstrated that the oxide layer strongly adhered to the underlying metal.

Sul et al -01[136] compared the oxide growth behavior on titanium surfaces in acid and alkaline electrolytes with different electrolyte concentrations, temperature (14-42℃), anodic forming voltage (20-130V), current forming density (5-40 mA/cm2), and agitation speed (250-800 rpm). The formed oxide surfaces were thoroughly characterized by AES and a Spectrophotometry system. It was concluded that colors were useful for thickness determination of titanium oxide and that each electrolyte presented an individual growth constant nm/V. Furthermore, a general trend that increased electrolyte concentration and temperature decreased anodic forming voltage, anodic forming rate and the current efficiency, while an increased current density and surface area ratio anode/cathode increased anodic forming voltage, anodic forming rate and current efficiency. The effects of electrolyte concentration, temperature and agitation speed were explained by the electrical double layer.

Sul et al-02[137] prepared anodic oxides by galvanostatic mode in acetic acid up to dielectric break down and spark formation (100-400V). The surfaces were characterized by Profilometry, AES, SEM, XPS, TF- XRD and Raman Spectroscopy. The results demonstrated a well characterized surface regarding surface roughness, oxide thickness, pore- size and distribution, chemical composition and crystal structure.

In Vitro

Takabe et al -00[108] compared anodized-heat treated titanium surfaces (CA/ß-GP) and titanium controls. The surfaces were characterized by Profilometry and Contact Angle Measurements. Initial cell (Rat Bone Marrow Stromal Cells) attachment, morphology and cytoskeleton were evaluated after 30, 60 and 120 minutes by Coulter counter (electrical conduction), SEM and CLSM, respectively. The anodized-heat treated surfaces were rougher, more hydrophilic and demonstrated increased cell attachment after 60 and 120 minutes compared to the controls. The cells showed a flattened surface with irregular edges and extended filipodium-like processes intimately adapted to the crystals of the surface. The actin filament was arranged parallel to the long axis of the cells and localized in the periphery.

Li et al -04[110] compared anodized-heat treated titanium surfaces (CA/ß-GP) with various anodic forming voltage (190-600V). The surfaces were characterized by Optical Interferometry, SEM-EDS, XTEM and XRD. Cell adhesion (MG-63 and Human Osteosarcoma Cells/HOS) after 3 days, proliferation after 7 days and differentiation after 10 days were evaluated by SEM, Hemocytometry and Spectrophtometry (ALP activity), respectively. The surface

roughness, oxide thickness and concentration of Ca and P ions increased with increasing voltage. In addition, there was a phase change from anatase to rutile. As a result the differentiation increased (>300 V), while the proliferation decreased (>190V). Preliminary results in vivo indicated increased removal torque values after 4 weeks for the anodized surfaces (270V).

Zhu et al-04[109] compared anodized titanium surfaces prepared in different electrolytes (CA/ß-GP and H_2PO_4) and anodic forming voltage (140-350V). The surfaces were characterized by SEM, Profilometry, XPS and Contact Angle Measurements. Cells attachment and spread (SaOS-2) after 1 and 2 hours, proliferation and differentiation after 1, 2 and 4 days were evaluated by immunohistochemistry (vinculin, phalloidin), Hemocytometry and Spectrophotometry (ALP activity), respectively. Cell attachment and proliferation increased with increasing voltage, while differentiation was similar or decreased. The cells on the anodized surfaces demonstrated a polygonal growth and lamellipodia, reflecting high motility, while the control demonstrated thick stress fibers and intense focal contacts.

Yang et al -04[141] compared anodized titanium surfaces prepared in an electrolyte (H_2SO_4) with different concentrations (0,5-3M) anodic forming voltage (90-180V), with and without subsequent heat treatment (600ºC 1h). The surfaces were characterized with SEM and TF-XRD. A simulated body fluid was used to evaluate the CaP nucleation capacity of the surfaces after 3 and 6 days. Apatite forming ability could be attained at 3 and 6 days by anodic oxidation > 90V and < 90V co-joined with heat treatment. Both the anatase and rutile was effective for apatite formation. No apatite formed on the surfaces without spark discharge (<90V) and heat treatment indicating that a certain thickness of the titanium oxide was required for apatite formation.

Kim et al -04[143] compared turned and anodized titanium surfaces (CA/ß-GP, 270V). The surfaces were characterized by Optical Interferometry, SEM and XRD. Cell (MG-63) adhesion and gene expression were evaluated after 12, 24 and 48 hours by Spectrophotometry (Crystal Violet) and Microarray technique, respectively. The anodized surfaces were rougher and displayed increased attachment of MG-63 osteoblast like cells without significantly affecting the gene expression.

Vanzilotta et al-06[140] compared CaP nucleation capacity in SBF of three surface modifications; etching and etching followed by either anodization or heat treatment.The surfaces were characterized by Profilometry, SEM-EDX and AAS, XPS before and after SBF soaking, respectively. The Ca ion concentration decreased in the SBF solution for all surfaces from day 1 to day 7. The heat treated and anodized surfaces demonstrated increased CaP nucleation capacity compared to the etched surfaces, while no differences were detected between the anodized and heat treated surfaces.

In Vivo

Larsson et al -94[120] compared machined titanium surfaces and machined electropolished with and without anodization (1M acetic acid 10 and 80V). The surfaces were characterized by SEM, AES and AFM. The surfaces differed with respect to surface oxide thickness (17-200 nm) and topography, although were similar with respect to surface composition. Bone-implant contact was evaluated in cortical bone in a rabbit model after 7 and 12 weeks. The results demonstrated decreased bone around the smooth electropolished surfaces compared to the machined surfaces with similar oxide thickness and anodized implants with thicker oxides after 7 weeks. It was concluded that a high degree of bone contact and formation were achieved by surface modifications with respect to oxide thickness and surface roughness. Furthermore, that a reduction in surface roughness influenced the rate of early bone formation.

Ishizawa et al -95[105] compared anodized titanium surfaces prepared in an electrolyte (CA/ß-GP, 350V) with different concentrations and with and without a subsequent heat treatment (300ºC , 2h) in a rabbit model. The surfaces were characterized by SEM. Turned titanium and a solid HA surface were used as positive and negative controls, respectively. The push out strengths and bone apposition increased after 8 weeks on the anodized-heat treated surface and were equivalent to HA ceramics. Furthermore, the anodized implants without heat-treatment showed increased push out strengths and bone apposition compared to the turned control surfaces. It was concluded that the good hard tissue compatibility of the implant surfaces might be attributed to the surface roughness and the possibly inhibition of titanium ion release.

Larsson et al -96[119] compared electropolished (smooth) and machined (rough) surfaces with (thick oxide) and without (thin oxide) anodization (1M acetic acid, 80V) after 1, 3 and 6 weeks. The surfaces were characterized by SEM, AES and AFM. At early stages, the smooth implants demonstrated decreased bone-implant contact compared to the machined implants irrespective of oxide layer thickness. At later stages, the thicker oxide layer increased the bone formation around the smooth surface, but not on the rougher machined surfaces. It was concluded that both topography, on the submicrometer scale, and the oxide thickness influenced the bone response to titanium surfaces. Furthermore, that reduction of surface roughness in the initial phase decreases the rate of bone formation.

Larsson et al -97[118] compared electropolished (smooth) and machined (rough) surfaces with (thick oxide) and without (thin oxide) anodization (1M acetic acid, 80V) after 1 year. The surfaces were characterized by SEM, AES and AFM. It was demonstrated that there were no significant differences between the differently prepared implant groups after 1 year. It was concluded that a reduction of surface roughness from R_q 30 nm to 3 nm, which in the initial phase decreases the rate of bone formation, had no influence on the amount of bone after 1 year in rabbit cortical bone

Ishizawa et al -97[106] compared anodized (CA/ß-GP) machined, grit-blasted and plasma-sprayed surfaces. A plasma-sprayed titanium surface and a solid HA surface were used as controls. The surfaces were characterized by SEM and XRD.Bone response was evaluated after 4 weeks in a dog model.
The anodized blasted implant showed increased bone formation compared to the smooth surface. Furthermore, the thin HA layer demonstrated quantitatively the same osteoconduction as the solid HA surface, however, with differed qualitatively.

Fini et al-99[112] compared etched (HF) titanium implants and anodized titanium implants (CA/ß-GP) prepared with and without heat-treatment. The surfaces were characterized by Profilometry, SEM, XRD and GD-OES.
Histomorphometric analysis demonstrated increased bone contact for the etched and anodized-heat treated surfaces compared to the anodized surfaces after 4 weeks, while the anodized-heat treated surfaces showed the highest values after 8 weeks in a rat femoral model.

Albrektsson et al -00[111] compared turned and anodized (TiUnite) titanium implants. The anodized implants demonstrated increased bone-implant contact and increased RTQ compared to the turned surfaces after 6 weeks in rabbit tibia and femur.

Gottlow et al -00[116] compared double etched (Osseotite) and anodized (TiUnite) implants. The anodized surfaces demonstrated increased bone-implant contact and stability by means of RFA and RTQ measurements compared to Osseotite after 6 weeks in rabbit femur and tibia.

Gottlow et al -00[115] compared double etched (Osseotite) and anodized (TiUnite) implants. The anodized implants demonstrated increased stability by means of RTQ after 10 weeks in dog mandible; however, there were no differences in bone-implant contact compared to the Osseotite implants.

Sennerby et al -00[123] compared insertion torque and stability of double etched (Osseotite) and anodized (TiUnite) implants. The anodized surface demonstrated an increased insertion torque, however, no differences in stability (RFA) after 3 weeks in rabbit tibia.

Henry et al-00[117] compared stability of anodized (TiUnite) and turned implants after 10 weeks in dog mandible. The anodized implants demonstrated a significantly increased RTQ compared to turned implants.

Rompen et al- 00[122] compared stability of anodized (TiUnite) and turned surfaces after 3 and 6 weeks in dog mandible. It was concluded that the anodized implants maintained higher primary stability during 6 weeks of healing compared to the turned controls.

Sul et al -02[132] compared anodized (acetic acid) and turned titanium implants with various oxide thicknesses (600-1000 nm and 17-200 nm, respectively) in rabbit tibia. The surfaces were characterized by Laser Scanning Profilometry, SEM, XPS, TF-XRD and AES. There were no differences in ALP and ACP activity between the surfaces with different oxide thickness. However, implants with an oxide thickness > 600 nm demonstrated increased bone-implant contact compared to the control surfaces. The increased bone response was ascribed the oxide properties including oxide thickness, pore size distribution, porosity and crystal structure.

Sul et al -02[130] compared anodized (acetic acid) and turned titanium implants with various oxide thickness (20-1000 nm) in rabbit tibia. The surfaces were characterized by Laser Scanning Profilometry, SEM, XPS, Raman spectroscopy, TF-XRD and AES. Implants with an oxide thickness > 600 nm demonstrated increased RTQ values compared to thinner layers, though there were no significant differences in RFA between the surfaces. The increased bone response were ascribed the oxide properties including oxide thickness, micropore configuration and crystal structure.

Sul et al -02[128] compared calcium ion incorporated anodized implants and turned titanium implants in rabbit tibia and femur. The surfaces were characterized by Laser Scanning Profilometry, SEM, XPS, TF-XRD, AES. The surfaces varied with respect to chemical composition, crystal structure and porosity but demonstrated similar surface roughness. The anodized calcium reinforced surface demonstrated increased RTQ values bone-implant contact and mineralization of the new bone after 6 weeks compared to turned implants. The results were ascribed the chemical composition (the Ca ions) of the implant.

Giaveresi et al -03[114] compared etched (HF) titanium surfaces and anodized (CA/ß-GP) titanium surfaces prepared with and without heat treatment. Machined surfaces and a plasma-sprayed HA surfaces were used as negative and positive control, respectively. The surfaces were characterized by Profilometry and SEM. After 8 weeks in sheep cortical bone the anodized and heat-treated surfaces showed increased push out force compared to the turned surface, while the etched surface showed decreased values compared to the machined surface. Highest values was demonstrated for the HA surface. Histomorphometric evaluation after 8 and 12 weeks revealed significantly decreased rates for the etched surface compared to the other surfaces. They concluded that there were no specific differences in behavior between the machined, anodized-heat-treated and HA surfaces.

Giaveresi et al -03[113] compared etched (HF) titanium surfaces and anodized (CA/ß-GP) titanium implant surfaces prepared with and without heat treatment. Machined surface s and a plasma-sprayed HA surfaces were used as negative and positive control, respectively. The surfaces were characterized by Profilometry and SEM. Histomorphometrical and micro hardness evaluations in sheep cortical bone after 8 and 12 weeks revealed that the anodized-heat treated surfaces had osteoconductive properties but it did not affect the surrounding bone in terms of bone remodeling or micro hardness.

Liang et al -03[121] evaluated bone bonding ability of anodized-heat treated implants (H_2SO_4, 155V + 600℃) in rabbit tibia after 4, 8, 16 and 24 weeks. The surface was characterized by FIB and FE-SEM. High bone bonding ability in early stages by means of de-attaching tests was observed for the anodized titanium surfaces compared to sodium free alkali-heat treated surfaces used in another study. The lack of improvement of bone bonding ability at later stages compared to the alkali-heat surface was explained by the low porosity of the anodized surface and furthermore, superficial apatite deposition into the pores.

Zechner et al -03[134] compared machined, anodized (TiUnite) and HA coated (Replace) implants. The anodized implants demonstrated similar histomorphometrical results in mandible of mini-pigs 3, 6 and 12 weeks as the HA coated implants, and furthermore, an increased bone response compared to the turned implant.

Son et al -03[124] compared anodized titanium implants (CA/ß-GP, 350V, 70A/m^2) prepared with and without heat treatment. The surfaces were characterized by SEM EPMA and XRD. Stability test and histomorphometrical evaluations were made after 6 and 12 weeks. There were no differences in bone-implant contact between the implants, however the anodized implants showed increased RTQ values at 6 weeks compared to the controls.

Sul et al -05[126] Compared anodized surfaces prepared with and without reinforced magnesium ions (anodized/Mg), although, with similar morphology. The surfaces were characterized by Optical Interferometry, SEM-EDS, XPS, TF-XRD. The anodized/Mg surfaces demonstrated increased RTQ values, fracture lines distant from the bone implant interface and ion concentrations gradient in rabbit femur after 6 weeks. It was concluded that this was positive evidence for the biochemical bonding theory.

Sul et al -05[133] compared magnesium ion incorporated anodized implants (anodized/Mg), and turned controls. The surfaces were characterized and evaluated by Optical Interferometry, SEM-EDS, XPS, TF-XRD. The anodized/Mg surface demonstrated increased RTQ and RFA values in rabbit femur after 6 weeks. The results were ascribed the chemical composition (the Mg ions) of the implants.

Sul et al -05[127] compared magnesium ion incorporated anodized implants (anodized/Mg), with various oxide thickness porosity, crystal structure and surface roughness. The implants were characterized by Optical Interferometry, XRD, XPS, SEM and AES. The highest removal torque values in rabbit tibia after 6 weeks were achieved with an oxide thickness of 1000-5000 nm, porosity of about 24%, surface roughness of about 0,8 µm S_a and 27-46 % S_{dr} and relative atomic Mg concentration of 9 %.

Salata et al-06[138] compared turned and oxidized titanium implants when placed in experimental bone defects with autogenous bone graft, with and without BMP-2. Results demonstrated no statistically significant differences between control and treated sites, for neither turned nor for oxidized implants by means of histomorphometry and implant stability tests (RFA) after 4 and 12 weeks in dog model. However, the oxidized implants demonstrated a significantly higher stability after 4 weeks compared to turned implants. It is concluded that oxidized implants

gained stability more rapidly and integrate with more bone contacts than implants with a turned surface when placed in bone defects.

Sul et al -06[125] compared magnesium ion incorporated anodized implants (anodized/Mg), TiUnite and Osseotite implants in rabbit tibia after 3 and 6 weeks. The implants were characterized by Optical Interferometry, XRD, XPS and FE-SEM. The anodized/Mg surfaces demonstrated increased RTQ values compared to the SLA surface after 3 weeks, while the Osseotite implants demonstrated significantly deceased values after 6 weeks compared to the other surfaces. Furthermore, histomorphometrical evaluations demonstrated increased new bone formation for the anodized/Mg surfaces compared to the others after 3 and 6 weeks. It was concluded that the comparatively rapid and strong osseointegration of the anodized/Mg implants enhanced the possibility of immediate/early loading of clinical implants.

Sul et al -06[139] compared turned and magnesium ion incorporated anodized implants (anodized/Mg), in rabbit tibia after 3 and 6 weeks. The implants were characterized by Optical Interferometry, XRD, XPS, SEM and AES. The anodized/Mg implants demonstrated increased RTQ values compared to the turned surfaces after 3 and 6 weeks. Additionally, the rate of osseointegration was increased for the andodized/Mg surface compared to the turned surface at both evaluation times. Bonding failure mainly occurred at the interface of the turned surfaces and in the immature bone for the anodized/Mg surfaces. It was concluded that the rapid and strong integration of bioactive anodized/Mg implants might encompass immediate/early loading of clinical implants.

Clinic

There are some commercially available implant systems, with TiUnite (Nobel Biocare, Gothenburg, Sweden) so far dominating the market. Anodized TiUnite implants have been clinically evaluated in approximately thirty articles since their launch in 2001 and the longest follow up period is 4 years[135]. The surface has been used in poor bone and in early loading situations where it has demonstrated good results in general with a success rate of about 95%.

Thin HA Sol-Gel Coated CP Titanium Surfaces

The sol-gel process involves the transition of a system from a liquid, the (colloidal "sol") into a solid (the "gel") phase. With further drying and heat-treatment, the "gel" is converted into dense ceramic i.e. ceramic is built starting from its molecular components in solution by a carefully controlled condensation reaction. Thin films can be produced on a piece of substrate by spin-coating or dip-coating[144]. The sol-gel process represents an alternative approach for HA coating preparations on titanium implants with potential advantages such as reduced thickness, uniform composition and lower processing temperatures[144]. Furthermore, it is a simple and cheap method and efficient for coating complex shapes[144]. TiO_2 sol-gel ceramics were the first coatings to be extensively studied for biomedical applications[145]. Studies of HA sol-gel coated CP titanium surfaces have so far mainly been performed *in vitro*[146-153] and only in a few cases *in vivo*[154]. *In vitro* HA has either been coated as a composite[148, 153] or as a double layer[150] with titania to obtain an increased bonding strength to the under lying substrate and furthermore to increase the corrosion resistance. Other approaches have been to coat the titanium surface with a composite[146] or double layer[149] of HA and FHA with the intention to improve integrity and longevity of the coating, since FHA has decreased solubility compared to HA. The HA composites have demonstrated increased bone cell differentiation[146-151] and occasionally increased bone cell proliferation[149, 151, 152] *in vitro*. An *in vivo* study has demonstrated increased bone response by means of increased bone implant contact and increased removal torque values[154]. The thickness of HA coatings made by sol-gel has so far been in the range of microns (1-10µm). Furthermore, the HA crystals have been obtained by change in phase on the surface after an additional hydrothermal treatment. In the present thesis the HA coatings evaluated were of nanometer thickness (<150 nm). Furthermore, the coating was obtained from a microemulsion that contained highly crystalline HA particles in the size range of 10-15 nm. In order to control the crystal size and crystal structure, i.e. its apatite structure, surfactant self-assembly was utilized, and by changing parameters such as temperatures and surfactant concentrations it was possible to produce the desired phase of HA. In the present thesis nano HA surfaces will be compared to other possibly bioactive surfaces *in vitro.*

References - Thin HA Sol-Gel Coated CP Titanium Surfaces

In Vitro

Ramires et al -01[153] compared sol-gel coated titanium surfaces with different titania/HA ratios (1:2, 1:1, 2:1). Titanium and TCP were used as controls. The surfaces were characterized by SEM-EDS. Cytotoxity tests performed demonstrated that the surfaces were biocompatible. The coated surfaces, especially titania/HA 1:1, demonstrated increased differentiation (ALP, collagen and osteocalcin) of MG-63 compared to the control, although with similar proliferation. It was concluded that titania/HA coating resulted in a bioactive surface due to presence of hydroxyl groups promoted Ca/P precipitation that improved the interactions with osteoblastic cells.

Kim et al -04[146] compared sol-gel coated (5µm) titanium surfaces with different FHA/HA ratios (FHA/HA ratios = 0, 0.25, 0.5, 0.75). TCP and titanium were used as controls. The surfaces were characterized by SEM, XRD and ICP-AES.
Increased concentration of FHA demonstrated lower dissolution rate after 14 days in SBF. An increase in FHA demonstrated a slight decrease in proliferation of MG-63 cells after 5 and 7 days. However, FHA/HA coatings increased differentiation (ALP) of HOS cells after 10 days compared to the control surfaces. It was concluded that the sol-gel coated surfaces improved the cell functions.

Kim et al -04[150] compared HA sol-gel coated (1 µm) titanium surfaces with and without an intervening titania layer hypothesized to increase bonding strengths to the under-laying substrate and decrease the corrosion rate. Titanium and Thermanox were used as controls.The surfaces were characterized by SEM and XRD.
Adhesion tests demonstrated that the double layer with titania increased the bonding strengths compared to a single layer of HA. Titania/HA coated surface demonstrated similar proliferation and increased differentiation (ALP activity) of HOS cells compared to control surfaces and titania coatings.

Kim et al -04[149] compared titanium surfaces coated (0,6-8 µm x2) with double layer of FHA (550ºC 30 min) and HA (550ºC 30 min) prepared by sol-gel treatment Titanium and HA were used as controls. The surfaces were characterized by XRD, FT-IR, ICP-AES, SEM and Laser Scanning Profilometry. The FHA/HA surface demonstrated biphasic dissolution behavior. The FHA/HA, FHA and HA surfaces demonstrated similar attachment after 2 and 6 hours and increased proliferation of HOS cells after 1, 3, 5 and 7 days compared to controls. Furthermore, differentiation (ALP,osteocalcin) was significantly increased after 5, 10 and 14 days on the coated surfaces compared to the CP titanium controls.

Kim et al -05[147] compared HA sol-gel coated (1 µm) titanium prepared at different temperatures (400, 500, 600ºC) and with different rate of heating (1 or 50ºC/min).
The surfaces were characterized by XRD, FT-IR, ICP-AES, SEM-EDS and Laser Scanning Profilometry. The HA films treated with higher temperature increased crystallinity, lower dissolution rate compared to controls and surfaces prepared with lower temperature, however similar roughness. Cell (HOS) attachment after 2 and 6 hours and proliferation after 2, 4 and 7 days were similar on control and coated surfaces. There was a slight increase in differentiation after 7 and 14 days (ALP, Osteocalcin) on the HA films compared to the controls. Increased heating rate demonstrated a 4-6 times rougher surface with a slightly higher dissolution rate, enhanced cell attachment, but no difference in differentiation.

Kim et al -05[148] compared sol-gel coated (<1 µm) titanium surfaces with different titania/HA ratios (10, 20, 30, 40 mol %). HA and titanium were used as controls.

The surfaces were characterized by XRD, SEM-EDS and Laser Scanning Profilometry. Adhesion tests showed that the HA/titania composites showed increased binding strengths (56 MPa) compared to HA (35 MPa). The composites demonstrated similar proliferation of HOS cells after 1, 3 and 5 days compared to control and HA. Furthermore, increased differentiation (ALP) after 7 days compared to the other surfaces with the highest concentrations obtained by 20% titania/HA.

Sato et al-05[152] compared PLGA/titania sol-gel coated titanium prepared with and without HA and subsequent heat treatment. The surfaces were characterized by XRD, EDS, AFM, ICP-AES SEM and Contact angle measurements. The adhesion of MG-63 cells were similar on the sol-gel HA compared to traditional HA surface and heat-treatment improved the adhesion.

Li et al-05[151] compared anodized titanium surfaces with and without an additional HA so-gel coating with different concentrations.
The surfaces were characterized by XRD, SEM-EDS and Laser Scanning Profilometry. Sol-gel treatment increased Ca and P concentration, while roughness did not change. The HA coated surfaces demonstrated an increased proliferation after 5 days and differentiation after 5 days (ALP) compared to the anodized surfaces.

In Vivo

Ramires et al -03[154] compared titanium surfaces coated (<10 µm) HA/titania and BG/titania by sol-gel treatment (550ºC 30 min).
The surfaces were characterized by a Laser Scanning Profilometry.
In vitro and *in vivo* results were in good agreement. *In vitro* results showed increased differentiation of MG-63 cells (ALP, osteocalcin , collagen) compared to control, while *in vivo* evaluation showed increased RTQ and bone-implant contact compared to the titanium control in rabbit femur and tibia after 12 weeks.

Clinic

So far, there are no commercially available Nano HA coated dental implant systems.

Proteins Covalently Immobilized to CP Titanium Surfaces

The aim of biochemical surface modifications is to induce specific cell and tissue response, i.e. to control the tissue implant interface. The biomolecules are administrated to the surface either by adsorption or by covalent immobilization. Covalent immobilization is hypothesized to provide an opportunity to align and expose the appropriate active site for a more rapid physiological response[155]. Furthermore, the coupling and cross linking is suggested to suppress the fast in vivo diffusion and degradation of the coating and thereby decrease the quantity required[155].

Polymeric substrates have been extensively studied because they possess abundant reactive functional groups for coupling, studies have been performed mainly in cardiovascular models, where the impact of interactions may be more obvious, with implications e.g. for the blood contact activation.

Early surface modifications of titanium surfaces were concerned with improvement of adhesion of soft tissue to provide a stable seal towards bacterial invasion.

Approaches in hard tissue implant research involve immobilization e.g. of the peptides (RGD), extra cellular matrix proteins (collagen) and growth factors (BMPs).

Modifications of metallic implants with RGD containing peptides have been performed by covalent immobilization, while proteins like collagen and growth factors have mostly been carried out by adsorption immobilization or embedding in degradable polymers such as PLGA.

A commonly used technique to covalently attach peptides and proteins to CP titanium surfaces is silanization (amino-functionalization)[156, 157] with additional coupling agents such as glutaraldehyde[158-160], IPN[161, 162] or Star-PEG[163]. Other techniques presented are based on polymers (PLL-g-PEG)[164, 165], multimeric phophonates[166] and gold-thiol chemistry[167, 168].

RGD is an amino acid sequence (Arg-Gly-Asp) in many ECM proteins that has been identified to mediate cell attachment to the integrin receptors. Binding activates signalling pathways, which are able to stimulate different cell functions like migration, proliferation, differentiation or matrix mineralization.

Some *in vitro* studies have demonstrated an increased differentiation and matrix mineralization with RGD coated titanium implants[161, 162, 168] , while others have failed to demonstrate convincing results[163, 164, 169].

Furthermore, RGD coated titanium implants have demonstrated early increased bone formation but no additional increase in stability *in vivo*[165, 167].

Collagen is the main component of ECM of bone and serves as scaffold for mineralization. Covalently immobilized collagen has been shown to increase bone cell response in vitro[170] and bone response *in vivo*[171-174].

On the contrary, it has been shown that native ECM produced by bone cells in vitro on titanium surfaces increased cell densities compared to covalently immobilized peptides and protein coating[175].

Few studies has dealt with covalently immobilized growth factors to titanium surfaces and there is no[176] or weak[177] evidence of increased bone response *in vitro* and *in vivo.* Bone cell attachment, collagen and growth factor production, however, appear in later stages of the healing phase.

In the present thesis attempts were made to intervene in earlier stages i.e. the inflammatory phase. Covalent immobilization of blood proteins such as fibrinogen and blood plasma on titanium surfaces has so far only been studied *in vitro*[178] and *in vivo* soft tissue models[158-160].

References – Proteins Covalently Immobilized to CP Titanium Surfaces

In Vitro Peptides

Xiao et al -97[156] were among the first to covalently immobilize (APTES, SMP) the RGDC peptide to CP titanium. The individual steps were semi-quantitatively characterized by XPS, Radio Labeling Techniques and Ellipsometry. It was concluded that the achieved surface coverage was expected to be enough for specific surface-cell interactions.

Barber et al -03[162] covalently immobilized (APTES/Interpenetrating polymer network - IPN) RGD from Rat bone sialoprotein. The surfaces were characterized by Contact Angle Goniometry and XPS.
Primary rat calvarial osteoblasts showed decreased attachment and spreading after 4, 24, 68 and 142 hours compared to TCPS and titanium, but significantly higher mineralization after 28 days compared to control titanium and the negatrive control surfaces IPN-RGE. It was concluded that the RGD surfaces to a greater extent promoted a more mature osteoblast than the titanium controls.

Barber et al -06[161] covalently immobilized (ATC/IPN) RGD from Rat Bone Sialoprotein with different concentrations. The surface was characterized by Fluorometry and XPS. Primary rat calvarial osteoblast attachment, spreading and differentiation were evaluated. The results demonstrated that surfaces with higher RGD densities (> 0.1 pmol/cm^2) showed significantly higher mineralization (ALP) at later stages compared to titanium, RGD with lower densities and IPN-RGE.
It was concluded that RGD peptide coated surfaces with certain densities enhanced the kinetics of differentiation.

Huang et al -03[168] compared two types of covalently immobilized (gold SAM) peptides, RGDC and RDGC. XPS and FTIR were used for surface characterization.
Primary calvarial osteoblasts were cultured and cell attachment, morphology, proliferation, and gene expression were assessed by Coulter counter, Immunofluorescence, Coulter counter and Northern blot, respectively. Four and 8 hours after culture, cell attachment was enhanced on the peptide surfaces compared to the control. Furthermore, osteoblasts on RGDC surfaces showed earlier osteocalcin mRNA expression (day 15) compared with controls (day 21). It was concluded that osteoblasts functions were enhanced on the RGDC coated surface, which might be effective in improving osseointegration for dental implants.

Senyah et al -05[169] compared attachment and morphology of MC3T3 –E1 osteoblast like cells on different immobilized (APTES) RGD peptides after 48 hours. The surfaces were characterized by AFM, SEM and CLSM.
Results demonstrated that the RGD sequence is not necessarily required to enhance adhesion of cells. Instead, the attachment was correlated to surface hydrophobicity caused by the different RGD peptides.

Auernheimer et al -05[166] presented a method for covalently immobilizing tailor made cRGD peptides through multimeric phophonates to titanium surfaces.
Characterization was made by Radio labeling and ELISA. Several advantages of the technique were presented; stable against enzymatic degradation, no risk of disease transmission, no risk of thrombosis formation.

Groll et al -05[163] evaluated covalently immobilized (Star-PEG) linear RGD coatings with different concentrations. The surfaces were characterized by Scanning force microscopy, Ellipsometry and Contact angle measurements. Adhesion and spreading of fibroblasts, SaOS and hMSC cells were recorded up to 30 days on the RGD surfaces, whereas no cell adhesion could be detected on unmodified Star PEG surfaces. Furthermore, increased RGD

concentrations increased the cell amount and spreading. Since differentiation of the hMSC cells after 14 days were comparable with TCPS, it was concluded that the PEG/RGD films did not negatively influence the differentiation process.

Schuler et al -06[164] compared cell adhesion and spreading patterns of epithelial cells, fibroblasts and osteoblasts on covalently immobilized (PLL-g-PEG) RGDSP with different concentrations, RDG and titanium control (SLA). The surfaces were characterized by AFM, SEM and XPS. Fibroblast attachment increased on the smooth surfaces, while there was an opposite tendency for the osteoblasts. The epithelial cells did not follow any regular pattern. In general, attachment and spread increased on the RGD containing surfaces compared to RDG and PEG. Furthermore, osteoblast attachment increased with increasing RGD concentrations resulting in similar attachment as control titanium at densities ≥ 0,67 pmol/cm^2.

In Vitro Proteins

Nanci et al -97[155] presented a method to covalently immobilize (APTES, glutaraldehyde) ALP and albumin to titanium surfaces. The surfaces were characterized by SEM, XPS and AFM. Evaluation by immunohistochemistry revealed that the proteins were linked at biologically relevant densities, and retained their enzymatic activity and antigenicity.

Jennissen et al -99[157] presented a method to covalently immobilize (APS/CDI) labeled model proteins (125I-ubiquitin) to CP titanium powder and polished as well as anodized disc surfaces. On the anodized surfaces model protein were coupled in 2-3 fold concentrations compared to polished surfaces. Furthermore, the biocoating technique was applied to rh-BMP-2 on titanium implants with the aim of constructing specific juxtracrine bone cell-reactive interfaces (Jennissen, H.P et al -95 and -97).

Pham et al -04[175] compared titanium surfaces coated with covalently immobilized RGD peptid (APTES), fibronection (APTES) or native ECM secreted by the SaOS-2 cells for 4 days. CP titanium was used as control. The removal of cells by NH_4OH was verified by LDH and ALP. The surfaces were characterized by SEM-EDS and AFM. An increased cell density was demonstrated for the native ECM surfaces after 4 hours. However, it was concluded that it could be excluded that cells adhered to remnants of cell surface proteins.

Muller et al -06[170] evaluated collagen covalently immobilized (APS/EDC/NHS) on surfaces prepared with different oxides (etched, anodized). The surfaces were characterized by XPS, AFM and Profilometry. The concentrations of interfacial bonds and the stability of cross-linked proteins increased with increasing oxide layer (OH groups). Furthermore, the cross-linked collagen layer improved cellular response (Human osteoblast-like cells/MG-63) in vitro.

In Vivo Peptides

Ferris et al -99[167] compared implants with covalently immobilized (gold-thiol chemistry) RGD peptides and control gold coated titanium surfaces in rat femur. The study did not include any non specific control. No surface characterization was available. Pull out tests and histomorphometry were performed after 2 and 4 weeks.
There were no significant differences between the surfaces when tested mechanically after 2 and 4 weeks. However, after 4 weeks the RGD implants demonstrated a significant increase in bone thickness. It was concluded that the RGD coated surface might enhance osseointegration.

Germanier et al -04[165] compared the SLA surface with and without covalently immobilized (PLL-G-PEG) RGD, RDG in a mini-pig model. Bone implant contact was increased after 2 weeks, while the surface after 4 weeks showed decreased bone implant values compared to the other surfaces. It was concluded that the RGD coated SLA surface promoted enhanced osseointegration during early stages.

Schliephake et al -05[174] compared machined titanium surfaces, covalently immobilized (anodic polarization/EDC) collagen and collagen coated implants with high and low concentrations of an additional immobilized (acrylat) RGD peptide in dog mandible. After 1 month, BIC was significantly enhanced only in the group of implants coated with the higher concentration of RGD peptides. Volume density of the newly formed bone was significantly higher in all implants with organic coating. No significant difference was found between collagen coating and RGD coatings. After 3 months, BIC was significantly higher in all implants with organic coating compared to implants with machined surfaces.

Bernhardt et al -05[179] compared titanium implants and titanium implants covalently immobilized (anodized) with either collagen type I, type III, or immobilized (phosphonate) RGD peptide in femur of goats. Histomorphometry and Micro CT were used for evaluation. All three coatings demonstrated a significant increase in bone volume compared to the uncoated controls after 5 and 12 weeks with the highest results for the collagen coatings. The coating with the RGD-sequence showed only a slight improvement compared to the control surfaces. While collagen type I demonstrated increased response in denser bone, collagen type III, appeared to be the more effective coating in areas of lesser bone density.

In Vivo Proteins

Jansson et al -01[158] compared machined and alkali-heat treated implant surfaces with and without a covalently immobilized (APTES/EDC/NHS) blood plasma coating in soft tissue rat model after 7 and 28 days. The thickness of the fibrous capsule, the fluid space width between implants and fibrous capsule, and formation of blood vessels were evaluated. The results demonstrated the thinnest fluid space for the plasma clot coated porous titanium surface compared to the untreated surfaces controls. The number of vessels and proportion of vessels in the fibrous capsule increased with time for the treated implant surfaces compared to the control titanium surfaces. It was concluded that the healing process around titanium could be modulated by porosity and thin pre-prepared plasma coatings.

Jansson et al -02[159] compared the release of inflammatory reactions around machined and alkali-heat treated implants with and without a covalently immobilized (APTES/EDC/NHS) blood plasma coating inserted subcutaneously in rats after 3 and 24 hours. Ex vivo PMA-stimulated oxygen radical production, cell recruitment, TNF-alpha secretion and cell-type pattern in the exudates around the porous plasma-coated implant were evaluated. It was concluded that the healing process around blood plasma coated titanium surfaces were more sham-like than references without blood plasma coating.

Morra et al -03[171] evaluated covalently immobilized (acrylic acid grafting/EDC/NHS) collagen coated implants. The surfaces were characterized by XPS, ATR-IR and AFM. In vitro cell (SaOS-2) demonstrated decreased proliferation and no difference in differentiation compared to the untreated surface after 1, 4 and 10 days. However, treated surfaces showed increased bone response after 4 weeks in rabbit femur and no adverse effect in rabbit soft tissue after 12 weeks.

Morra et al -05[173] compared bone micro-hardness around covalently immobilized (plasma deposited acrylic acid grafting/EDC/NHS) collagen coated implants in rabbit femur after 4 weeks. The surfaces were characterized by XPS. The micro-hardness measurements demonstrated improved bone maturation and mineralization at the interface of collagen coating compared to untreated CP titanium.

Morra et al -06[172] evaluated covalently immobilized collagen (acrylic acid grafting/EDC/NHS) coated anodized titanium implants. The surfaces were characterized by XPS and SEM. The collagen coated surfaces showed increased bone-implant contact after 4 weeks in rabbit femur and enhanced cell growth of human mesenchymal cells after 12, 24 and 72 hours.

Becker et al -06[176] compared bone formation around sandblasted acid etched titanium implants prepared with and without covalently and non-covalently bound rhBMP-2 in a dog model. The bone-implant contact and bone densities were evaluated after 4 weeks. The covalently and non-covalently bound rhBMP-2 implant surfaces demonstrated a similar but increased bone response compared to control implants. It was concluded that rhBMP-2 covalently and non-covalently immobilized by this method seemed stable and promoted direct bone apposition in a concentration dependent manner and that an optimal method for BMPs still is lacking.

Seol et al -06[177] evaluated implants with covalently immobilized (APTES/EDH/NHS) synthetic receptor motif mimic BMP-2 (10 amino-acids). The surface was characterized with XPS and Gamma counting (radioactivity).
In vitro test were conducted by osteoblast like MC3T3-E1 cells to evaluate proliferation after 1, 7, 14, 21, 30 days and differentiation after 10 days. In vivo tests were conducted in a dog mandible model at 4 weeks. The results showed increased cell activity and bone response on the BMP-coated surfaces compared to the control.

Clinic

So far, there are no commercially available covalently immobilized protein coated dental implant systems.

Aims

- To investigate the significance of surface orientation for bone tissue response *in vivo.*

- To investigate bone response *in vivo* to non-bioactive titanium (anodized) and possibly bioactive (alkali-heat treated, covalently immobilized protein coatings) titanium implant surfaces.

- To investigate early inflammatory response *in vitro* to CP titanium surfaces with non-bioactive (anodized) and possibly bioactive (anodized/Mg) titanium implant surfaces.

- To investigate early inflammatory response *in vitro* to titanium surfaces coated with candidate pro-inflammatory (fibrinogen) and anti-inflammatory (catalase) proteins.

- To investigate calcium phosphate nucleation capacity and subsequent bone cell response *in vitro* to four possibly bioactive titanium implant surfaces (alkali-heat treated, anodized/Mg, fluoride and nano HA coated).

Materials and Methods

Implant and Sample Preparation

Implant Design

Commercially pure titanium (grade 3) screw shaped implants were used in Study I and II, while commercially pure titanium (grade 3) circular discs were used in Study III, IV and V.

Surface Preparations

Paper :	I	II	III	IV	V
1. Turned	11		72	72	
2. Turned + fibrinogen				72	
3. Turned + fibrinogen + blood plasma		11			
4. Turned + fibrinogen + catalase				72	
5. Blasted	11		72		64
6. Fluoride etched					38
7. Alkali-heat treated (AH)		11			38
8. Alkali-heat + fibrinogen + blood plasma		22			
9. Anodized (TiUnite)		11	72		
10. Anodized (TiUnite) +fibrinogen + blood plasma		11			
11. Anodized/Mg ion incorporated (TiMgO)			72		38
12. Nano HA					38
Total number of surfaces :	22	66	288	216	216

Table 1: Titanium surface modifications and number of differently modified surfaces per study

Turned

Commercially pure titanium discs and implants machined by a turning process.

Turned + Fibrinogen

Turned implants and discs coated with a monolayer (Study II) or eight layers (Study IV) of covalently immobilized rabbit fibrinogen, respectively. The surfaces were pre-conditioned with 3-aminopropyltriethoxysilane (APTES)
and glutharaldehyde[155, 158]. Between each layer of fibrinogen, cross-linking was achieved by incubation in PBS solution, containing ethyl-dimethyl-aminopropylcarbodiimide (EDC, Sigma, USA) N-hydroxy-succinimide (NHS, Sigma, USA). For detailed information about the protein coating preparations, see Study III and IV.

Turned + Fibrinogen + Plasma

Turned implants coated with a monolayer of covalently immobilized rabbit fibrinogen (Sigma) and additionally four layers of rabbit blood plasma (Bacteriological laboratory of Sweden). The surfaces were preconditioned with 3-aminopropyltriethoxysilane (APTES) and glutharaldehyde[155, 158]. Between each layer of blood plasma, cross-linking was achieved by incubation in a HEPES buffer, containing thrombin and $CaCl_2$. For detailed information about the protein coating preparations, see Study II.

Turned + Fibrinogen + Catalase

Turned implant coated with eight layers of covalently immobilized rabbit fibrinogen (Hyphen Biomed, Haemochrom Diagnostica), Sweden and additionally two layers of catalase. The surfaces were preconditioned with 3-aminopropyltriethoxysilane (APTES) and glutharaldehyde[155, 158]. Between each layer of protein coating, cross-linking were achieved by incubation in a PBS solution, containing ethyl-dimethyl-aminopropylcarbodiimide (EDC, Sigma, USA) N-hydroxy-succinimide (NHS, Sigma, USA). For detailed information about the protein coating preparations, see Study IV.

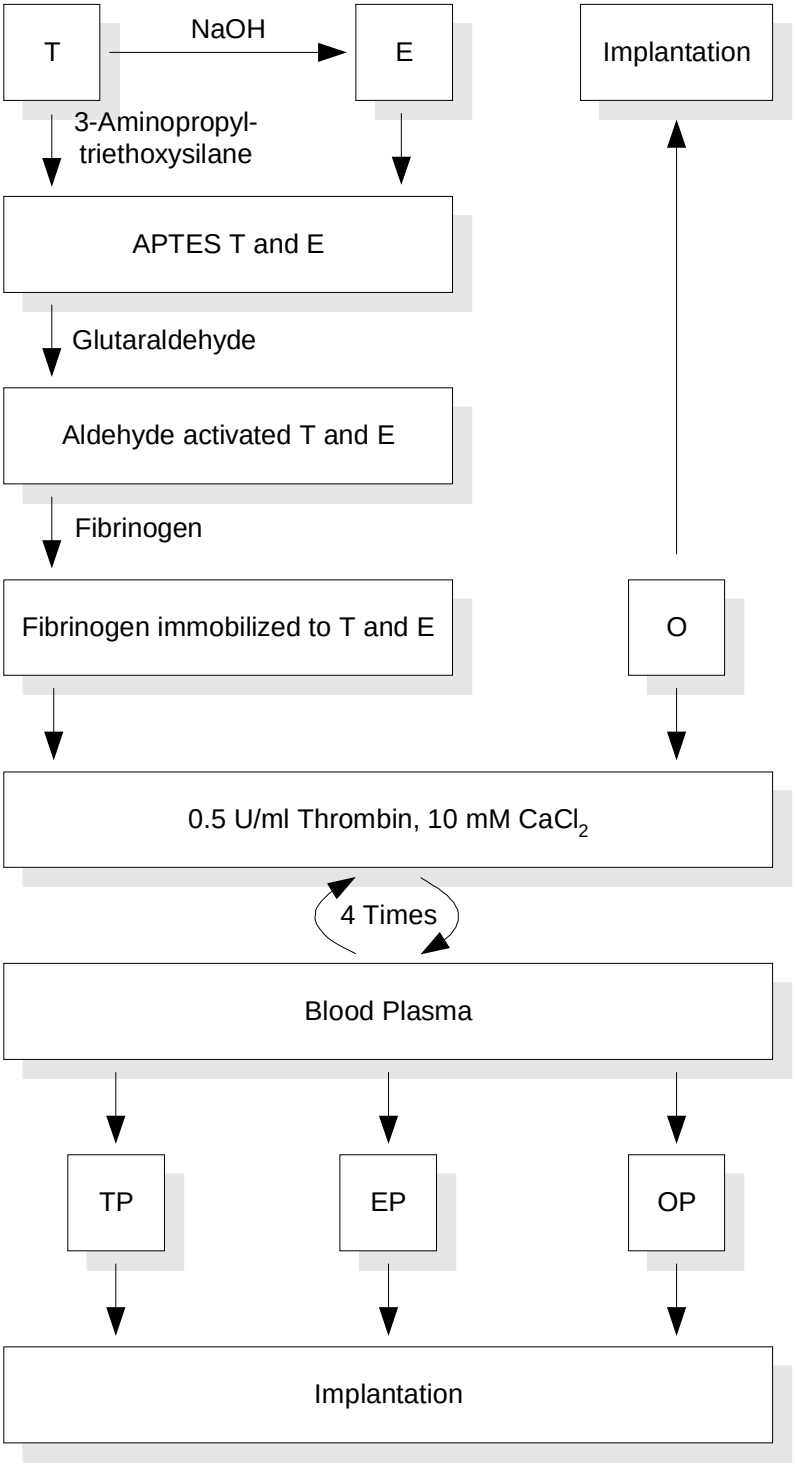

Figure 1: Preparation schedule for implants surfaces in Study II: turned implant surface (T), blood plasma coated turned implant surface (TP), alkali-heat treated implant surface (E), blood plasma coated alkali-heat treated implant surface (EP), anodized/TiUnite implant surface (O) and blood plasma coated anodized/TiUnite implant surface (OP).

Blasted

Turned implants blasted either with Al_2O_3 particles with a medium grain size of 75 µm or with TiO_2 particles with a medium grain size of 7.5-12.5 µm.
Air pressure during the blasting process was 0.35 MPa[44].
The idea with the small TiO_2 particles was to create a blasted surface that was similar to a turned surface with respect to S_a, though, that differed with respect to S_{cx} value and in this way enable evaluation of surface orientation in a controlled manner. The turned surfaces were anisotropic demonstrating a dominant direction of the surface topography, while the blasted surfaces were isotropic with no main direction of surface topography.

Fluoride Etching

Turned discs blasted with Al_2O_3 (75 µm) immersed in an aqueous solution of NaF[79].

Alkali-Heat Treatment

Turned implants (Study II) and turned discs blasted (Al_2O_3 powder 75 µm, Study V), immersed in an aqueous solution of 5 M NaOH for 24 hours at 60℃, followed by gentle rinsing in distilled water before driying for 24 hours at 40ºC.
The implants were additionally subjected to hydrothermal treatment at 600ºC by increasing the temperature by 5ºC/minute in air in an electrical furnace.
The implants were kept at 600ºC for one hour and, thereafter, allowed to cool to room temperature in the furnace[85, 180].

Alkali-Heat Treatment + Fibrinogen + Blood Plasma

Alkali-heat treated implants[85, 180] coated with a monolayer of covalently immobilized rabbit fibrinogen and in addition four layers of rabbit plasma clot films.
The surfaces were preconditioned with 3-aminopropyltriethoxysilane (APTES) and glutharaldehyde[155, 158]. Between each layer of blood plasma, cross-linking was achieved by incubation in a HEPES buffer containing thrombin and $CaCl_2$. (Figure 1)

Anodized (TiUnite*)*

TiUnite™ (Nobel Biocare AB, Göteborg, Sweden) implants (Study II) and discs (Study III) prepared by electrochemical anodization[115] with a TiUnite like surface, provided by the manufacturer.

Anodized (TiUnite) + Fibrinogen + Plasma

TiUnite™ (Nobel Biocare AB, Göteborg, Sweden) implants coated with a monolayer of covalently immobilized rabbit fibrinogen and in addition four layers of rabbit blood plasma clot films. The surfaces were preconditioned with 3-aminopropyltriethoxsilane (APTES) and glutharaldehyde[155, 158]. Between each layer of blood plasma, cross-linking was achieved by incubation in a HEPES buffer, containing thrombin and $CaCl_2$. (Figure 1)

Anodized/Mg Ion Incorporation (TiMgO)

Turned (paper III) and blasted (Al_2O_3 powder 75 µm) (Study V) discs were prepared by the electrochemical anodization method[136, 137] in a mixed electrolyte containing magnesium ions using Micro Arc Oxidation (MAO) in galvanostatic mode. The electrochemical cell was composed of two platinum plates as cathodes and the titanium anode at the centre. Current and voltages were continuously recorded at intervals of one second by an IBM computer interfaced with a DC power supply. (Figure 2)

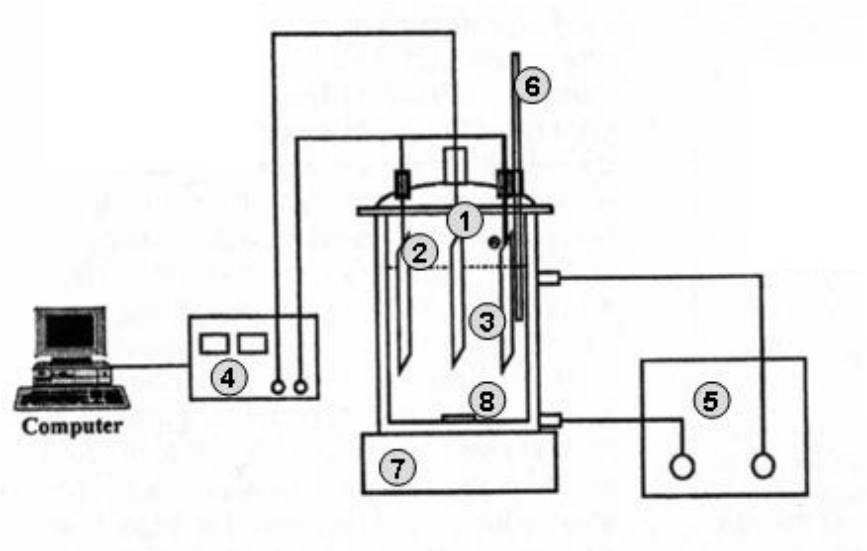

Figure 2: Schematic illustration of Anodizing Apparatus: 1.Ti anode 2. Pt cathode 3.Power supply 4.Cooling system 5.Thermostater 6.Stirrer 7.Stirring bar (Sul Y.T. Thesis 2004)

Nano Hydroxyapatite Coating

Turned discs were blasted with Al_2O_3 (75 µm) and dipped in a stable sol, which contained surfactants, water, organic solvent and crystalline nanoparticles of hydroxyapatite (approximately 10 nm) with a Ca/P ratio of 1.67. The discs were dried for half an hour in open air, followed by a hydrothermal treatment at 550ºC for 5 minutes in nitrogen atmosphere.

Cleaning Procedure

In Study I, III and V the implants were ultrasonically cleaned in diluted Extran MA01 and absolute ethanol, respectively, and dried at 60ºC for 24 hours.
In Study II and IV the implants were washed at R.T. in 99.5% ethanol (Kemetyl, Sweden) in an ultrasonic bath during 5 minutes. The ethanol was changed for distilled water and the procedure was repeated. Finally, the surfaces were dried in flowing N_2 and exposed to UV-light (UV-cleaner from Novakemi, Sweden) during 5 minutes.The surfaces were additionally tested for the presence of endotoxin by Limulus Amebocyte Lysate assay (E2 108; Sigma USA, LAL-test Sahlgrenska University Hospital, Sweden).

Surface Characterization

Topographical Analysis

All surface modifications were topographically evaluated in triplicate and each disc was measured on three areas, while the screw was measured on 9 sites of the threaded area, 3 tops, 3 valleys and 3 flanks.
Thereafter one height, one spatial and one hybrid parameter were used to numerically describe the roughness[43].

S_a = Arithmetic mean height deviation from a mean plane.
S_{cx} = Mean spacing between the surface peaks along the x-axis.
S_{ds} = Density of summits, i.e. the number of summits of a unit sampling area.
S_{dr} = Developed interfacial area ratio, i.e. the ratio of the increment of the interfacial area of a surface over the sampling area.

Confocal Laser Scanning Profilometry

TopScan 3D®, (Heidelberg Instruments, Heidelberg) is based on the confocal laser scanning principle. A small laser spot helium/neon laser beam (λ:633 nm, Ø:1 µm) scans the surface, and a wide aperture objective focuses the laser onto the surface, and thereby the focus of light is adjusted depending on the on sample surface topog-

raphy. Only light reflected from the focal plane reaches the point detector for further computer evaluations, while the light not reflected from the focal plane is removed with two blocking filters (pinholes). Simultaneously measuring the reflected laser light and the coordinate position of the laser spot enables measurements of surface topography. Additionally a Gaussian filter is applied to separate roughness from form and waviness. The optimal lateral and vertical resolution of the instrument are 0.5 µm and 6 nm respectively. Furthermore, maximal measurement area and vertical range are 2x2 mm and 108 µm respectively. (Figure 3)

Optical Interferometry

MicroXam™, (Phase-Shift, Tucson, Arizona, USA) is an optical interferometer based on the principles of interference; light (λ :550 nm) is directed towards the sample surface and interference fringes between the incident and reflected light are detected at different positions depending on the surface topography.

The optimal lateral and vertical resolution of the instrument are 0.3 µm and 0,05 nm respectively. Furthermore, maximal measurement area and vertical range are 5.3x4 mm and 5 mm, respectively. (Figure 4)

Scanning Electron Microscopy (SEM)

For the SEM analysis, a LEO Ultra 55 FEG (Zeiss, Oberkochen, Germany) operating at 10kV was used. All samples were examined without surface sputtering. Micrographs were recorded at different magnifications at several randomly chosen areas.

Chemical analysis

X-ray Photoelectron Spectroscopy (XPS)

Surface chemistry was analyzed by XPS (PHI 5000C ESCA System, Perkin Elmer Wellesley, USA) with an operating angle of 45° at 300W using a Mg *K* exitation source. An average was calculated from two separate analyses at approximately the center of the disc and at the edge of the disc for two discs.

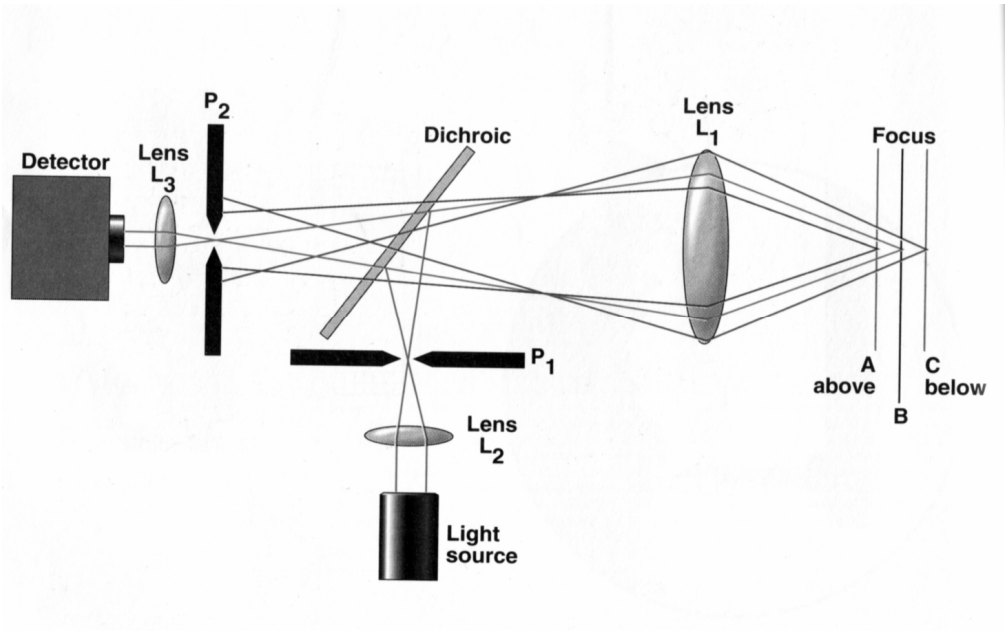

Figure 3: Schematic illustration; Principle of Laser Scanning Profilometry

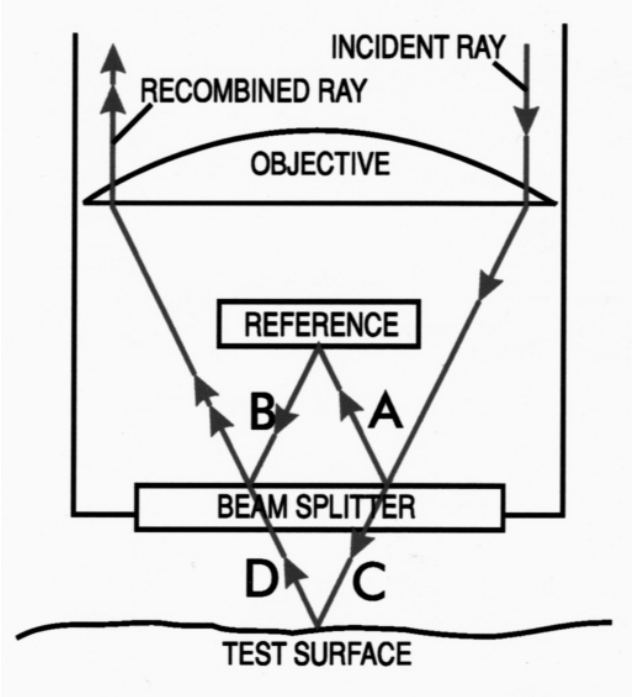

Figure 4: Schematic illustration; Principle of Optical Interferometry (ADE Phase Shift Technology)

Study Design

In Vitro

Simulated Body Fluid Immersion

Simulated body fluid (SBF) is a solution with ion concentrations approximately equal to those of human blood plasma. The revised SBF (r-SBF) described by Oyane and co-workers[181] was prepared by dissolving various salts in 1000 ml distilled water. HEPES was dissolved in 100 ml distilled water before being added to the solution, and the final pH was adjusted to 7.4 at 37ºC with 1.0 M NaOH. Each specimen was immersed in 25 ml r-SBF in separate sealed polystyrene vials and kept at 37ºC. After immersion, the SBF immersion the specimens were thoroughly rinsed with distilled water and then dried at room temperature. For details, see Study V.

Cell Culture Monocytes

Buffy coats from human blood doors were subjected to a two-step gradient centrifugation according to the method of Pertoft and co workers[182]. Mononuclear cells obtained by centrifugation were washed in HBSS and centrifuged a second time to extract a pure fraction of monocytes[183]. The isolated cells, containing mainly monocytes, were harvested, washed, counted and re-suspended in supplemented RPMI 1640 at a concentration of 10^6 cells/ml. Half the numbers of discs were immediately treated with lipopolysaccaride (LPS) to evaluate the effect of exogenous stimuli, while the other half the number of discs were left without any stimuli and served as controls. Discs with cells were cultured in 37ºC, 100% humidity and 5% CO_2. For details, see Study III or Study IV.

Cell Culture Osteoblasts

Human mandibular bone specimens without any clinical or radiographic evidence of pathology were obtained from healthy patients. After culture of the bone fragments for 3–4 weeks in a humidified atmosphere of 95% air, cells that had migrated from the bone fragments were harvested and characterized using three different assays[63, 64]. Only cells expressing high levels of 1,25-$(OH)_2D_3$-responsive alkaline phosphatase (ALP) activity and with the capacity to synthesize osteocalcin were used in the study. A total of 2×10^4 cells/cm^2 were seeded on each titanium surface. The

minimum essential culture medium, alpha modification (α-MEM; Gibco, Grand Island, NY, USA), supplemented with penicillin/streptomycin solution and L-glutamine was used. During the experiments 10% fetal calf serum (FCS) was added to the medium. In all experiments, cells were used at first passage. For details, see Study V.

In Vivo

Animals and Surgical Technique

The local committee at the University of Göteborg Sweden approved the animal studies. Adult female New Zealand White Rabbits were used in the experiment.

Presurgery: General anaesthesia was induced by intramuscularly injections of Hypnorm® (Janssen Pharmaceutical LTD, Oxford, England) and intraperitoneal injections of Stesolid Novum® (A/S Dumex Denmark). If required during surgery, additional doses of Hypnorm® and Stesolid Novum® were administered. The hind legs were shaved and antibiotics were administered prophylactically. Immediately before surgery Xylocain® (Astra, Sweden) was injected to each insertion site.

Surgery: The operations were performed under aseptic conditions. The skin and fascia were opened in separate layers and the periost was gently pushed aside. All implant sites were prepared using low rotatory speed and irrigation of saline. In Study I, one test and one control implant was inserted into the distal condyles of left and right femurs respectively. In Study II one implant was inserted into the distal femoral metaphysis and two into the proximal tibial metaphysis, and each animal served as its own control. All implants were anchoraged in one cortical layer only. (Figure 5)

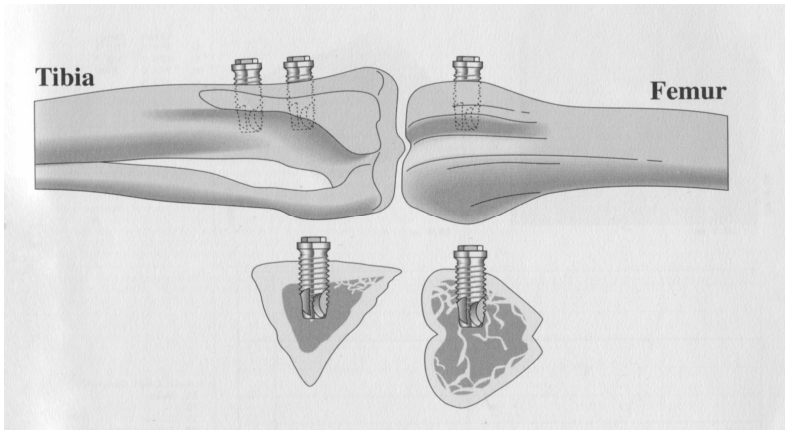

Figure 5: Schematic illustration; Principle of implant placement in rabbit tibia and femur.

Postsurgery: All rabbits received analgesia subcutaneously (Temgesic™, Reckitt Coleman, Hull, England). The animals were kept in separate cages immediately after implant insertion with the purpose to control the healing process and, as soon as the circumstances so allowed, they were free to return to room were they were kept uncaged in large groups. The rabbits were fed with standard pellets and had free access to tap water. Full weight bearing was allowed immediately after surgery. After complete healing time the animals were sacrificed with an intravenous overdose injection of Pentobarbital (Apoteksbolaget, Malmö, Sweden).

Evaluation Methods

In Vitro

Simulated Body Fluid

Calcium Phosphate Formation

The CaP formation was evaluated topographically by Interferometry (see page 44) and chemically by XPS (see page 44). Furthermore, bone cells were cultured on the immersed surfaces and subsequent cell attachment, osteocalcin and TGF-β production was evaluated (see page 49).

Cell Culture Monocytes

Cell Viability

Cell viability was estimated by analyzing the content of lactate dehydrogenase (LDH) in the culture medium. Damaged cells release the cytoplasmatic enzyme LDH, where LDH mediated conversion of pyruvate to lactate in the presence of NADH and 2, 4-dinitrophenylhydrazine. The concentrations of NADH were measured colorimetrically (Sahlgrenska University Hospital, Sweden) and thus the amount of LDH could be determined.

Tumor Necrosis Factor-α and Interleukin-10

Cytokine concentrations in the harvest supernatant were assessed using a commercially available ELISA assays (Quantikine®, R&D Systems, UK). The standard and the samples (TNF-α or IL-10) were added to the antibody-coated well, followed by

the addition of a secondary antibody conjugated to horseradish peroxidase (HRP). Bound enzymes labeled antibodies were detected through a chromogenic reaction and absorbance were measured with a micro plate reader (Vmax, Molecular Device, UK). The concentrations of TNF-α and IL-10 in the samples were determined by interpolation from the standard curve. (Figure 6). For details, see Study III and IV.

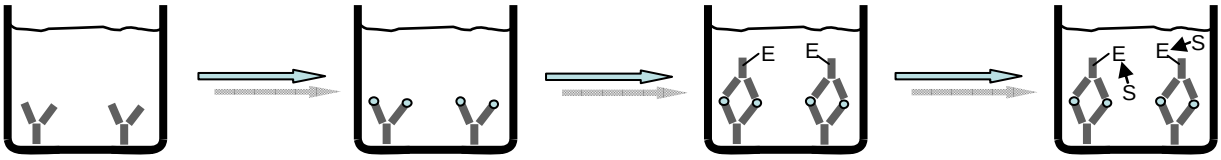

Figure 6: Schematic illustration; Principle of ELISA

Cell Attachment and Differentiation

Numbers of adhered and differentiated cells were evaluated by immunohistochemistry. The discs with the adherent cells were fixed in absolute ethanol for 15 min –20ºC after the different culture periods. The cells were pre-incubated to reduce non-specific binding and then incubated with monoclonal antibodies RM3/1 and 27E10 (Biogenesis, UK). Bound antibodies were detected by sequential incubation with biotinylated secondary antibody, and FITC-conjugated avidin (Amersham, UK). The cells were immunolabelled and mounted with Vectashield DAPI (4,6-diamidino-2-phenylindole) (Vector lab., Burlingame, USA) and examined in Nikon Mikrophot FXA epifluoroscence microscope. Photos of DAPI and 27E10 or RM3/1 positive cells were taken in five randomly selected areas on each disc. The area fraction of all adhered cells (DAPI, RM3/1 and 27E10 positive stained cells) was counted on the photos using the software Easy Image Measurements (Tekno Optik AB, Göteborg, Sweden). Thereafter mean values were calculated. For details, see Study III and IV.

Cell Culture Osteoblasts

Cell Attachment

Cell morphology and attachment were evaluated by scanning electron microscopy (Jeol JSM-7400F SEM, Tokyo, Japan) operating at 10 kV. Furthermore, the ability of cells to reduce the tetrazolium salt 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) to a formazan product (an indication of mitochondrial integrity and

activity), which can be interpreted as a measure of cell number was quantified by spectrophotometry where the optical density of the resulting solution was read at 570 nm.

Osteocalcin

Osteocalcin concentrations in the harvest supernatant were assessed using a commercially available ELISA kit (BioSource International, Inc, USA). The standard and the samples were added to the antibody-coated well, followed by the addition of a secondary antibody conjugated to horseradish peroxidase (HRP). Bound enzymes labeled antibodies were detected through a chromogenic reaction, and absorbance was measured with a micro plate reader. The osteocalcin concentrations in the samples were determined by interpolation from the standard curve. (Figure 6)

Transforming Growth Factor β_1

The concentrations of TGF-β_1 in the harvest supernatant were assessed using a commercially available ELISA kit (Amersham Pharmacia Biotech, Sweden). The standard and the samples were added to the antibody-coated well, followed by the addition of biotinylated primary antibody and streptavidin-conjugated horseradish peroxidase (HRP). Bound enzymes labeled antibodies were detected through a chromogenic reaction, and absorbance were measured with a micro plate reader. The TGF-β concentrations in the samples were determined by interpolation from the standard curve. (Figure 6)

In Vivo

Histomorphometry

All implants were removed in blocs with surrounding bone tissue, fixed in 4 % neutral buffered formaldehyde, dehydrated in alcohol solutions and embedded in light curing resin (Technovit 7200 VLC, Kultzer & Co, Germany). Cutting and grinding was performed as described by Donath[184]. The final sections were made approximately 10 µm thick, and stained with toluidine-blue. Morphometrical measurements were made in a light microscope using a Leitz Microvid unit. The rate of bone-to-metal contact (BMC) and bone inside the threaded area (BA) were calculated. Results were presented as the mean value of all threads around the entire implant (BMC and BA) and

the mean value of the three best consecutive threads (BMC3 and BA3). All light microscopic calculations were made with a 10x magnification objective and an eyepiece of 10x magnification.

Resonance Frequency

Changes in implant stability during healing were measured by resonance frequency analysis (RFA). This method has previously been described by Meredith[185] and gives quantitative data of bone/implant interface stiffness. A small transducer was attached to the implant immediately after implant insertion and immediately before sacrifice of the rabbits. Measurements were performed parallel and perpendicular to the long axis of the rabbit femur. A mean value of one parallel and one perpendicular measurement was calculated for each implant. (Figure 7)

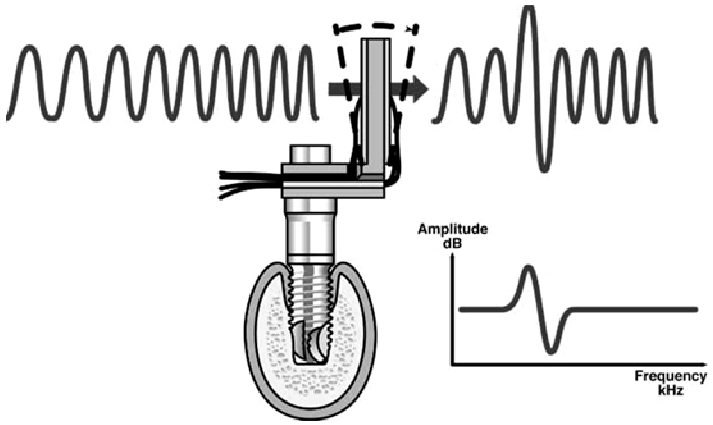

Figure 7: Schematic illustration; Principle for Resonance Frequency Analysis

Statistics

The non-parametric Wilcoxon signed rank test was used at a significance level of $p<0.05$ for histomorphometrical evaluations and for the resonance frequency analysis in Study I and II.

The non-parametric Mann-Whitney U- test and Kruskal Wallis were used for statistical evaluation at a significance level of $p<0.05$ in Study III, IV and V.

Results

Surface Characterization

Topographical Evaluation

Laser Scanning Profilometry and Optical Interferometry

Results from the topographical analysis are summarized in Table 2 on page 57.

In Study I the turned (anisotropic) and blasted (isotropic) surface received the intended similarities regarding the topographical height parameter (S_a) and differences in spatial descriptive parameter (S_{cx}). (Figure 8)

In Study II the anodized surface (TiUnite) demonstrated a rougher surface (S_a, S_{dr}), while the alkali-heat treated surface demonstrated a smoother surface compared to the turned control. Furthermore, an additional protein coating, in general, made the surfaces smoother. (Figure 9)

In Study III the anodized (TiUnite) surface demonstrated a rougher surface (S_a, S_{dr}), while the anodized (TiMgO) and turned surface demonstrated a smoother surface compared to the blasted surface. However, for the spatial descriptive parameter, density of summits (S_{ds}) the TiUnite surface showed the lowest value while the TiMgO showed the largest value.

In Study V all surfaces demonstrated similar roughness (S_a, S_{dr}).

Chemical Evaluation

X-Ray Photoelectron Spectroscopy

In Study V the surfaces contained no significant amounts of Ca and P except for the nano HA and TiMgO surface that demonstrated about 15 Atom% of Ca and P and P respectively (see Figure 11 on page 59).

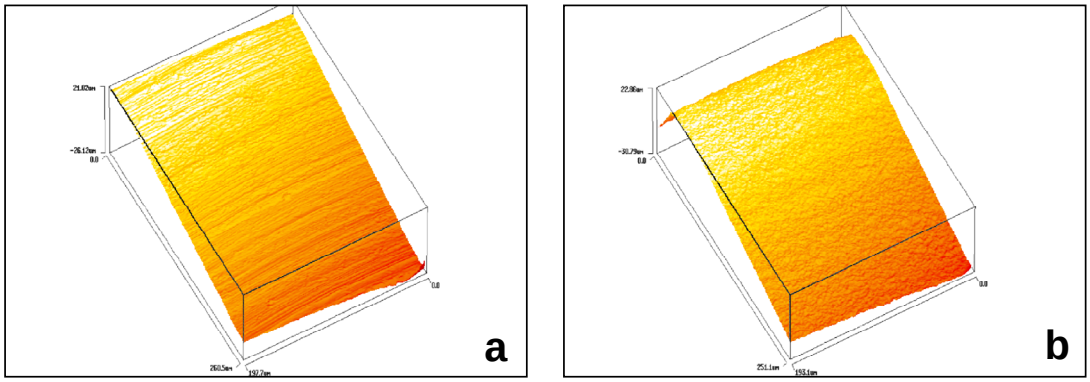

Figure 8: Optical Interferometer images from Study I demonstrating **a)** an anisotropic surface where the turning process has given the surface a clear orientation. **b)** an image of an isotropic surface where the blasting procedure has removed the turning marks and the surface has no longer an orientation.

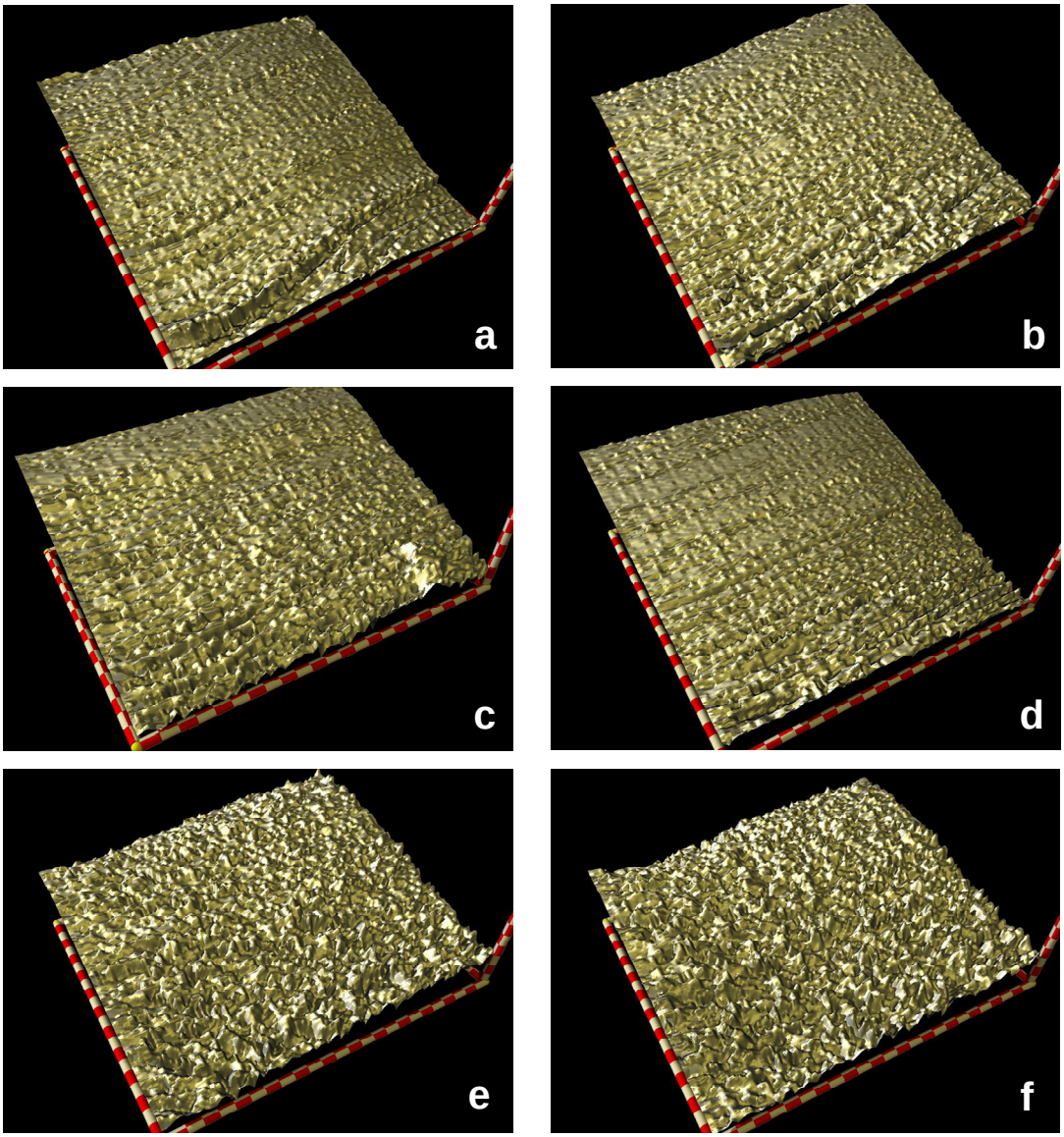

Figure 9: Laser Scanning Profilometer Images from Study II demonstrating **a)** turned **b)** turned and blood plasma coated **c)** alkali-heat treated **d)** alkali-heat treated and blood plasma coated **e)** anodized (TiUnite) and **f)** anodized (TiUnite) blood plasma coated.

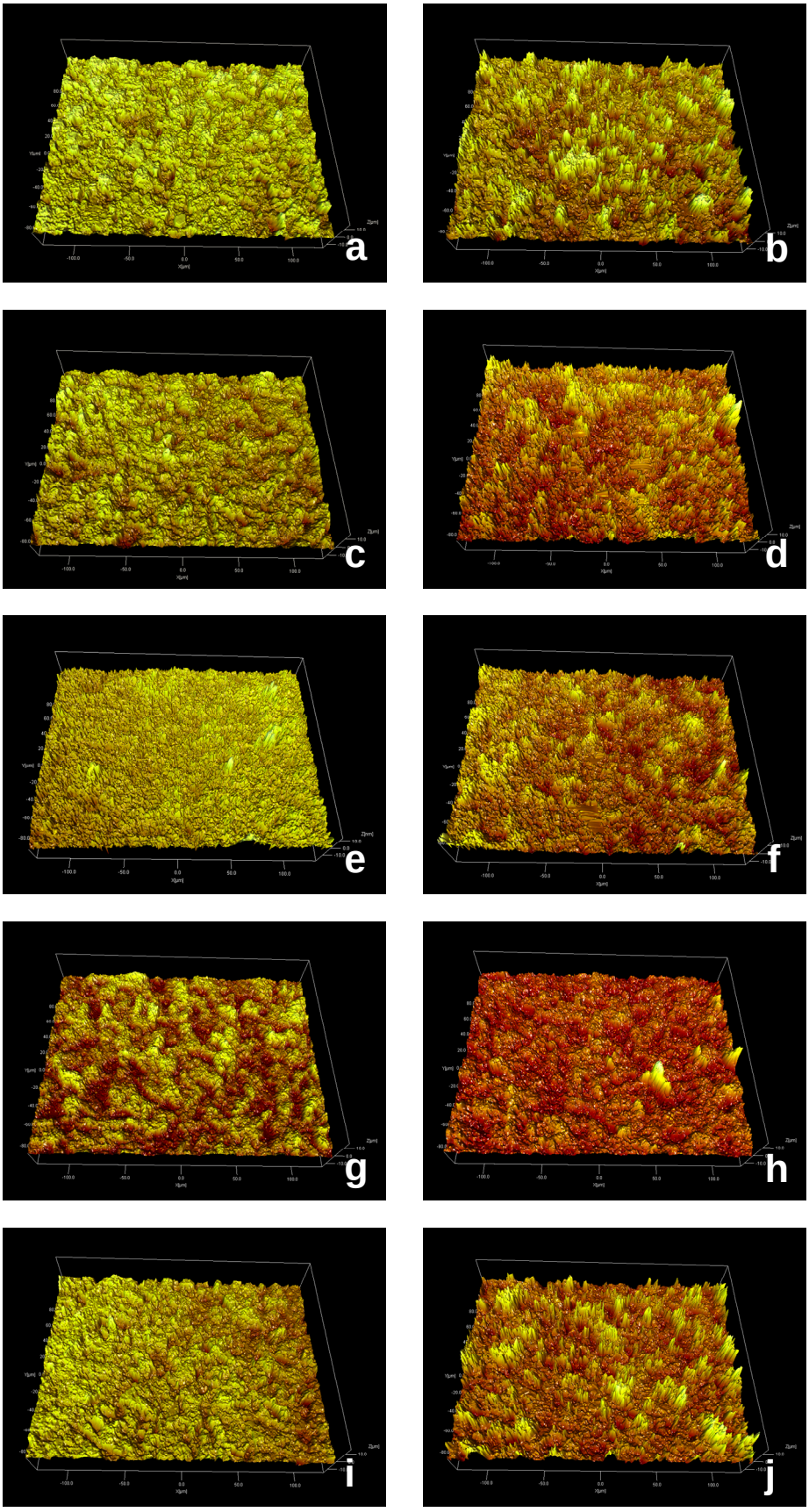

Figure 10: Optical Interferometer images of **a)** blasted surface **b)** SBF treated (72 hours) blasted surface **c)** Fluoride surface **d)** SBF treated (72 hours) Fluoride surface **e)** Alkali-heat treated surface **f)** SBF treated (72 hours) Alkali-heat treated surface **g)** Anodized/Mg surface **h)** SBF treated (72 hours) Anodized/Mg surface **i)** Nano HA surface **j)** SBF treated (72 hours) Nano HA surface.

		Surface topography			
		Sa (µm)	Scx (µm)	Sds (μm^{-2})	Sdr (%)
Turned	I	0.70±0.1	9.40±1.7	-	21±7
	II	0.79±0.2	9.35±1.7	-	26±17
	III	0.19±0.03	-	0.07±0.02	4±1
Turned + fibrinogen + blood plasma	II	0.56±0.1	8.59±0.8	-	16±5
Blasted	III	1.02±0.04	-	0.08±0.02	36±2
	V	0.97±0.04	-	0.12±0.03	31±2
Blasted (“isotropic”)	I	0.78±0.2	10.25±1.5	-	20±6
Fluoride etched	V	0.91±0.05	-	0.11±0.05	27±1
Alkali-heat treated	II	0.68±0.3	8.74±1.5	-	19±11
	V	0.94±0.08	-	0.15±0.02	37±5
Alkali-heat+ fibrinogen + bloodplasma	II	0.55±0.2	8.72±1.1	-	15±6
Anodized (TiUnite)	II	1.08±0.2	10.98±0.8	-	37±9
	III	1.13±0.18	-	0.06±0.01	53±7
Anodized (TiUnite) + fibrinogen + bloodplasma	II	1.20±0.2	10.88±0.7	-	42±12
Anodized /Mg	III	0.27±0.03	-	0.14±0.01	11±2
	V	0.96±0.06	-	0.12±0.01	31±2
Nano HA	V	0.92±0.09	-	0.11±0.05	29±3

Table 2: Three screws and three discs of each surface modification were analyzed, where each screw was measured at 9 areas and each disc was measured at 3 areas. The mean values and standard deviations (±) of each surface modification are summarized above.

In Vitro Evaluation

Simulated Body Fluid

CaP Formation

Topographical Evaluation (Optical Interferometry)

In Study V there was a tendency for all surfaces to become smoother after 12 and 24 hours in SBF. Furthermore, a significant increase in surface roughness (S_a, S_{dr}), was demonstrated for all surfaces except the porous TiMgO and AH surface after 72 hours in SBF, but on the other hand, they demonstrated the highest (S_{ds}) values. (Figure 10)

Chemical Evaluation (XPS)

After 12 hours all surfaces, except the blasted ones, displayed an increase in Ca and P content, particularly so for the fluoride surface.

After 24 hours, all surfaces demonstrated a continued increase of Ca as well as P. except for the blasted ones that still demonstrated only Ca.

However, after 72 hours the blasted surface demonstrated the largest amount of Ca and P of all surfaces (Figure 11). For all surfaces, the content of Ca increased more than P during the first 24 hours, but after 72 hours all the surfaces showed a Ca/P ratio of approximately 1 (Figure 12). This was especially apparent for the blasted surface, which until 24 hours had no P content on the surface at all. The TiMgO surface contained some Mg and the F surface contained a high content of fluoride, although, these elements dropped fast and after 12 hours it was only around 1 Atom% for both surfaces.

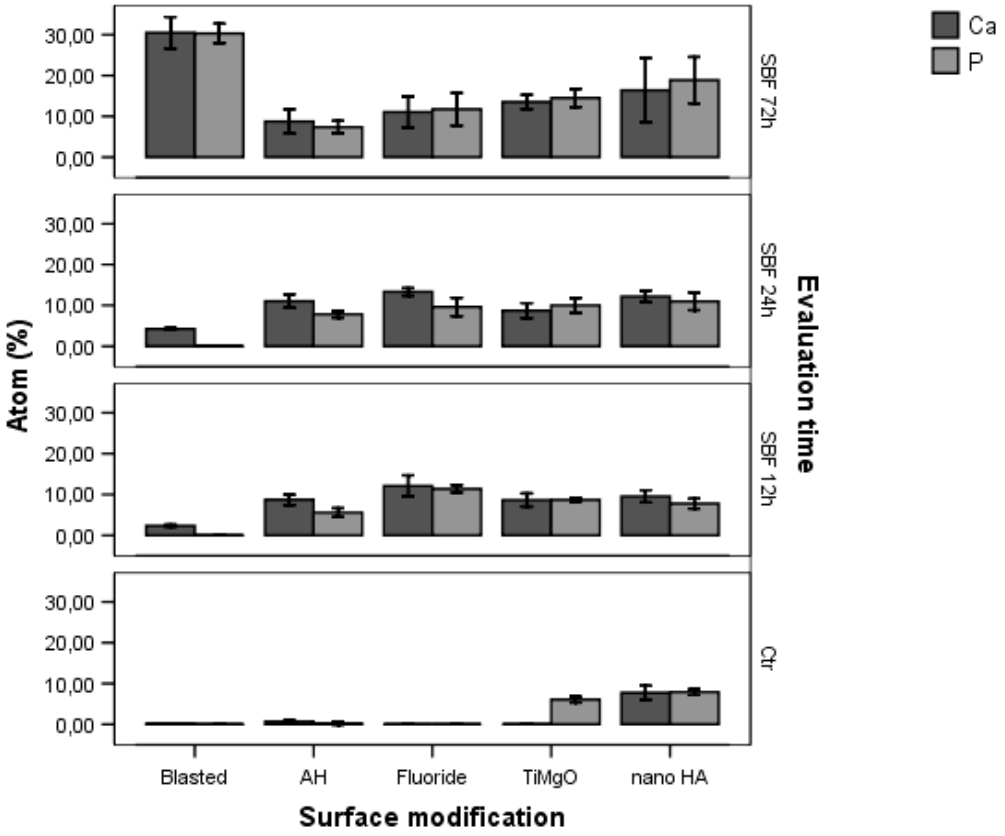

Figure 11: Atom % of Ca and P on the differently modified surfaces after 12, 24 and 72 hours of SBF treatment.

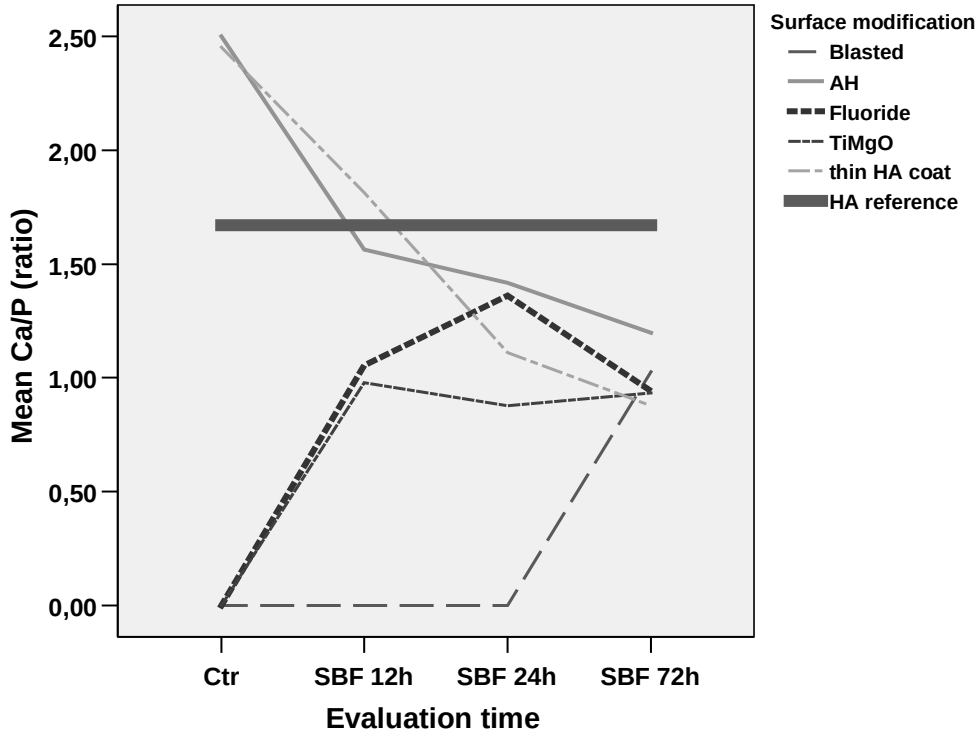

Figure 12: Ca/P ratio on the differently modified surfaces after 12, 24 and 72 hours of SBF treatment.

Cell Culture Monocytes

Viability

Generally, in Study III and IV, the results demonstrated, low concentrations of LDH i.e. high viability for the adhered cells on all tested surfaces (within the range of 0.8-1.6 µkat/l).

However, an increase in LDH concentrations was achieved after LPS stimulation and after 72 hours of culture compared to 24 hours in Study III. Similar observations were made for cells on catalase coated surfaces after 24 hours and on turned and fibrinogen coated surfaces after 72 hours in Study IV.

The results of the LAL test showed no endotoxin in the samples (<0.05ng/ml).

Cell Attachment and Differentiation

The number of cells and their differentiation are summarized in Figure 13 on page 61.

The total number of adhered cells differed significantly between the different, modified surfaces in both Study III and IV, with the largest number of cells on the anodized (TiUnite) surface in Study III and on the catalase coated surface in Study IV at both evaluation times.

A decrease in total number of cells was seen over time (from 24 hours to 72 hours) for all surfaces in Study III and IV except for the catalase coated ones in Study IV.

Furthermore, there were no statistical differences between stimulated and unstimulated wells.

In general, the percentages of differentiated cells (27E10, RM3/1) were low.

In Study III there were no differences between the differently modified surfaces, however, in Study IV the unstimulated catalase coated surface demonstrated an increased number of 27E10 positive cells after 72 hours.

In Study III acute monocytic phenotype 27E10 marker dominated after 24 hour while in both studies the expression of the chronic RM3/1 dominated after 72 hours. Furthermore, in Study IV the number of 27E10 positive cells dominated on stimulated surfaces compared to the unstimulated for all surface modifications and evaluation times.

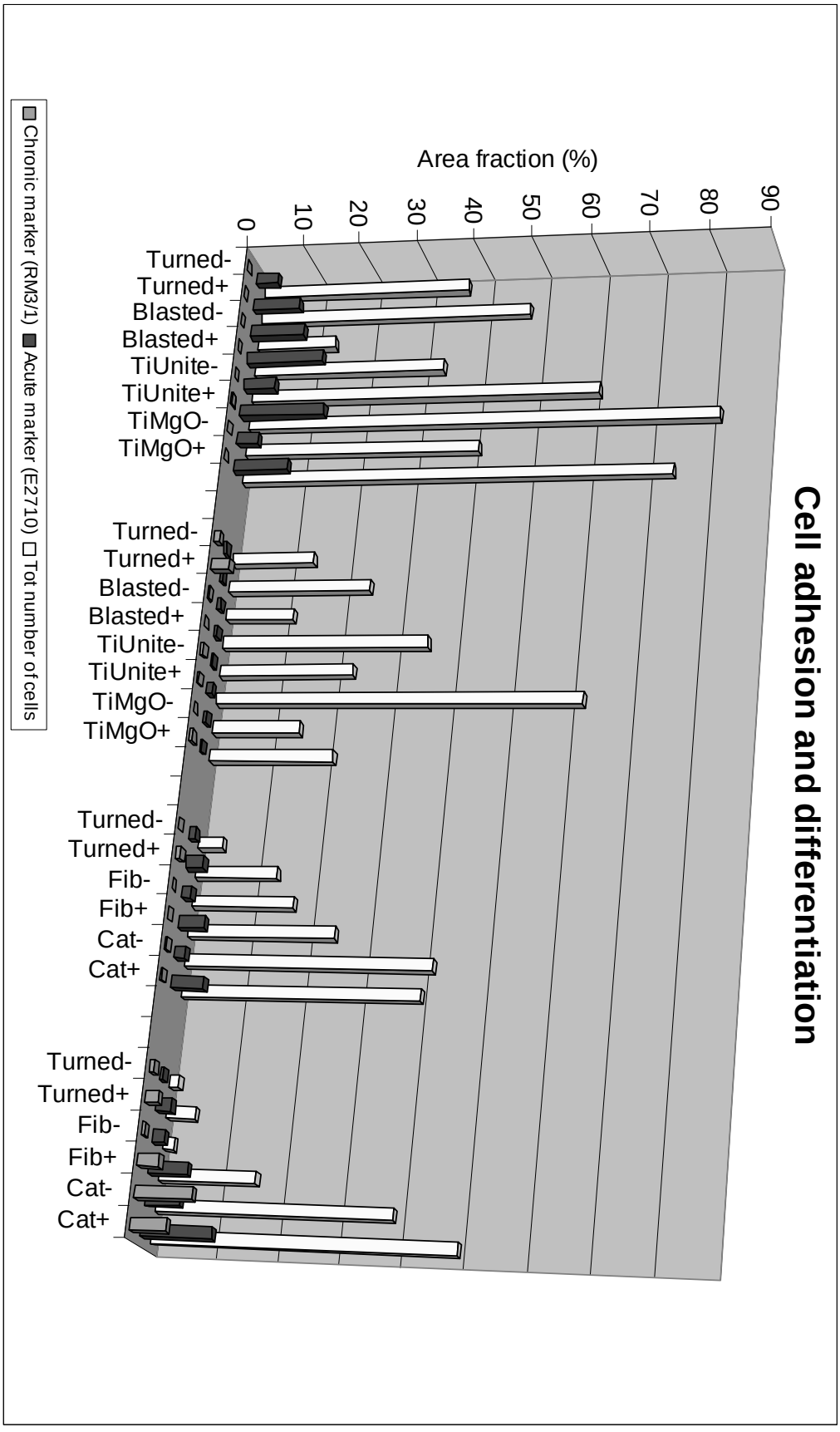

Figure 13: Mean area of total adherent and differentiated cells per measured area (%) on the discs with the different modified surfaces after 24h and 72h with (+) and without (-) LPS stimulation.

Tumor Necrosis Factor-α

The TNF-α cytokine release is summarized in Figure 14 on page 63.

In Study III there were no significant differences in release of TNF-α from cells on the differently modified surfaces.

In Study IV the release of TNF-α from the cells differed significantly between the differently modified surfaces. Cells on non-stimulated catalase coated surfaces and stimulated fibrinogen coated surfaces induced, at both evaluation times, the highest TNF-α concentration.

In addition, LPS stimulated cells produced significantly higher concentrations of TNF-α than the unstimulated cells on the different surfaces except for the catalase coated surface in Study IV.

Cells on both stimulated and non-stimulated surfaces showed significantly higher concentrations of TNF-α after 24 hours than after 72 hours.

Interleukin-10

The IL-10 cytokine release is summarized in Figure 15 on page 63.

In Study III there were no significant differences in release of IL-10 from cells on the differently modified surfaces, whilst in Study IV the release differed significantly. Mononuclear cells on non-stimulated catalase coated surfaces induced, at both evaluation times, the highest IL-10 concentrations.

Additionally, the LPS stimulated cells demonstrated significantly higher values compared to the unstimulated cells on the tested surfaces except for the catalase coated surface in Study IV.

No differences were observed between 24 hours and 72 hours, except for stimulated cells on the turned surface that decreased IL-10 production with time.

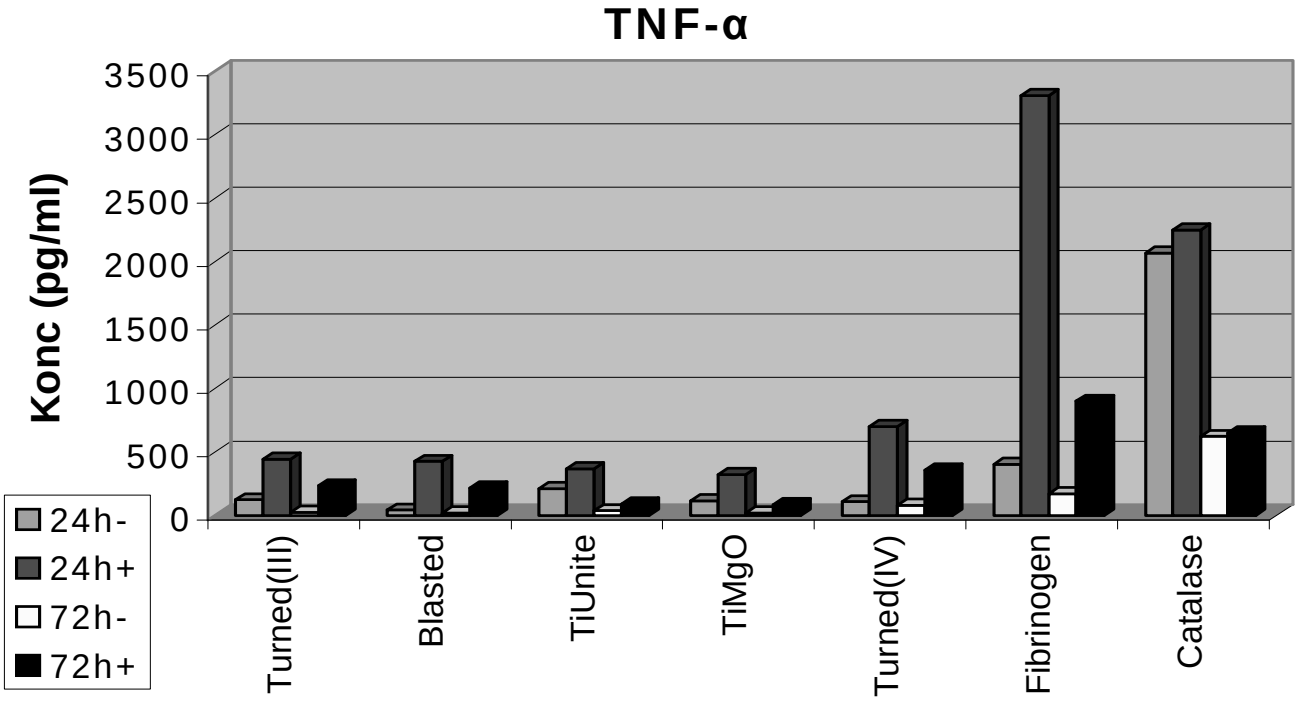

Figure 14: Mean TNF-α concentrations (pg/ml) in the culture medium around the discs with the differently modified surfaces in Study III and IV after 24h and 72h with (+) and without (-) LPS stimulation.

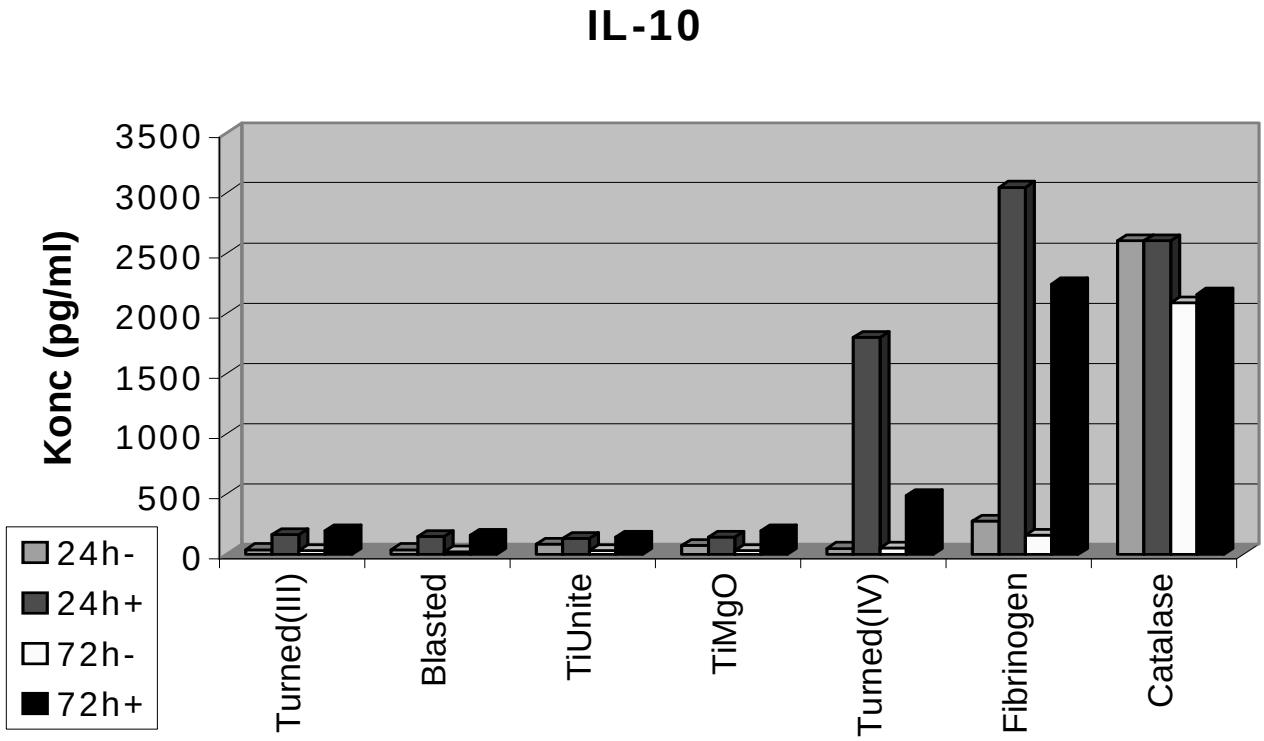

Figure 15: Mean IL-10 concentrations (pg/ml) in the culture medium around the discs with the differently modified surfaces in Study III and IV after 24h and 72h with (+) and without (-) LPS stimulation.

Cell Culture Osteoblasts

Cell Attachment

The amounts of attached cells in Study V are summarized in Figure 16 on page 65.
The differently modified surfaces demonstrated similar amounts of attached cells.
The blasted control surface demonstrated significantly higher values than the SBF treated AH and Fluoride surfaces, while the SBF treated nano HA and blasted surface demonstrated significantly higher values than the SBF treated AH surface. There were no significant differences in cell attachment between the blasted and SBF treated blasted surfaces. (Figure 19a-f and Figure 20a-f)

Osteocalcin

The osteocalcin concentrations in Study V are summarized in Figure 17 on page 65.
The SBF treated blasted surface demonstrated significantly increased osteocalcin concentrations compared to the blasted, SBF treated Fluoride and TiMgO surfaces. Furthermore, the blasted, SBF treated nano HA and the AH surfaces demonstrated significantly higher concentrations than the SBF treated Fluoride and TiMgO surfaces. As mentioned above there were significant differences in osteocalcin production between the blasted and SBF treated blasted surfaces.

Transforming Growth Factor-ß

The TGF-ß concentrations in Study V are summarized in Figure 18 on page 65.
The SBF treated blasted and nano HA surface demonstrated significantly higher TGF-ß concentrations compared to the other surfaces while, the SBF treated TiMgO, and Fluoride surfaces showed significantly lower concentrations compared to the other surfaces. The blasted control demonstrated significantly higher concentrations compared to the SBF treated Fluoride and TiMgO surfaces and similar concentrations to the SBF treated AH surface. Furthermore, there were significant differences in TGF-ß production between the blasted and SBF treated blasted surface.

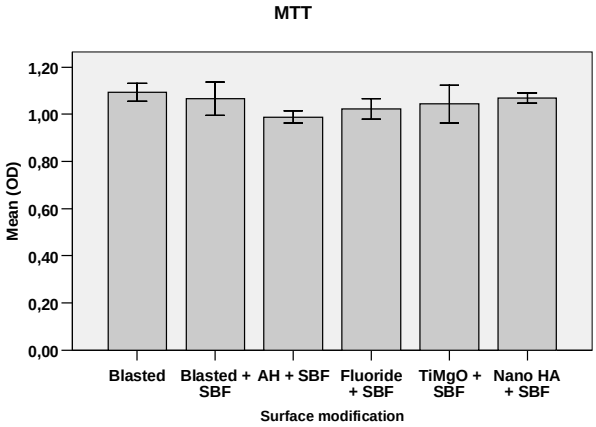

Figure 16: Cell attachment after 3 hours on discs with the differently modified surfaces (Study V).

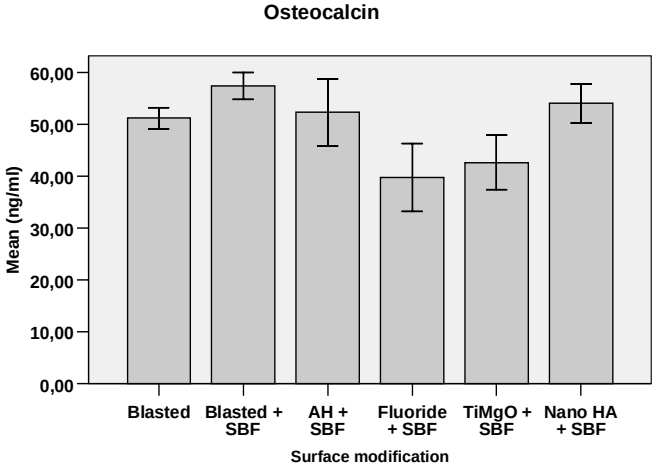

Figure 17: Mean osteocalcin concentrations (ng/ml) in the culture medium around the discs with the differently modified surfaces (Study V).

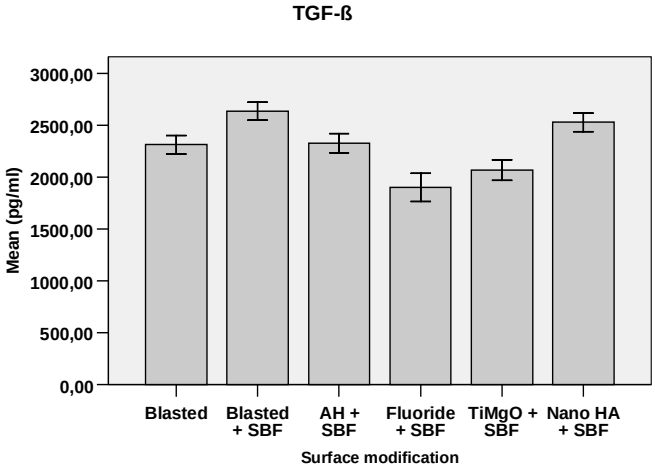

Figure 18: Mean TGF-β concentrations (pg/ml) in the culture medium around the discs with the differently modified surfaces after 10 days (Study V).

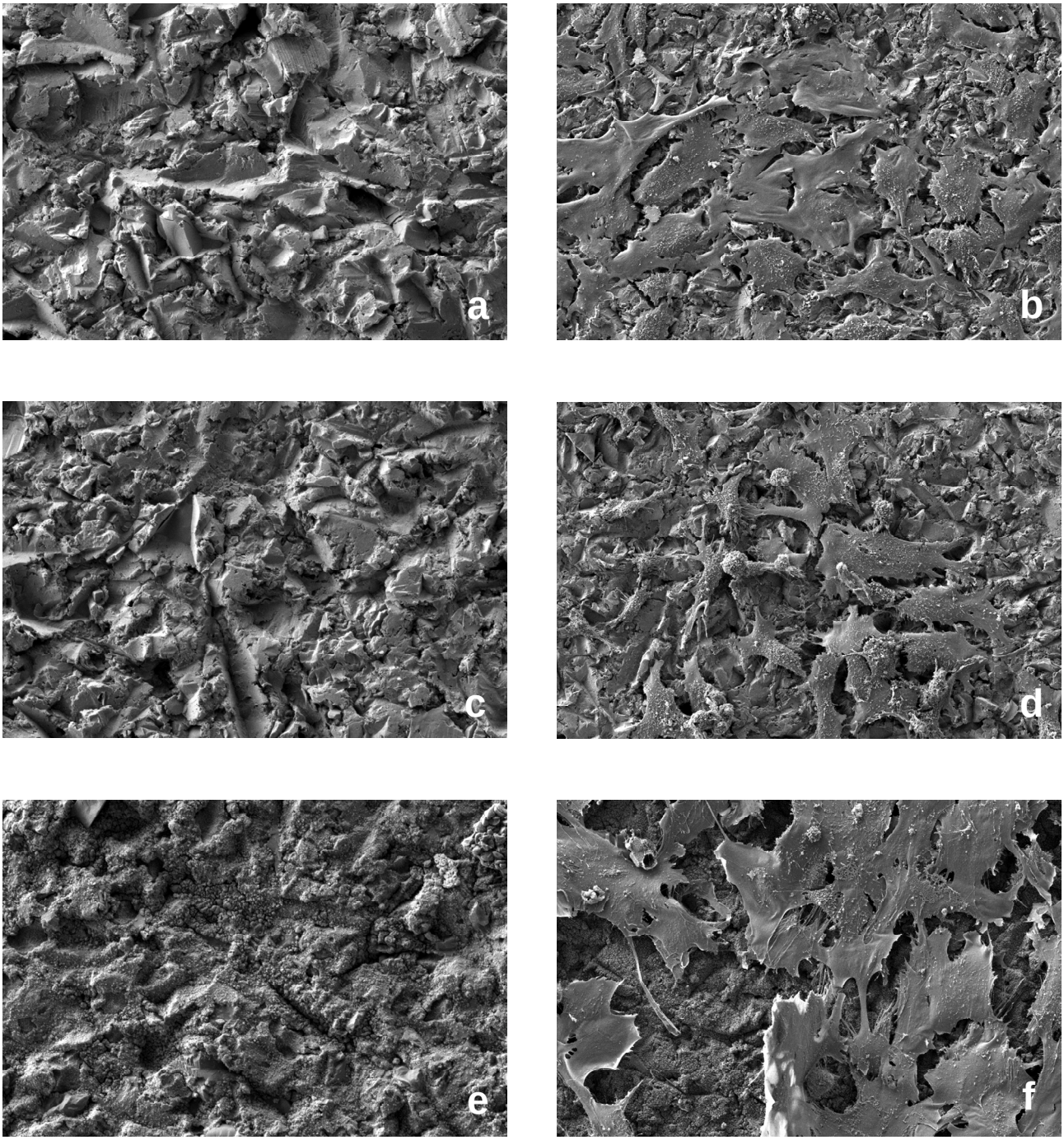

Figure 19: SEM images demonstrating control at x1000 magnification (left column) and cell attachment after 3 hours at x500 magnification (right column).
a,b) Blasted Surface
c,d) Blasted Surface treated in SBF for 72 hours
e,f) Fluoride Surface treated in SBF for 72 hours

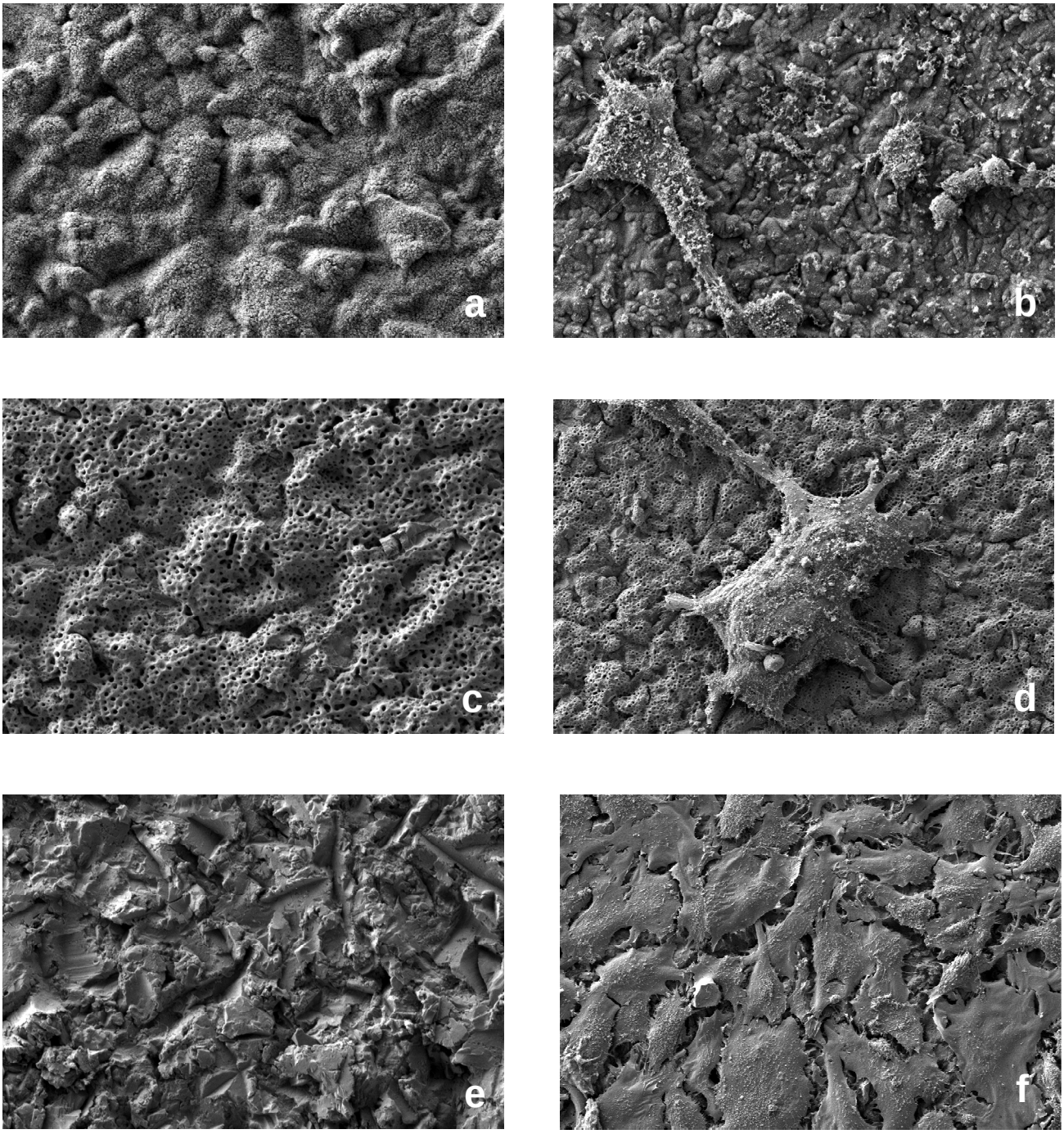

Figure 20 SEM images demonstrating control at x1000 magnification (left column) and cell attachment after 3 hours at x500 magnification (right column).
a,b) Alkali-heat treated Surface treated in SBF for 72 hours
c,d) Anodized/Mg Surface treated in SBF for 72 hours
e,f) Nano HA Surface treated in SBF for 72 hours

In Vivo Evaluation

Resonance Frequency Evaluation

Results from the resonance frequency analysis (RFA) are summarized in Figure 21. In Study I and II increased stability were achieved for all surface modifications after 12 and 4 weeks respectively. However, no significant differences were demonstrated between the differently modified surfaces either at insertion or removal time, except for the anodized tibial implants that demonstrated increased values compared to etched and turned implants. In Study II tibial implants demonstrated higher RFA values both at time of insertion and removal compared to femoral implants.

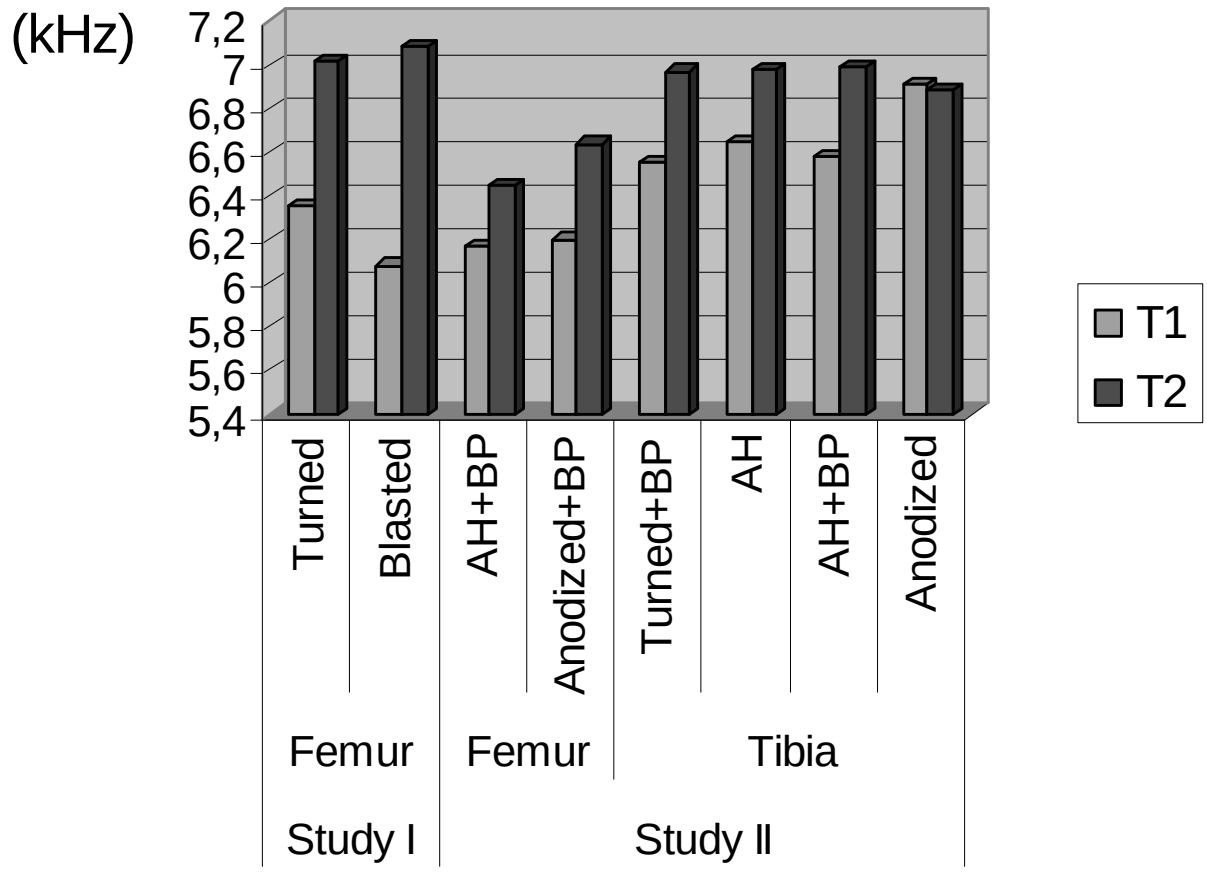

Figure 21: Mean Resonance Frequency measurements for each surface modification calculated at time of insertion (T1) and before removal(T2) after 12 weeks (Study I) and 4 weeks (Study II) (9 and 11 animals, respectively).

Histomorphometric Evaluation

Light microscopic investigations of the ground sections revealed no adverse reactions around the implants, independently of surface preparations.

Macrophages and multinucleated giant cells were observed in small numbers in close contact with both surface preparations. The results from the morphometrical analysis are summarized in Figure 22. In Study I and II there were no significant differences between the differently modified surfaces regarding bone-implant contact, bone area inside threads, or best three consecutively threads.

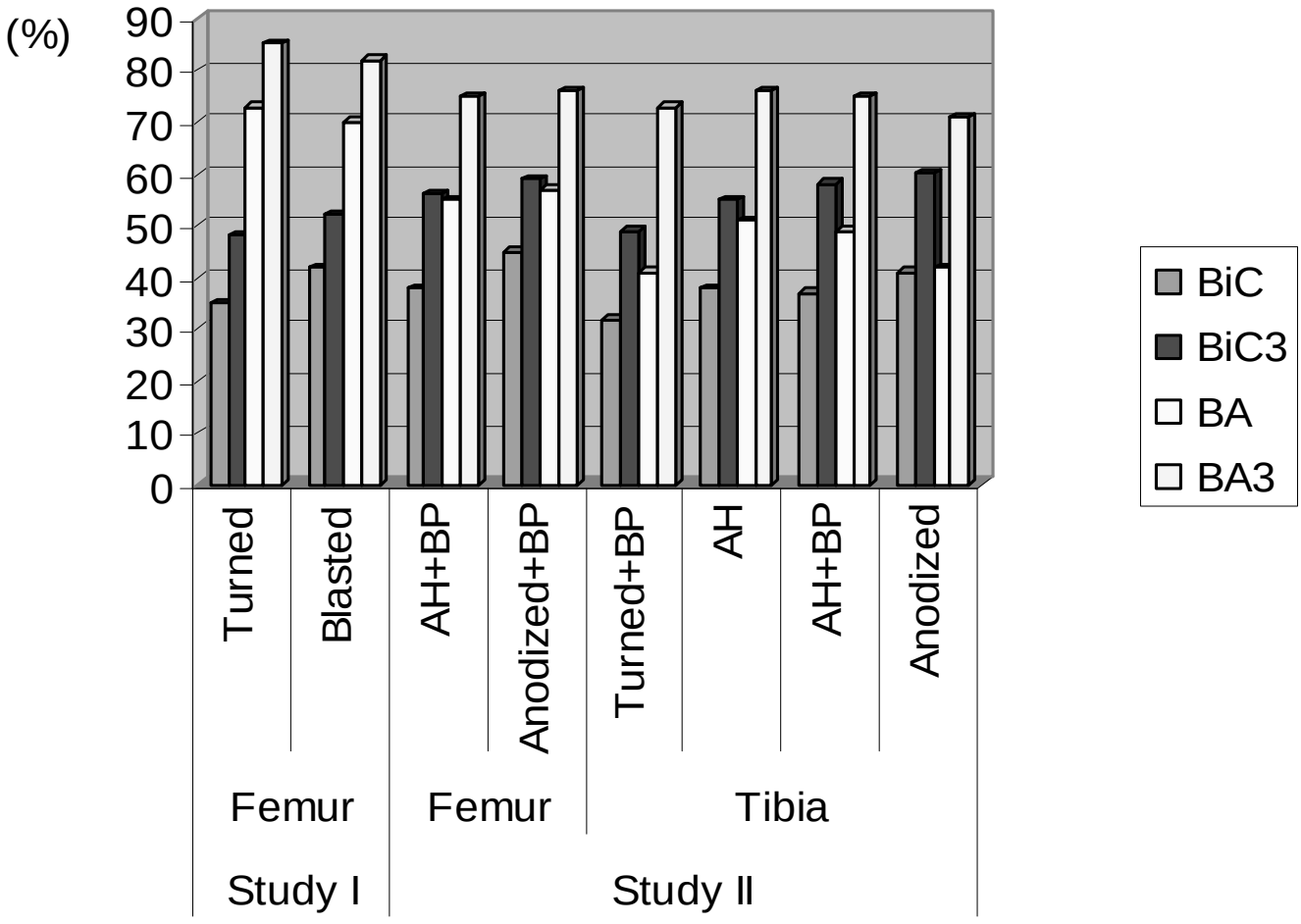

Figure 22: The rate of bone-implant contact (BiC), bone area inside the threads (BA) calculated on each side of the implant. The best three consecutive thread bone-to-metal contact and area inside threads (BMC3 and BA3) represents the situation at the cortical passage after 4 weeks of insertion (9 and 11 animals, respectively).

Discussion

Materials and Methods

Implant and Sample Preparation

Surface Preparations

Thirteen differently modified titanium surfaces were characterized and evaluated in the present thesis. The methods used; blasting, alkali and acid etching, anodizing, sol-gel coating, and immobilization of different proteins, all being modern techniques to prepare implant surfaces for possibly improved bone response.

Some of the surface modifications were evaluated in one study, while others were tested repeatedly. It is tempting to evaluate numerous surface modifications in an investigation such as this, however the parameters need to be controlled, and an adequate number of specimens are needed for statistical reasons, hence a limitation of tested surface alterations are necessary.

Surface Characterization

The surface roughness was evaluated with different techniques in the present thesis. The Optical Interferometer used in Study III and IV is a further development of the Confocal Laser Scanning Profilometer used in Study I and II. The methods have approximately the same lateral resolution while the Interferometer has larger maximal measuring area and maximal vertical range. Not only for being faster, the Interferometer has also a higher optical vertical resolution. While the interferometer has problems in measuring surface irregularities with slopes exceeding three degrees for surfaces with low reflective capacity, there is a tendency for the Confocal Laser Scanning Profilometer to overestimate the surface roughness of surfaces with high reflectance.

Three parameters; one height, one spatial and one hybrid parameter were presented as recommended by Wennerberg and Albrektsson[43]. In Study I and II the spatial parameter where represented by Sdr while in Study III and IV Sds, was preferred as spatial parameter due to the development of the calculation program and change in the use of 3D parameters.

If precisely the same preparation techniques are being used, surface roughness values will be greater for screw shaped implants compared to discs. Surface roughness

of a screw shaped implant is the sum of measurement made on the top, flank and valley. The flank often resembles the disc, while the tops of the screw usually have a rougher profile.

Cleaning Procedure

The cleaning procedure varies between different laboratories. The protocol used in our laboratory is carefully tested and used in the industry for commercially available implants for clinical application.

Study Design

In Vitro

Simulated Body Fluid Model

Simulated body fluid (SBF) is an acellular solution, with ion concentrations most similar to human blood plasma. The principle was introduced in 1980 when Hench demonstrated that calcium phosphate (CaP) films formed on the surface of Bioglass *in vivo* could be reproduced *in vitro* by a Tris buffer solution[186]. Others failed to demonstrate CaP *in vitro* with the Tris buffer on AW glass-ceramic despite *in vivo* bioactivity, and the additional SiO_2 rich layer formed on the Bioglass was thought to be the explanation. Kokubo and co-workers succeeded in having an *in vitro* response comparable with that *in vivo* on AW glass-ceramics by the “Original SBF”. Later Kokubo and Hench independently confirmed CaP layers in SBF on Bioglass as well. The method has since been used as an *in vitro* screening test for predicting *in vivo* bioactivity of materials. Qualitative and quantitative correlations between *in vitro* and *in vivo* apatite formation have recently been presented in a review article by the group[187].

The recipe for the SBF has been changed several times since Kokubo introduced it in 1990[188]. In the original SBF there were no SO_4^{2-} ions as in human blood plasma. This was corrected in the next version, the “c-SBF” or “SBF”[189]. Furthermore, in the revised version, the r-SBF[181] the Cl^- and HCO^{3-} concentrations were increased and decreased, respectively, to levels of human blood plasma. In the newest version the “n-SBF” levels of HCO^{3-} are decreased to avoid spontaneous precipitations when

preparing the solution[190]. In the present thesis the r-SBF was used because, the ion concentrations most mimicked human plasma.

Cell Culture Model

Cell culture studies may be used to interpret *in vivo* results, to get more detailed information about mechanisms regarding the material-cell interaction.

To mimic natural conditions human cells were used in the present thesis. Moreover, by using primary cell types the ability to adhere and differentiate was maintained. However, results from primary cultures is more prone to vary because of inter individual variations between donors, while results from cell lines are more reproducible. Furthermore, there is a risk of the primary cell types becoming differentiated during recruitment.

The monocytes were separated from Buffy-coats by a method described by Pertoft and co-workers[182], while the osteoblasts were separated from human mandibular bone specimens by a method described by Beresford and co-workers[191]. Both methods are routinely used at the laboratory in Gothenburg and Bergen, respectively. The purity of the monocytes are according to the protocol approximately 75%, while the purity of the osteoblasts are approximately 70%.

The evaluation times in Study III, IV and V were based on results achieved from previous studies[31, 61].

In Vivo

Animal Model

The rabbit model has been extensively used and is well documented not at least in several PhD thesis published by the dept of Biomaterials. The cortical bone of rabbit tibia has similarities with mandibular bone in humans, while femur has similarities with maxillary bone. However, human jawbone is of intramembranous origin, while rabbit femur and tibia are of endochondral origin. The rabbit bone has additionally a bone turnover that is 2-3 times higher than that of human bone[7]. Furthermore, when interpreting the results, considerations have to be taken that the implants are not dynamically loaded.

In rabbit, new bone is present after a few weeks, while a full bone cycle is approximately 3 months[5]. The evaluation times of 4 and 12 weeks in Study II and I, thereby, represented early and late healing time, respectively.

Evaluation Methods

In Vitro

Cell Number and Differentiation

Cells in five selected areas in 1/3 (DAPI) of the discs and five x 2 selected areas of 2/3 (DAPI/RM3/1 or E2710) of the discs were photographed (totally 1800 pictures/study) and subsequently evaluated. The computer based program the "Easy Image measurement" was used to rationalize the evaluation, but since the areas of the DAPI stained cell nucleus are measured without respect for overlapping cells, there is a risk of under estimation of the surfaces with the largest amount of cells.
Furthermore, the possibility to compensate for size discrepancy of cell nucleus on differently modified surfaces by dividing the surface area of one nucleus with the total nuclei area was not utilized. Yet, the method enables evaluation the amount of cells and differentiation on the same disc. Same investigator made all evaluations and re-measurements were made to improve accuracy.

Cytokine Assays

The assay kits used in Study III, IV and V are routinely used in laboratories and it is an accurate and reliable method.

In Vivo

Histomorphometry

Ground sections were prepared according to the method described by Donath and co-workers[184], a technique extensively used in our laboratories since 1984.
The standardized way of cutting and grinding to an approxemate sample thickness of 10-15 µm, is important to avoid overestimation of bone contacts. However, the technique is time consuming and since it does not permit serial sectioning, the most cen-

tral section is generally used for histomorphometrical quantifications. The histomorphometric parameters bone-implant contact and bone area are routinely used at our laboratories and all measurements with the computer based light microscope were performed by the same person in a blinded manner.

Resonance Frequency Analysis

The RFA is a bending test that measures the stability of the implant in bone in a non-destructible manner. It is frequently used as a supplementary evaluation method to histomorphometry in *in vivo* studies; furthermore, it has been demonstrated to predict the outcome of implants in the clinical situation[192]. Since the equipment measures the interfacial stiffness in the transducer beam vibration direction, the transducer was placed both parallel and axial to the bone in order to minimize measurement errors. Care was taken not to entrap soft or hard tissue since this may cause false results.

Statistics

In the present thesis, mostly non-parametric methods were used for statistical analysis, since the number of animals and human donors was low and may not be normally distributed. In Study I and II the non-parametric Wilcoxon signed rank test was used since the animal model had a study design with paired observation, while in Study III, IV and V, the Mann Whitney test (comparison of 2) and the Kruskal Wallis test (>2) were used since the cell culture model had a study design with unpaired observations.

Results

Study I

In Study I no significant differences in bone response were demonstrated between the iso- and anisotropic modified surfaces.

The hypothesis of surface orientation being of importance was based on results from a study made by Ivanoff and co-workers[65] where blasted micro-implants in a human test model demonstrated an increased bone-implant contact compared to the turned implants despite similar average roughness (top, valley, flank). Hallgren and co-workers additionally had demonstrated the importance of both the height and the spatial parameters when comparing surfaces prepared with a laser micro-machining technique[66]. Furthermore, there had been some *in vitro* studies showing that soft tissue cells responded differently to surface orientation[193, 194].

However, the results from the present study did not support the surface orientation being of any significance. Instead the results indirectly supported evidence of numerous *in vitro*[51, 57, 61] and *in vivo*[44, 195] studies demonstrating surface roughness by means of average height deviation being of importance for implant bone integration. The specially designed turned micro-implants used by Ivanoff[65] demonstrated very rough tops resulting in average roughness values highly exceeding what is expected for a turned surface. If only the flanks had been considered, the blasted implants indeed demonstrated the roughest surface, instead indicating that the roughness of the flanks might be of more importance for implant incorporation than the tops and valleys. However, one cannot rule out that surface orientation designed in a certain way might affect the bone cell response, furthermore, that there were no qualitative differences of the bone implant contact between the implants.

Study II

In Study II no significant differences in bone response were demonstrated when comparing the anodized and alkali-heat treated surfaces with and without protein coating. The anodized and the alkali heat treated surfaces had in previous studies demonstrated increased bone cell response *in vitro*[49, 92, 93, 110] and increased bone response in vivo[97, 98, 105, 106] compared to control surfaces.

Related studies on immobilized proteins to control and induce specific cell and tissue response had so far mainly been made *in vitro* and in the cardiovascular field. Furthermore, the effect of blood plasma coating for bone formation only had been tested by dip coating implants in platelet rich plasma before insertion.

The hypothesis of a more favourable bone response with an additional blood plasma coating was based on the result from an *in vivo* study in a soft tissue model where the titanium surfaces with covalently immobilized blood plasma demonstrated more sham-like inflammatory response than uncoated implants[159].

The proposed bioactive alkali-heat treated surface demonstrating similar bone response compared to the anodized surface despite a smoother surface. This is interesting since other studies has demonstrated these parameters to be of importance for improved bone integration[44, 195]. However, it may be that the increased but not significant results for the anodized surface compared to the alkali-heat treated surface had been more pronounced in cancellous bone.

The lack of significant results from the blood-plasma coating may depend on lack of effect of the coating or that it was not possible to further reinforce the effect of the anodization and alkali heat treatment. The lack of effect of the immobilized plasma could be explained by clot coatings cracked wholly or partly at implant installation, the coating being too thin or that the composition was not optimal. For amplification of wound healing, the presence of blood cells such as platelets may be needed.

Study III

In Study III there were no significant differences in inflammatory cell response *in vitro* between the differently modified titanium surfaces except for inflammatory cell attachment with the largest amount of cells on the anodized surfaces. The potentially bioactive TiMgO surface demonstrating a similar response to the TiUnite like anodized surfaces despite the former having a surface roughness of a smoother character, of some interest since the macrophage have been observed to be "rugophilic"[46-50]. Under these circumstances, it was surprising that the blasted surfaces showed low cell adherence. However, comparisons with other studies have to be made with caution since other materials, cell types, evaluation methods etc have been used[46-48, 50]. In yet other cases it is difficult to compare results since surface characterization has been omitted[49]. Furthermore, the concomitant increase in porosities and change in physicochemical properties of the thickened oxide of the anodized surfaces may contribute as well. This would explain the substantial cell adherence on the TiMgO surface after 24 hours despite its quite smooth surface (S_a). Osteoblasts seem to have a higher adherence to Mg ion reinforced ceramic surfaces[196] and this may be valid for the cells in this study as well. An explanation may be the supply of Mg ions, since early adhesion to a biomaterial surface is mediated by integrins, and integrin function depends on divalent cations such as Mg ions[197]. Except for the change in physicochemical properties and increased porosity because of the thickened oxide, the surface expressed a high value for peaks per area unit, which may be favorable for cell attachment. The decreased amount of adhered cells and TNF-α and constant concentration of IL-10 for all surfaces over time is in accordance with results from Suska and co-workers who showed that inflammatory response to titanium is transient compared to copper[20, 23].

The generally low differentiation rate may be explained by that maximum expression of the two phenotypes occurred later, that the antigen was missing on the cells or that the surfaces did not have the capacity to stimulate the cells to differentiate. In the present study the cytokine production did not reflect the cell accumulation on the surfaces, although the contribution from the cells of the supernatant is unknown.

Study IV

Study IV proved it possible to influence the early inflammatory response *in vitro* by covalently immobilizing protein coatings to titanium surfaces.

The fibrinogen coated surface demonstrated a stronger inflammatory response than the control which is consistent with other studies, where fibrinogen has been shown to enhance the adhesion and activation of the inflammatory cells[10]. In fact, results have demonstrated that the amount of fibrinogen absorbed to a surface corresponds to the magnitude of the inflammatory response in terms of inflammatory cell recruitment[198].

Some mechanisms whereby fibrinogen appears to initiate the acute inflammatory response to implants were described by Tang and co-workers, and recently the major pro-inflammatory amino acid sequence of fibrinogen was identified to interact via the integrin CD11/CD18 on the cells membranes[199].

Mononuclear cells cultured on the catalase coated surfaces demonstrated the strongest inflammatory response, opposed to the hypothesis of producing a coating with anti-inflammatory characteristics. Indeed, an increase in the anti inflammatory cytokine IL-10 production was achieved, especially compared to the amount released from cells on unstimulated titanium and fibrinogen coated surfaces, although additionally TNF-α and cell attachment were increased.

Moreover, while cells on the titanium and fibrinogen coated surfaces were sensitive to LPS stimulation, adherent cells on catalase coated surfaces were not.

This suggests that catalase itself is a potent mediator for the inflammatory response by mononuclear cells, a highly surprising finding.

The two catalase layers were covalently immobilized to the surface via fibrinogen and an explanation may be that the catalase coating did not fully cover the fibrinogen influencing the inflammatory cell response. This seems unlikely since a double layer of catalase was bound above the cross-linked fibrinogen layer.

The possibility to selectively use the anti-inflammatory capacity of catalase or other substances thereby needs to be further evaluated.

Study V

In Study V the bioactive AH, Fluoride, TiMgO and nano HA surfaces, gave rise to an earlier CaP formation than the blasted control surface. However, after 72 hours the blasted surface demonstrated the largest amount of CaP formation. Since surface roughness values were similar for the blasted and bioactive surfaces, the time dependent difference in induction of CaP formation may be related to surface chemistry. Early induction of CaP formation on bioactive surfaces is in agreement with other studies, where anodized surfaces have been reported apatite formation after a few days[87, 88]. The blasted surface demonstrating the largest amounts of formed CaP after 72 hours was surprising. While Takadama and co-workers did not use any control when characterizing the early apatite formation on the alkali-heat treated surface[87, 88], other groups claim either no precipitation of apatite on the titanium surface[141] or apatite formation after two months[200] when using different SBF solutions. Consequently, the differences may be due to the later version of simulated body fluid, the revised SBF[181], solutions used in the present study. Furthermore, in the present study the SBF was used not only as an evaluation method, but also as a coating for evaluation of subsequent cell attachment, production of osteocalcin and transforming growth factor-β_1. Cell attachments corresponded neither to the surface roughness nor to the amount of formed CaP, since the smoothest untreated blasted surface attracted most cells. This is not in agreement with other studies where osteoblast in general has been demonstrated to be rugophilic[51, 57, 61]. Even though the “solid” surface roughness is taken into consideration no correlations can be made since the surface roughness from start was very similar for all surfaces. The production of osteocalcin, and transforming growth factor-$\beta_{1,}$ on the contrary, was to a large extent correlated to CaP formed and subsequently the SBF treated HA and blasted surfaces demonstrated an increased osteocalcin and TGF-ß production compared to the other surfaces. Increased cell differentiation related to the CaP formation is in accordance with previous studies[92, 93], although, cell production of TGF-ß on these four bioactive surfaces has, to the knowledge of the author, not been evaluated before. Bone response (cell attachment, osteocalcin and TGF-β production) on the blasted controls were similar or increased compared to the SBF treated Fluoridated, AH and TiMgO surface.

Conclusions

- Surface orientation had no effect on bone response *in vivo* after 3 months of implantation.

- Possibly bioactive titanium implants (alkali-heat treated, covalently immobilized protein coatings) did not significantly increase bone response compared to non-bioactive titanium implants (anodized) *in vivo* after 1 month of implantation.

- The non-bioactive (anodized) and possibly bioactive (anodized/Mg) titanium surfaces demonstrated an increased inflammatory cell attachment compared to the turned and blasted control surfaces *in vitro*, however, otherwise there was a similar early inflammatory cell response.

- The covalently immobilized protein coatings influenced early inflammatory response *in vitro*; however, catalase instead of fibrinogen demonstrated the strongest inflammatory response.

- The possibly bioactive surfaces (alkali-heat treated, anodized/Mg, fluoride and nano HA coated), induced earlier calciumphophate formation than the non-bioactive (blasted control), however, only the possibly bioactive SBF treated nano HA implants, demonstrated a subsequent increased bone cell response *in vitro* compared to the non-bioactive blasted controls.

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