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Improved titanium and steel implants:
Studies on bisphosphonate,
strontium and surface treatments

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Cover: Calcium nucleation on bioactive TiO₂ surface, visualised with SEM

During the course of the research underlying this thesis, Paula Linderbäck was enrolled in Forum Scientium, a multidisciplinary doctoral programme at Linköping University, Sweden.

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- Ur glädjen föds kreativiteten -

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PAPERS I-IV

ABSTRACT

Purpose: The general aim of this thesis was to increase the understanding of biomaterial surface modifications and local delivery of osteoporosis drugs for bone integration. We therefore (i) characterised and investigated model surface coatings for controlled drug delivery in a rat tibia screw model (ii) elucidated the effect of surface treatment for activation of complement system *in vitro*.

Materials and methods: Bisphosphonate was immobilised directly to implant surfaces by two methods. In the first method, bisphosphonate was bound via a crosslinked fibrinogen layer to titanium surfaces. In the second method, stainless steel screws were first dip coated in a TiO₂ sol-gel, and thereafter incubated in simulated body fluid (SBF). The so prepared thin calcium phosphate layer on titania bound then bisphosphonate directly with high affinity. The drug release kinetics was determined *in vitro* by ¹⁴C marked alendronate that was quantified with scintillation techniques. The screws were inserted in the metaphysis of rat tibia and the mechanical fixation monitored by screw pullout measurements after 2 or 4 weeks of implantation. In order to compare two different osteoporosis drugs, bisphosphonate and strontium ranelate, stainless steel and PMMA screws were inserted in the tibial metaphysis of rat for 4 and 8 weeks. Bisphosphonate was then delivered subcutaneously and strontium ranelate orally during the whole implantation period. The mechanical fixation was analysed by pullout force measurements, and bone architecture studied by micro-computed tomography (μ CT). The immune complement activation on sol-gel- and smooth titanium surfaces was analysed in human blood plasma before and after annealing of titanium at 100-500°C or upon UVO-treatment for up to 96 hours.

Results: Bisphosphonate coated screws enhanced the screw pull out force after 2 weeks of implantation by more than 30% (fibrinogen coating) and by 93% after 4 weeks (sol-gel derived TiO₂ coating). Systemically administered bisphosphonate enhanced the mechanical screw fixation after 4 weeks by more than 96% and after 8 weeks by more than 55% as compared to strontium ranelate treated animals ($p = 0.00$). Strontium ranelate treatment did not show significant improvement of screw pullout force after 4 and 8 weeks, compared to

control. The immune complement surface deposition from blood plasma vanished irreversibly after Ti heat treatment at 250-300 °C during 30 minutes or after UVO exposure for 24 hours or longer. Tentatively, changes in surface water/hydroxyl binding upon heat- and UVO treatments were observed by XPS and infrared spectroscopy.

Conclusions: The results show that fixation at short implantation time (weeks) of orthopaedic implant can be enhanced by immobilised bisphosphonate on stainless steel or titanium implants. Systemic delivery of strontium ranelate showed no significant effect on implant fixation in rat tibia, and we hypothesise therefore that strontium ranelate will not become a power tool to increase the early implant fixation, but may be beneficial at longer times. Heat annealing or UVO-treatment of titanium surfaces change the surface hydroxylation, leading to decreased immune complement deposition from blood plasma.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Studier på förbättrad förankring av metalliska benimplantat

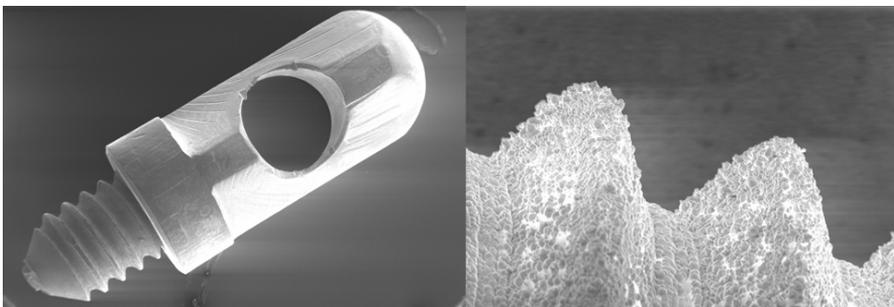
Det är idag möjligt att ersätta en förlorad tand eller ett skadat knä med ett nytt och på så sätt återge rörelseförmågan eller kroppsfunction till en skadad individ. Till och med en ung idrottare med brutet ben kan få möjlighet att fortsätta sin idrottskarriär. Detta är ett enormt framsteg inom den medicinska vården och för dessa människor. Men det händer ibland att någonting går snett och implantaten läker inte som de ska. Orsakerna därtill kan vara flera, såsom infektion eller överbelastning, och det är viktigt att fortsätta forskningen kring hur främmande material reagerar i kroppen och hur man förbättrar läkningen av ortopediska och dentala implantat. I denna avhandling har vi dels vidareutvecklat implantatytor med förbättrad fixation i ben, dels studerat titanoxidens grundläggande immunologiska reaktioner i blodplasma.

En viktig del av vårt medfödda immunförsvar är komplementsystemet, ett system av plasmaproteiner, som kan aktiveras på främmande ytor. De mer än 30 proteinerna i det mänskliga komplementsystemet har till primär uppgift att försvara oss mot mikroorganismer, men också mot annat icke-kroppseget material, såsom biomaterial. En del material aktiverar komplementsystemet snabbt och andra långsammare. Mycket av mekanismerna är fortfarande oklara och det är därför viktigt att forska om detta. Vi jämförde i en del av denna avhandling komplementaktivering på titanytor som preparerats på olika sätt; utan behandling, UV-bestrålning eller efter mild uppvärmning. Vi fann att UV-bestrålade och värmda ytor aktiverade komplement i betydligt mindre grad än ytor utan behandling, sannolikt beroende på förändringar i Ti-oxidens struktur och kemi.

En snabbare integration av benimplantat har några kända fördelar:(i) En tandpatienten kan börja äta fast mat tidigare. Eller för en gammal människa med en bruten höft är det av stor betydelse om hon kan gå några månader tidigare. Livskvalitén ökar då betydligt. (ii) Problemen vid inläkning av benimplantat uppstår oftast under de första veckorna och en påskyndad läkning minskar risken för reoperation.

För att förbättra benläkningen har vi bundit fast en liten mängd osteoporosläkemedel, bifosfonat, på implantat yta med två olika beläggningar: 1. ett tunt proteinlager eller 2. TiO_2 med calciumfosfat (CaP)-beläggning. Skruvarformade implantat opererades in i råttans ben där de satt i 2 eller 4 veckor. Vi såg då tydligt att bifosfonat stärkte skruvens fäste. Det betyder i sin tur att mer ben nybildas runt implantat med bifosfonat. Vi jämförde också två olika osteoporosläkemedel, bifosfonat och strontiumranelat och deras effekter kring implantat. Strontiumranelat är ett relativt nytt läkemedel och har visat sig ha dubbel effekt: den saktar ned bennedbrytande celler och stödjer benbildande celler. I denna studie gav vi dessa läkemedel systemiskt till råttor under 4 och 8 veckor, och observerade ingen tydligt effekt av strontiumranelat. Bifosfonat däremot ökade skruvens fixation redan efter 4 veckor. Mikroskopisk analys visade betydligt mer nytt ben hos bifosfonatbehandlade råttor än råttor som behandlats med strontiumranelat.

I alla delstudier vi genomfört har vi lärt oss något nytt. Vi har hittat nya immobiliseringsmetoder för bifosfonat, och observerat hur detta kan förbättra läkningen runt implantat. Att strontiumranelat, som troddes ha stark tidig påverkan på benläkning visade sig ha svagare effekt än bifosfonat, det var överraskande. Likaså, att olika behandlingar av titanytor gav olika immunreaktioner var oväntat, och kan ha betydelse för titanets reaktioner med blod, samt för vävnadsläkning i allmänhet.



Figuren visar en rostfri skruv (vänster) och gängorna med vår calciumfosfat (CaP)-beläggning (höger). Skruvens längd är 1 cm, gängornas längd är ca 3 mm och yttre gängdiametern 1.7 mm

LIST OF PAPERS

- Paper I** Wermelin K, Aspenberg P, Linderbäck P, Tengvall P.
Bisphosphonate coating on titanium screws increases mechanical fixation in rat tibia after 2 weeks
J Biomed Mater Res A. 2008 Jul;86(1):220-7
- Paper II** Linderbäck P, Areva S, Aspenberg P, Tengvall P.
Sol-gel derived titania coating with immobilised bisphosphonate enhances screw fixation in rat tibia
J Biomed Mater Res A. 2010 Aug;94(2):389-95
- Paper III** Linderbäck P, Harmankaya N, Askendal A, Areva S, Lausmaa J, Tengvall P.
The effect of heat- or ultraviolet treatment of titanium on complement deposition from human blood plasma
Biomaterials. 2010 Jun;31(18):4795-801. Epub 2010 Apr 3
- Paper IV** Linderbäck P, Agholme F, Wermelin K, Närhi, T, Tengvall P, Aspenberg P
Weak effect of Strontium ranelate in early bone screw fixation
Submitted

MY CONTRIBUTIONS TO INCLUDED PAPERS

Paper I Bisphosphonate coating on titanium screws increases mechanical fixation in rat tibia after 2 weeks

I participated in the evaluation of controlled release, produced screw coatings, participated in *in vivo* study and authoring of the paper.

Paper II Sol-gel derived titania coating with immobilised bisphosphonate enhances screw fixation in rat tibia

I participated in the planning of project, performed all parts of *in vitro* and *in vivo* studies, and authored the paper.

Paper III The effect of heat- or ultraviolet treatment of titanium on complement deposition from human blood plasma

I participated in the planning of project as well as production and characterization of coatings, participated in the authoring of paper.

Paper IV Weak effect of Strontium ranelate in early bone screw fixation

I contributed to planning of the project; Performed *in vivo* study and large part of characterization; results analysis and authoring of paper.

INTRODUCTION

Bone

Bone is composed of two main components: an organic extracellular matrix mainly responsible for the mechanical flexibility and toughness of bone, and an inorganic mineralised substance serving as mechanical reinforcement and body Calcium Phosphate (CaP) deposit. The human skeleton (Greek *skeletos* = "dried-body", "mummy") serves as a scaffold that support organ functions, anchors muscles, tendons and ligaments, participates in the humoral and cellular regulation and turnover, and protects organs such as the brain, lungs and heart. The organic matrix of bone, approximately 20% w/w, consists mainly of collagen type I and smaller amounts of types V and XII. Smaller amounts of non-collagenous proteins, such as osteocalcin, osteonectin, and bone sialoprotein is also imbedded in the organic matrix and have different functions in the regulation of bone mineralisation and bone cells (1). The inorganic part of bone consists mainly of hydroxyl apatite (HA) crystallites accounts for approximately 65% of wet weight and 50% bone volume. Compared to pure inorganic hydroxyapatite, apatites in biological systems are calcium-deficient and always carbonate saturated . Apatite crystallites are plate-like and approximately 20 nm by 3-7 nm in size and serve also as an ion reservoir. The apatite crystallites are incorporated within and between the organic extracellular matrix components. The water content of bone is about 10%, and the cell fraction around 2% (2).

On the macroscopic level, the skeleton is composed of two types of 3D-structures, cortical and trabecular bone. 80% of the skeleton mass is cortical bone with load bearing and protective function in the exterior part of all types of bone. The structure of the outer part (periosteum) is smooth, white and solid in appearance. The trabecular part of bone is porous and located beneath the cortical bone (endosteal part). The open porous network of trabecular bone gives place for cells, blood vessels and bone marrow (Figure 1). At least four types of bone cells, osteoclasts (bone resorbing cells), osteoblasts (bone building up cells), mesenchymal stem cells (MSCs) and osteocytes participate in the remodeling of bone.

Osteocytes are mature osteoblasts and orchestrate the remodeling via chemical and biomechanical cues. MSCs become during the early phase of bone trauma and inflammation (hours to days) differentiated to osteoblasts. Active osteoblasts secrete collagen fibrils and other bone proteins to form osteoid, the organic and unmineralised part of extracellular matrix. They become upon the subsequent mineralization totally embedded in bone and differentiate to osteocytes. Osteocytes are the most abundant cell type in bone and they communicate with osteoblasts and other osteocytes. Reactions to chemical stimuli are mediated via channels called canaliculi. Osteoclasts are hematopoietic lineage multinucleated cells residing on bone surfaces. Activated osteoclasts bind to bone surface located vitronectin via its membrane located integrins. In the resorption zone hydrogen ions (H^+ , acid) are secreted by osteoclasts and the bone mineral, CaP, is dissolved. In addition to the formation of solid bone, the *de novo* bone is supplemented with blood vessels that sprout during the regeneration process.

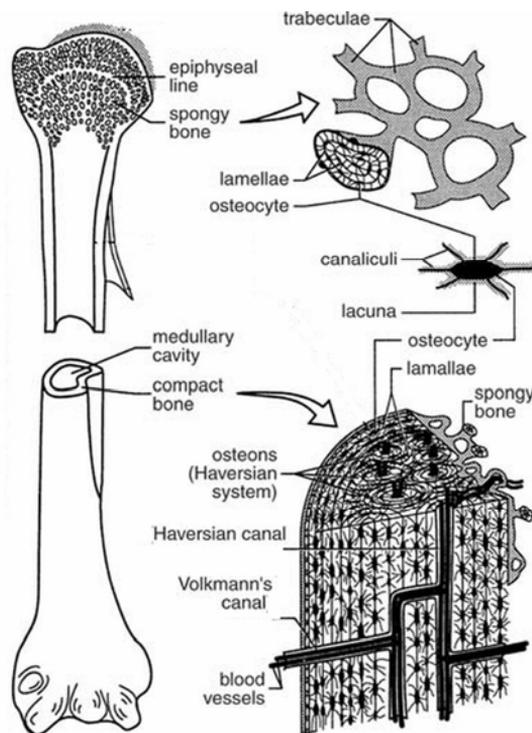


Figure 1. Bone

The remodeling of cortical and trabecular bone begins thus with resorption followed by bone build-up by differentiation of MSCs to osteoblasts that are attracted to the cavities formed by osteoclasts, and fill these first with non-mineralised bone, an osteoid. The osteoid is subsequently mineralised and new bone is eventually formed. These dynamic interactions among the bone cells and cell-derived molecules at fracture sites ensure a complete regeneration of bone (1).

Wound healing around bone implants

Wound healing around bone implants can be divided into primary and secondary healing, depending on how well the prostheses micromotion is controlled. The primary fracture regeneration/repair is similar to healing of closely apposed fractured bone edges that heal without the formation of intermediate soft callus. Bone remodeling occurs then similar to normal bone and throughout lifetime. Bone fractures that are not rigidly stabilized during repair, go through a secondary phase repair with intermediate granulation tissue prior to bone formation at the interface. As many of the orthopaedic or odontological implants are not mechanically well stabilized, the healing follows a secondary phase repair (3).

The wound healing process begins with hemostasis, and blood plasma and cells are located around the implant. This is followed by the formation of a fibrin network with embedded platelets, white cells and erythrocytes. Macrophages become soon formed from blood monocytes and are located during the early phase (up to 2 weeks) of healing close to the implant surface (4). During hemostasis, acute inflammation takes place as part of the healing process, and with increasing time various signal molecules can be found at the wound site. Inflammatory cells, such as neutrophils and monocytes, are recruited within minutes to hours to the implant site. Acute inflammation continues then 2-3 days, when monocytes slowly differentiate to macrophages that, as mentioned above, reside in the healing site for up to a couple of weeks. Tissue repair is observed by increasing cell differentiation and proliferation and extracellular matrix (ECM) synthesis. The provisional ECM is the blood clot, called the coagulum. It is, when mechanical stability is lacking replaced by early granulation tissue and a vascularised structure is formed around the implant after 3-4 days (5).

After 2 weeks pluripotent mesenchymal stem cells originating from bone marrow differentiate to osteoblasts. Osteoblasts fill a gap in the fractured bone tissue with an osteoid, which eventually becomes mineralised. The new mineralised bone is formed, but does not always unite completely with the old bone (3).

While the gap formation often occurs with inert biomaterials, osteoconductive and osteoinductive implants are faster integrated by so called 'contact repair'. The direct contact allows organized lamellar bone form directly across the fracture line. The necrotic bone is then removed by osteoclasts prior to new bone formation. Osteoclasts resorb 20-40 μm bone per day, and the cutting cones provide nutrients to tissues to be repaired. Behind the cutting cone, osteoblasts replace bone at the non-contacting wound edges by producing an osteoid, mainly composed of collagen I and IV. When osteoids become mineralised through deposition of hydroxyapatite crystals, new lamellar bone is formed (6).

As most bone fractures are not rigidly supported after injury, the unmineralised tissue undergoes a secondary repair phase (3). The healing starts then with ECM-rich bridges and intramembranous ossification produced by osteoblasts, which together give a minor support around the wound. When fibroblasts and undifferentiated mesenchymal cells arrive to location after 3-4 days through the circulation, the wound starts to gain mechanical strength. While in the primary healing osteoprogenitors differentiate to osteoblasts, they differentiate first to chondrocytes during the secondary healing process. A collagen-rich callus is formed by chondrocytes and fibroblasts around the fracture, and serves as a matrix for the vascular network and as a fracture support. After approximately 2 weeks after fracture, the soft callus begins to mineralise to hard callus. The bone formed through collagen mineralisation is called woven bone. Osteoblasts, which are now located on the surface of bone, secrete osteonectin and osteopontin, and thereby providing nucleation sites for calcium phosphate deposition (1, 3, 7).

After 3 weeks of healing, the cartilage has transformed to bone and the implant or healed bone edges are stabilised. However, the newly formed bone does not reach the same level of mechanical strength and load support as the uninjured bone. This is obtained when the woven bone has become a more compact lamellar bone.

Primary remodeling for stabilised bone is coupled to restoring of tissue strength (gap healing) and to a repair process (direct contact). As the cutting cones mature during contact remodeling, lamellar bone becomes ring-shaped with blood vessel canals inside. Lamellar bone that was deposited along the long axis of injury during the primary gap repair serves as a matrix. Haversian canals are remodeled and osteoblasts synthesise new lamellar bone until the tunnel is closed. The newly formed bone is still weak, and it may take several years of remodeling to reach the strength of uninjured bone (3).

The secondary bone remodeling differs from the primary remodeling mainly from the extensive removal of partly mineralised callus, which was formed during the secondary repair phase. Woven bone is removed by osteoclasts, while osteoblasts produce new lamellae bone. Mechanical stress in mineralised tissue generates signals at the cellular level, and affect that way to increased bone production. While a bone remodeling cycle may take 6-12 months or so, the newly formed mineralised tissue has close to same mechanical strength as the old, uninjured bone (6) .

Wound healing in bone differs from other tissues in that there is no scar formation in healed bone. A successful bone implant insertion leads thus to formation of a tissue that closely resembles the original, i.e. bone is regenerated.

Bone-implant interface

All implanted non-self materials elicit a host tissue response. Thus, no material implanted into a living tissue is biologically inert. The bone-implant interface appearance and more specifically integration dynamics depend largely on implant chemistry, load patterns, design and surface topography. An adverse response can end up in a local infection or chronic inflammation, or larger, systemic signs of hypersensitivity, elevation of implant material in blood (dissolution), or lymphatic particle transport. The ultimate complications at tissue-implant interfaces are extended infection, exuberant or defective healing, adverse local tissue reaction, and implant failure.

When a bone-titanium implant interface is studied at the electron microscope ultrastructural level, a 20 to 500 nm thick amorphous layer is sometimes observed. This layer is not observed within fibrous tissue, and is therefore associated with an organic bone matrix (8, 9). This non-mineralised layer can be successfully avoided by osteoinductive and osteoconductive surfaces and good physical bone-implant contact.

Bioinert fixation

When implant materials are considered as chemically inert (such as stainless steel, titanium, and zirconium) and the interface is not chemically or biologically bonded to a tissue, a progressive development of a thin, non-adherent fibrous capsule may develop in soft tissues. Its thickness depend on factors such as condition of the host tissue, reactivity of the implant material and micromotion and mechanical loading at the interface (10). Micromotion results in the development of a non-adherent fibrous capsule and can lead to deterioration of the host tissue interface and function of the implant (11). Titanium is considered as a low reactive metallic implant material, and many investigations have confirmed cellular responses to titanium as compatible and advantageous. Suska and co-workers compared the early biological response towards (cP) titanium and non-biocompatible copper in an *in vivo* rat study and found that (cP) titanium caused a different signal molecule dynamics in the adjacent host tissue (12). This was suggested to be indicative of a benign osseointegration process. However, several groups studying bone-titanium contact at the ultra-structural level have observed a thin amorphous non-calcified layer at the Ti-implant interface (13, 14). In dental failure cases the jaw volume, bone quality, and overload are indicated as the three major determinants for late implant failure in the Brånemark system (15).

Biological fixation

Animal experiments with varying micro- and nano implant surface topographies have encouraged new thinking to increase and accelerate new bone formation around implant surfaces. Measured surface roughness (R_a) at the order of 1-5 micrometers is suggested optimal for bone ingrowth and has a strong reinforcing effect on porous implants (9). Also, the optimal pore size for bone ingrowth is indicated in the range 100-500 μm , giving rise to a “biological fixation” (10). However, such implants show a lower compressive strength than implants without micro- or sub-millimeter porosity.

Bioactive fixation

Until the introduction of so called 'bioactive' implant materials the surface reactivity of implants was assumed as a side effect and even an unwanted property. This is, however, no longer the case and several implant modifications suggest that bone regeneration around implants can be actively guided. The most prominent bioactive fixation can be obtained via the preparation of thin CaP films (most often hydroxyl apatite) on bone implants. The common denominator of CaP containing implants is their ability to release Ca^{++} -ions. Other surface active modifications may include immobilisation of osteoporosis drugs, modulators of inflammation, modulators of the several of Wnt signaling pathways, hormones, bone morphogenetic proteins (BMPs), other growth factors, pro-angiogenetic factors, and insulin.

When a bioactive implant is inserted to bone, a series of biophysical and biochemical reactions take place. CaP coated implants are ascribed in several studies the ability to form an interfacial 'chemical bond' with the surrounding bone. This fixation is called "bioactive fixation" (10). Most literature dealing with bioactive fixation is focused on hydroxyapatite (16-23). In one study the investigators reported that bone integrated directly onto hydroxyapatite through an organic-free transition layer comprised of biological apatite with a thickness up to 1000 nm (24). Recently investigators reported good bone growth also on bioactive glasses. In one study in rabbit femoral bone, the bone volume was significantly higher on bioactive glass coated samples compared to non-glass coated already after 2 weeks of implantation, and after 12 weeks osteoconducting properties were suggested for bioglass surfaces (25). The outermost layer of hydroxyapatite consists of carbonate-apatite crystal that is reported to be beneficial for new bone formation, mainly due to the slow release of Ca -ions. A common characteristic for bioactive glasses and bioceramics is their time-dependent change of the implant surface (26). Such surfaces form carbonate-apatite crystals, adsorb proteins and provide a platform for bone growth via upregulation of osteoblasts and down regulation of osteoclastic activity (27-29).

Blood plasma - implant interface

Coagulation at artificial surfaces

Blood that contact artificial surfaces deposit a plasma protein layer and initiate eventually a coagulation cascade via the intrinsic pathway (30). The pathway is activated by negatively charged surfaces. The extrinsic or tissue pathway is, however, not activated by artificial materials. As many biomaterials possess negative charge at physiological conditions, blood contact may lead to an unwanted thrombus formation at the surface, especially in applications such as artificial grafts, dialyzers or heart valve implants. Modifications of implant design, surface chemistry and other material characteristics may alter the haemostatic behavior. For future, a more thorough understanding of the impact of blood clotting on general biomaterial behavior and implant integration in tissues is of importance.

Immune complement system

Also the humoral part of immune response may be activated when blood contacts a biomaterial. It is composed of blood plasma proteins, mainly the complement cascade system, but is also coupled to the coagulation system and the cellular part of the immune system, and immune cells such as T-cells, B-cells, dendritic cells and white cells. The surface mediated activation of the complement system influence blood platelet and leukocyte activation and affects the recruitment and adhesion of other inflammatory cells to the implant site, i.e. the early phase of wound healing.

The complement system can be described as a developmentally old rapid and effective protein mediated surveillance system that promotes the clearance of body debris, regulation of inflammatory reactions and healing and cellular immune attacks against infections agents and “non-self” elements. These elements can be pathogenic, such as bacteria, viruses, fungi, or maybe a foreign material that should be eliminated from the host effectively by cellular or humoral activities. It is known till date that the complement cascade constitutes of more than 30 distinct plasma and cell membrane bound proteins. The activation can follow three separate but interacting pathways: classical, alternative and lectin pathways, as shown in figure 2 (31, 32).

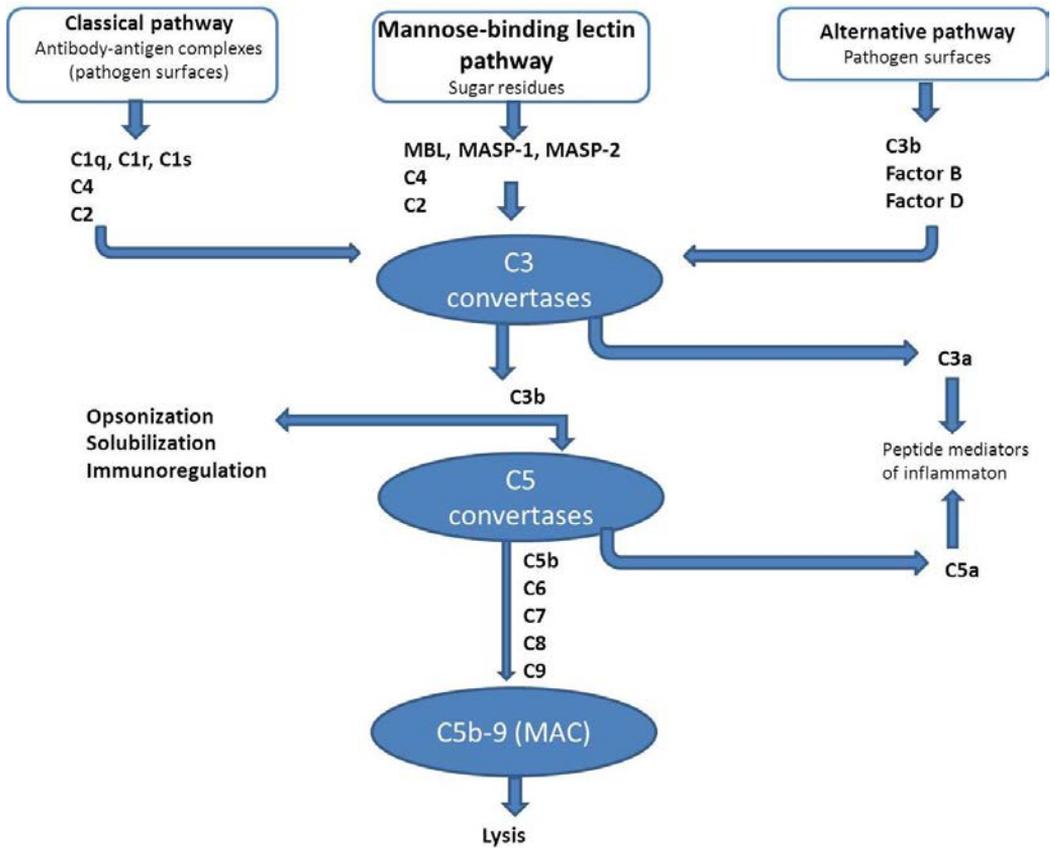


Figure 2. Overview of the three activation pathways of complement. All three activation pathways lead to the formation of C3 convertases, although the activation mechanisms are different. Plasma C3 is cleaved by a soluble or surface bound C3-convertases to soluble C3a and soluble or surface bound C3b. A C5-convertase may then be formed and leads to the cleavage of C5, and if not protected, assembly of membrane attack complexes (MAC) and generation of soluble inflammatory mediators.

These three complex pathways form cleavage products (such as C3b, C4b and iC3b), which often bind to particles, surfaces, bacteria and immune complexes. Also soluble complement protein fractions (polypeptides), often called anaphylatoxins, such as C3a, C4a and C5a, are generated at the same time. Both the soluble and surface bound polypeptides bind to specific receptors of cells such as neutrophils, monocytes, macrophages, fibroblasts and smooth muscle cells, thereby inducing various cellular responses and systemic effects of complement

activation. The C-system is thus also a part in inflammation and the normal wound healing process. The complement system is in severe cases known to participate together with the cellular part of the immune system in the killing and rejection of non-accepted transplanted organs.

One of the critical control points in the activation of the complement system is the proteolytic cleavage of complement factor 3 (C3) to C3b and C3a. This is done by two multisubunit enzyme complexes, called C3 convertases. The cleavage of C3 may in turn give rise the buildup of C5 convertases that cleave complement factor 5 (C5), leading to the formation of the membrane attack complex (MAC) that disrupts the membrane integrity of pathogens (33, 34). The complement system is controlled or inhibited by soluble factors I and H and C4 binding protein that down-regulate convertases, and by C1-inhibitor that down regulates C1 complexes. The main cell membrane bound control proteins are decay accelerating factor (DAF, accelerates decay of C3/C5 convertases), complement receptors 1 and 2 (CR1 and CR2, regulation of C3b breakdown and B-cell functions) and CD59 (that prevents MAC-formation on homologous cells).

Immune complement activation by biomaterials

Although the immune complement activation at artificial surfaces has been studied for almost 30 years it is not yet fully understood. Chenoweth et al. suggested a blood - surface interaction model for large extracorporeal machine surfaces (35, 36), a model that most likely is of general validity. He proposed basically that large nucleophilic surfaces activate complement via the alternative pathway, and negative surfaces down regulate complement (Figure 3). The degree of activation could then be defined by quantitation of soluble C3a-fragments and surface bound C3b fragments. It is known that the thickness of an adsorbed protein layer varies over a time, and depend on surface characteristics of a biomaterial (35). It was long suggested that biomaterials activate through the alternative pathway, when C3b binds to a nucleophilic group (such as $-OH$ and $-NH_2$) of a surface (37). Edwin et al. suggested that hydroxyl rich surfaces activate complement, and prompt the accumulation of inflammatory cells (38). However, also complement activation via the classical pathway is shown relevant in biomaterial applications (33, 34, 39).

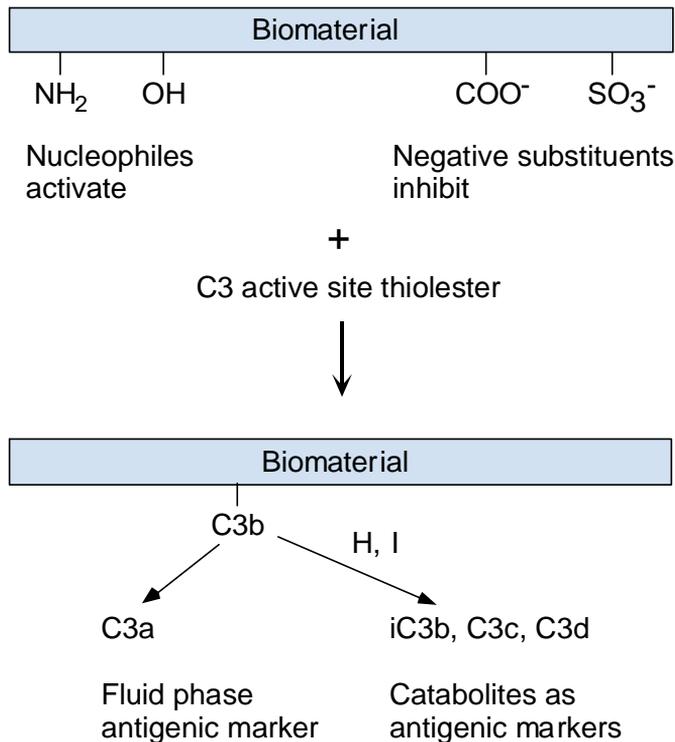


Figure 3. Schematic diagram summarizing the molecular mechanisms of complement alternative pathway activation at (large) protein coated artificial surfaces (38)

Certain hydroxylated surface structures bind IgG from plasma or body fluids and activate thereafter C via the classical pathway. Bioincompatibility, as a clinical consequence of complement activation during extracorporeal circulation has led to several studies on how complement becomes triggered by artificial materials. Andersson et al. reported that heparin coated surfaces reduced blood activation upon contact with artificial materials (40). They demonstrated increased surface blood biocompatibility, in terms of coagulation, complement and platelet activation, by increasing surface concentration of heparin to 6-12 pmol AT/cm². The commercially most well-known artificial immune shadowing preparation is surface PEGylation, a widely used technique to escape immune clearance of micron- and nanosized particles during targeted drug delivery. Increased knowledge of how implanted materials affect complement activation, cell recruitment and differentiation, and wound healing is of

importance and the development to find optimal surfaces for each tissue application is of prime interest for many research groups.

Biomaterials

One early clinical application of a biomaterial was invented in 1759 by Hallowell when he united the edges of a lacerated brachial artery using a wooden peg and a twisted thread (41). History teaches us also that ancient people have used metals for tooth replacements, and wooden leg replacements have been used after extremity amputations. Thus, replacements of body functions or parts are not a historical novelty. At a national Institutes of Health Consensus development conference held in 1987, a biomaterial was defined as "materials intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body" (42).

Bone anchored implants

The musculoskeletal system must endure strong mechanical forces, and expose a harsh chemistry. Therefore, tough requirements are necessary for bone implant materials and these are largely determined by the top mechanical loads and physical/chemical conditions during the early phase of healing, inflammation. Current commercial replacement materials for teeth and bone include metals, polymers (natural or synthetic), corals, human bone (processed cadaver bones), animal bone (processed cow bones), corals and coral derived synthetic ceramics (calcium phosphates, calcium sulfates, calcium carbonate, bioactive glasses), and composite materials (43-45). The most common materials in total hip arthroplasty are metal alloys based on CoCr, titanium and stainless steel (SS). These are traditionally cemented with polymethylmetacrylate (PMMA). Titanium screws are common in dental replacement applications and bridges are made of CoCr and SS. Noncemented fixation with granules and biological fixation with bioceramic coatings, such as hydroxyl apatite are still under development (41).

The success of orthopaedic implant fixation is highly dependent on the selected material. Metal implants, such as stainless steel, CoCr, and titanium-based alloys are, as indicated above, the most commonly used metallic materials in total bone replacement and fixation due

to their good biocompatibility and excellent mechanical properties that meet requirements for load-bearing applications (46). Several types of stainless steels are commercially available although in practice the most common is 316L (ASTM F138, F139), grade 2. It consists 60-65 % iron with significant alloying additions of chromium (17-20 %) and nickel (12-14 %), and in order to reduce *in vivo* corrosion less than 0.030 % (wt %) carbon (47). Commercially pure (CP) titanium (ASTM F67) and extra low interstitial (ELI) Ti-6Al-4V alloy (ASTM F136) are the two most common titanium-based implant material, with a titanium content of 98.9 % / 88.3-90.8 % (48).

Bioceramics

Ceramics are hard and brittle non-metallic inorganic materials. They include amorphous and crystalline- and glass-ceramics. Ceramics in orthopaedic surgery can be divided into several subgroups depending on their load bearing properties, surface chemistry (bioinert vs. biodegradable) and *in vivo* reactivity (bioactive). Bioceramics in orthopaedic can generally be classified into two large subgroups: (i) technical high strength ceramics that are used in load bearing applications. To this group belongs e.g. zirconia in dental caps and ball heads in hip prostheses and (ii) calcium phosphate based ceramics (e.g. hydroxyapatite) in bone regeneration applications (49).

Hydroxyapatite (HA) based materials can be prepared as sintered structures, as granules, as composites or as coatings. Most synthetic HA has the stoichiometry $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. In contrast to this, human bone do not have a pure HA stoichiometry, and ions like CO_3^{2-} and trace amounts of Na^+ , Mg^{2+} , Fe^{2+} , Cl^- , or F^- are included in the crystal structure. The excellent biocompatibility of synthetic hydroxyapatite paired with the bone-like composition and structure and osteoconductivity has led to increased clinical use over the last 30 years (24, 50). The predeposition of hydroxyapatite on bone implant surfaces is believed to promote mineralisation and chemical bond formation at bone-implant interface (osteinduction). Pure synthetic HA in the bulk shape possesses however, severe mechanical limitations, so the emphasis has shifted to the preparation of (submicron-micron) thin HA coatings on metals for load-bearing applications (16, 41). These are then less brittle.

Implant surface treatments and improved osseointegration

Surface modifications to improve early bone fixation

There is an increasing interest in the composition and structure of titanium- and titanium alloy oxides. Titanium is a highly reactive material and forms in atmosphere a thin outmost oxide layer (Ti(IV)O_2 , thickness 2-10 nm). The oxide provides a good corrosion resistance during physiological conditions (47). However, titanium is often separated from bone by a thin non-mineralised layer, resulting in a poor integration or direct adhesion to bone. To enhance a true adhesion a variety of surface modifications have been suggested.

In the early 70's scientists introduced a concept of porous metal biomaterials that allowed bone ingrowth into the porous interface. The porosity was reported to influence cell adhesion and morphology, proliferation and differentiation (45, 51). The porous orthopaedic devices are most often surface blasted and acid etched, and with sintered CoCr beads or diffusion bonded fiber metal meshes. Titanium is cancellous-like structured, or plasma sprayed onto various solids, e.g. onto CoCr and SS. Buser et al. studied cylindrical titanium implants with different surface modifications (electron polished, sandblasted, acid pickled HF/HNO₃ with medium or high grit, titanium plasma sprayed and hydroxyapatite coated). They observed that the bone-implant interface integration in miniature pig femur metaphyses and tibia was significantly improved with increasing surface roughness (52). More recently the desire for increasingly porous surfaces for enhanced bone ingrowth paired with low surface friction characteristics and availability for a coating has led to the development of novel highly porous metal interfaces. These have porosity in the range 100-600 μm and an overall porosity of 60-70 % (53, 54). In one study with implantable chambers in dogs, high porosity titanium interfaces were compared to titanium and cobalt chrome beads and revealed then enhanced bone ingrowth, that may contribute to an enhanced long-term implant survival (54). An open-cell tantalum construct was recently presented in the field of orthopaedic surgery. The mechanical properties show a low modulus of elasticity (~ 3 GPa) while allowing the support of physiological load and bone ingrowth (55). Porous tantalum was thus indicated bioactive *in vitro* and *in vivo* and showed enhanced bone ingrowth in a canine study, indicating higher shear fixation strength than reference implants with lower volumetric porosity.

Table I. Selected inorganic surface modification studies on titanium implants

<i>Surface modification</i>	<i>Purpose of the study</i>	<i>Model</i>	<i>Author and year*</i>
Heat treatment	Bone response to heat treated implants	Rat, 4, 5, 6, 10 and 35 days	Hazan et al. 1993
Blasting	Bone response to 3 different surface structures	Rabbit 12 weeks	Wennerberg et al. 1995
Anodic oxidation	Bone response to different surface structures and oxidation	Rat, 1, 3 and 6 weeks	Larsson et al. 1996
Bioactive glass vs. HA coating	Comparison of bioactive glass and HA coating	Rabbit, 4, 8, 12 and 16 weeks	Wheeler et al. 2001
Acid etching	Evaluation of difference between acid etching and machined surfaces	Rabbit, 12 weeks	Sung-Am Cho et al. 2003
Alkali- and heat treatment	Bone response to chemically modified implant surface	Rabbit, 3, 6 and 8 weeks	Nishiguchi et al. 2003
HA coating, plasma spraying	The influence of different crystallinity of HA coatings	Dog, 3 months	Xue et al. 2004
Sol-gel derived glass coating	In vivo behavior of sol-gel derived, bioactive glass coating	Rabbit, 4 months	Gil-Albarova et al. 2004
Chemical with nano topography	Early bone response to chemically and nanotopographically modified surfaces	Rabbit, 4 weeks	Meirelles et al. 2008
UV-treatment	Bone response to UV treated titanium	Cell, Rabbit 2 weeks	Sawase T, et al. 2008
Laser modification	Bone response to laser micro- and nanostructured surfaces	Rabbit, 8 weeks	Palmquist et al. 2009

*(56-66)

As bone and tissues contact the oxidised metal or ceramic surfaces, it has been quite natural to try to improve integration by modifying this oxide layer or to immobilise active substances onto it. In some cases oral- or subcutaneous delivery was preferred to demonstrate proof of case. Several research groups have reported of enhanced bone-bonding ability with calcium phosphate or hydroxyapatite immobilized to implant surface (18-20, 22, 23, 67-70). Sul et al. studied the effect of incorporated magnesium ions on oxidized screw shaped implant surfaces, implanted in rabbit for periods of 3 and 6 weeks. They found that oxidized implants

with incorporated Mg ions are rapidly and strongly integrated in bone (71). Strontium incorporated to hydroxyapatite matrix has also reported to enhance implant osseointegration in ovariectomized rats (72). Taxt-Lamolle et al. reported recently that electro-implementation of fluoride enhanced osseointegration properties of titanium implants in the cortical bone in tibia of rabbit (73).

Sol-gel derived titania coating

Unlike traditional methods, the sol-gel technique allows for the control of surface homogeneity and purity of ceramic materials made from chemical precursors (salts) at low temperatures. The structure of the material can be controlled by choice of appropriate parameters for the chemical reactions. Sol-gel derived materials have usually high porosity and surface area in the range of 100-1000 m²/g.

A sol is a dispersion of small colloidal particles suspended in a liquid and disperse by Brownian motion within the fluid matrix. Colloids are suspensions of particles with a diameter of 1 -1000 nm (74). Gelation of the sol leads to formation of wet gel that is an interconnected solid three-dimensional matrix within an interconnected liquid phase. Upon drying a gel becomes a dry gel, called xerogel, and can be further processed with a heat treatment to form a (nano)porous ceramic.

Typical precursors for the preparation of sol-gel metal oxides are metal organic compounds or an aqueous solution of inorganic salts dissolved in alcohol or water. The precursors undergo two process steps: hydrolysis and polymerisation by condensation. In hydrolysis the hydroxyl ion becomes attached to the center atom of a cluster (such as Ti or Si).

Depending on the amount of water and catalyst present in a sol, all or only part of the alkoxy groups are substituted by hydroxyl groups. Partly hydrolyzed molecules act as attacking molecules for linking with the second molecule and during this condensation reaction water or alcohol is released. The reactions go on through polymerisation and lead to formation of larger entities of molecules and form finally a three-dimensional network.

There exist several advantages of sol-gel synthesis when the film deposition is considered as one of the most important preparation steps. The sol-gel film can be deposited onto an implant e.g. by dip-coating, spinning, sputtering or spraying. By appropriate chemical modifications of precursors, control maybe achieved over the rates of hydrolysis and

condensation and colloid particle- and pore size, and porosity and pore wall chemistry of the final material. The chemical conditions are mild, and extreme pH conditions can be easily avoided which give the possibility to entrap biological molecules, such as proteins, enzymes, or even whole cells into the matrix and still retain their functionalities. Further, sol-gel derived materials have a relatively high OH content in the outermost layers, which is regarded as a benefit in terms of bioactivity (74).

Sol-gel derived titania coatings show bioactivity both *in vitro* and *in vivo* (75-78). It was suggested that the nano-scale topography is related to charge and charge density of the surface and that, rather than porosity, pore size or surface area influence hydroxyl apatite formation on titania (77). The nano-scale topography of the outermost surface layer can be modified by change in annealing temperature, aging time of the sol, and by addition of coating layers (78). The optimal pore-size distribution for apatite formation was reported to be between 5-50 nm, with the peak height > 1 nm (79).

Controlled drug delivery by biomaterials

There exist several goals in controlled drug delivery. One of them is to target the drug of interest to a particular place, tissue, organ or cell type of the body. Other goals may be to control the release rate, and optimize the effective treatment duration and drug concentration in circulation (80). Various types of controlled release systems exist today, ranging from implants that release drugs via surface erosion mechanisms with duration up to 5 years, to osmotically driven pills that may release drugs during months at a constant release rate (81). Common examples of drugs or molecules released from materials include e.g. antibiotics to combat biomaterial related infections, anti-inflammatory drugs to modulate wound healing, growth factors to up- or down regulate specific cell or tissue growth, cell binding peptides and proteins such as fibronectin, fibrinogen, vitronectin and laminin to attract and bind specific cells to surfaces, anti-cancer drugs, anticonceptives for prevention of pregnancy, osteoporosis drugs to slow down the bone remodeling rate, gene vectors, statins, etc.

Controlled drug release occurs mainly by one of three main mechanisms: (i) in diffusion controlled devices the drug is generally uniformly distributed through the carrier material (such as polymer), or the drug is surrounded by a barrier membrane. The drug diffuses then

out through the carrier polymer or through the membrane (ii) in case of a chemical reaction mediated release mechanism the drug is released when a carrier material is degraded by water (hydrolysis) or chemical reactions or, alternatively, the drug can be attached to the host material by a covalent bond that is hydrolytically cleaved or released by enzymatic cleavage. (iii) Solvent activation and transport applies either an osmotically driven process, which subsequently pushes the drug out from the matrix, or the drug is released from a hydrogel by swelling and increased diffusion constant of the drug (80).

Drug delivery (of bisphosphonate) in the present thesis is believed to occur via molecule surface exchange processes or via hydrolysis.

Table II. Studies on various pharmaceutical agents bound to orthopaedic implant surfaces, examples.

<i>Surface immobilised drug</i>	<i>Purpose of the study</i>	<i>Model</i>	<i>Author and year*</i>
Alendronate incubation to HA	Bone density, amount of peri-implant bone	Dog, 4 weeks	Meraw et al. 1999
Zolendronate. Incubation to HA	Bone ingrowth and density, size and number of bone islands	Dog, 12 weeks	Tanzer et al. 2005
Pamidronate incubation to HA and Ca ion	Bone implant contact	Dog, 4 and 12 weeks	Yoshinari et al. 2002
Zolendronate mixed in poly(D,L)-lactide matrix	Cell viability	Human osteoblasts or osteoclast-like cells	Greiner et al. 2006
rhBMP-2, PLGA matrix	Effect of PLGA matrix carrier for rhBMB-2, new bone formation	Rat, 3 weeks	Bessho et al. 2002
bFGF in Matrigel	Survive of bFGF, bone implant fixation	Ovariectomized Rat, 21 days	Gao et al. 2009

*(82-87)

Bisphosphonate

Bisphosphonate (BP) is a geminal (structurally similar) of pyrophosphate, a natural metabolite of bone. The two structurally different BPs, one N-BP and one without nitrogen act via different mechanisms, the former via inhibition of farnesyl diphosphate during protein prenylation and the other by disturbance and affected cell ATP synthesis and metabolism (88, 89). They are used for the treatment of different bone resorbing diseases, such as osteoporosis, tumor-associated osteolysis, and hyperparathyroidism (90-92). They contain a $P-R_1CR_2-P$ backbone structure (cf. pyrophosphate $P-O-P$), which allows a great number of possible variations, either by changing the two lateral chains on the carbons (R_1 and R_2) or by esterification of phosphate groups. Bisphosphonates bind strongly to bone mineral, apatite.

Today, many different compounds of the bisphosphonate family are commercially available for the treatment of bone diseases (Figure 4). Each bisphosphonate has its own chemical, physiochemical, and biological characteristics, and vary thus greatly in efficacy (90). The length of the aliphatic carbon chain and addition of a hydroxyl group to the central carbon atom at position 1 increases the potency of bisphosphonates, by enhancing its affinity to bone. Furthermore, derivatives with an amino group at the end of the side chain, the length of the side chain, and a nitrogen containing ring structure increase further the activity, by regulating the potency of bone resorption (93).

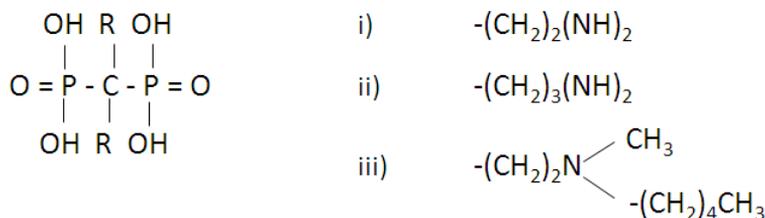


Figure 4. The chemical structure of bisphosphonate and structures of bisphosphonate side chains for pamidronate (i), alendronate (ii) and ibandronate (iii)

Bisphosphonates act via two separate mechanisms: inhibition of bone resorption when given at high doses (89). The overall effects can be divided into three levels: (i) reduction of bone turnover, (ii) osteoclast inhibition at cellular level and (iii) actions at molecular level. At the

tissue level, the actions of all bisphosphonates are similar, both new bone formation and old bone reduction is decreased, although the latter process dominates. Thus a decrease in bone turnover leads to a decrease in bone loss. The effect is due to a decrease in the generation of new bone remodeling units and a decrease in the depth of the erosion cavities (94, 95). At the cellular level, the main bisphosphonate target cell is the osteoclast, and its inactivation decrease bone resorption. Bisphosphonates reduce the bone resorption directly and/or indirectly through the inhibition of osteoclast recruitment to bone surfaces, inhibition of osteoclast activity on the bone surface, and shortening of the osteoclast life span (89). Bisphosphonates generate changes in osteoclast cytoskeleton and in ruffled border of active osteoclasts, indicating that under certain conditions bisphosphonates can enter osteoclasts but not other cells in bone and bone marrow (96-99). Cellular uptake of bisphosphonate drugs require fluid-phase endocytosis and is enhanced by Ca^{2+} ions, whereas transfer from endocytic vesicles into the cytosol requires endosomal acidification (100).

Furthermore, bisphosphonates act by shortening the life span of osteoclast, possibly through apoptosis. The possible action at the molecular level also include action on cell surface receptors and bisphosphonate uptake by cells, where it interacts with an enzyme or other molecule, affecting the cellular metabolism by inhibiting protein tyrosine phosphatase (89).

Bisphosphonates have been given orally or locally in order to improve the fixation of orthopaedic implants. Oral treatment with clodronate, risedronate, alendronate and/or zoledronate have shown improved fixation of pins, screws and knee prostheses (101-104). Various coatings have been developed to immobilise bisphosphonate to implants, and with the aim to enhance the fixation to the surrounding new bone. The most common way to apply bisphosphonate to implant surfaces is adsorption to hydroxyapatite, as bisphosphonates possess high affinity to calcium minerals (82, 105-112).

Strontium ranelate

A relatively new anti-osteoporotic drug, strontium ranelate (protelos), is proposed to induce, in contrast to other anti-osteoporotic drugs (like bisphosphonate), simultaneously both an anti-resorption and bone forming effect (113-120). Strontium is a trace metal in human body and in its physiologically stable divalent cationic form (Sr^{2+}). The ion has a relatively high affinity to bone and may replace Ca in apatite crystals by ion exchange (121, 122). Strontium ranelate contains two Sr^{2+} ions and ranelic acid (Figure 5).

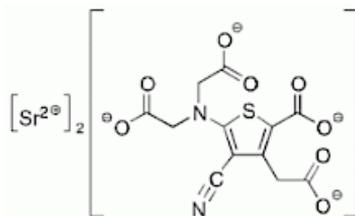


Figure 5. Distrontium 5-[bis(2-oxido-2-oxoethyl)amino]-4-cyano-3-(2-oxido-2-oxoethyl)thiophene-2-carboxylate

The effect of strontium ranelate is not fully understood but it is suggested activate Ca sensing receptors on osteoblasts (123). Moreover, the beneficial action of strontium ranelate on bone tissue quality (density) may be the results of a modification of the bone matrix, as it has been shown that strontium ranelate stimulates the synthesis of bone collagen *in vitro* (118, 124). Several *in vitro* and *in vivo* studies suggest that strontium increases the number of osteoblasts, and decreases the number and activity of osteoclasts, decreases bone resorption and maintains a high de novo bone formation in osteoporotic rats, thereby preventing bone loss (113, 115, 117, 118, 125). Strontium ranelate has also shown increased lumbar spine and hip bone mineral density, and reduced the incidence of vertebral and non-vertebral fractures during long term treatment of postmenopausal osteoporotic women (126-129). A couple of studies show strontium ranelate related improvement of implant osseointegration, and indicate then improvement of bone microarchitecture around the implant (124). The *in vivo* kinetics of bone turnover upon administration of Sr-ranelate is largely unknown, and especially so around bone anchored metal implants.

AIMS OF THESIS

The overall aim was to study and improve titanium and stainless steel integration and healing in bone. We used for this purpose mainly differently prepared titanium surfaces and two different immobilised osteoporosis drugs.

The specific aims were to:

1. Study the biomechanical effects of locally delivered bisphosphonates in a screw model (Papers I and II)
2. Investigate and characterize a model surface coating for controlled delivery of pamidronate (Paper I) and alendronate (Paper II)
3. Elucidate the effect of surface treatment for the activation of complement system on titania model surfaces (Paper III)
4. Study the mechanical and biological effects of systemic strontium ranelate treatment on implant healing (Paper IV)
5. Compare the effect of systemically administered bisphosphonate and strontium ranelate in an implant model (Paper IV)

SUMMARY OF MATERIALS

Table III. Summary of materials used in this thesis

<i>Materials</i>	<i>Paper I</i>	<i>Paper II</i>	<i>Paper III</i>	<i>Paper IV</i>
Titanium screws	x			
Stainless steel screws		x		x
PMMA screw				x
Silica wafers	x	x	x	
PVD	x		x	
Fibr. coating	x			
Sol-gel TiO ₂ coating		x	x	
SBF		x		
Bisphosphonate ranelate	x	x		x
Blood plasma			x	

Implant screws

Titanium screws in paper I were fabricated by Nobel Biocare and the surface displayed an oxidized porous topography. The length and the outer diameter of threaded parts of the screws were 3 mm and 1.7 mm, respectively.

Stainless steel (316L) screws used in papers II and IV were 2.5 mm or 2.8 mm in length and had outer diameter of the threaded part of 1.7 mm and 1.6 mm, respectively.

In paper IV, polymethyl methacrylate (PMMA) screws with outer diameter of 1.6 mm and the threaded part was 2.8 mm. The screws were used to avoid metal artefacts during microCT- and histological analysis.

Silica

Spontaneously air oxidised and polished silicon wafers (1 0 0) with thickness of 0.5 mm were used for reference ellipsometry measurements and for the preparation of smooth ultrathin metal films. The wafers were cleaned as specified in papers I, II and III with a basic peroxide solution and acidic peroxide solution, respectively, resulting in a thin (approximately 1 nm) hydrated amorphous silicon dioxide (SiO₂) layer. Due to the similar hydroxylation of SiO₂ and TiO₂, the same immobilisation chemistry could be applied on both.

Surface coatings

TiO₂ coating by Physical Vapor Deposition (PVD)

Vacuum (melting) deposition of thin films of a material onto various surfaces is generally called physical vapor deposition (PVD). The source material is evaporated at high temperature in high vacuum, and the vacuum allows vaporised material (metal) to attach onto the colder substrate, and finally condense back to its solid state. The equipment includes a vacuum chamber and pump, and a metal wire as the energy source to melt the material under vaporisation (130).

The pressure in the evaporation chamber was below 1×10^{-8} Torr. Approximately 200 nm of optically smooth titanium was physical vapour deposited (PVD) onto clean silicon surfaces (papers I, and III).

Crosslinked fibrinogen coating

The screw coating technique in study I was prepared as described in the original article by Tengvall et al., with slight modifications (18). In short, after the oxidation of the implant surface, screws were immersed in amipropyl triethoxy silane (APTES) and followed by immersion in glutardialdehyde, to build an anchor for the attachment of fibrinogen via any of its amine groups (-NH₂). This was followed by activation of carboxyl groups (-COOH) of immobilised fibrinogen with EDC/NHS, and immobilisation of the next layer of fibrinogen via amine group binding to activated carboxyls. This way, 10 layers of fibrinogen were attached chemically to the surface and simultaneously slightly crosslinked with EDC/NHS

technique. The so fabricated fibrinogen network was approximately 50 nm thick, and allowed N-bisphosphonate, e.g. pamidronate, to be chemically immobilised to this matrix.

Sol-gel derived titania coating

The sol preparations in the present thesis work are described in paper I. Shortly, sol was prepared by mixing and stirring of two solutions. Solution 1 contained commercially available tetraisopropyl orthotitanate $[\text{Ti}((\text{CH}_3)_2\text{CHO})_4]$ dissolved in absolute ethanol. Solution 2 contained absolute ethanol, water and nitric acid (HNO₃, 65%).

A dip-coater (KVS Instruments, Helsinki, Finland) was used to make TiO₂ coating on stainless steel screws. A computer-controlled stepper motor stage was then used to perform the reproducible coating procedure. Substrates were dipped into the sol and drawn up with a speed of 0.7 mm/min. The sol coated screws were then heat-treated in a furnace at 500°C for 15 minutes. The dip-coating and heat treatment procedure was repeated twice.

Calcium phosphate coating

Simulated body fluid was prepared as described in detail by Kokubo and coworkers, but with a slight modification (131). In short, K₂HPO₄ were exchanged for KH₂PO₄, which resulted 0.7 mM higher PO₄ concentration in solution (Table IV). This led to a faster calcium mineral formation on sol-gel derived titania surfaces. Screws used in paper II were soaked in modified SBF at 37°C for 1-5 days to induce CaP formation on the surface of sol-gel derived titania coating.

Simulated body fluid

Spontaneous *in vitro* calcium phosphate (CaP) and bonelike apatite formation on implant materials is generally considered as an indicator of bioactivity of artificial materials. Different physiological salt solutions simulate the content of inorganics in body fluids. Several have been evaluated, but Kokubo's Simulated Body Fluid (SBF) is the most widely used (132, 133). The calcium and phosphate supersaturated SBF has an inorganic salt concentration close to that of human plasma, and the calcium phosphate and apatite deposited from SBF is similar to that of bone (Table IV), i.e. the Ca/P ratio can be adjusted to close to 1.67. The *in vitro* CaP deposition to surfaces with this method is suggested to be a good

indication of bioactivity *in vivo* (133), and the *in vitro* SBF immersion method is today a recognized method to predict a materials' bone induction capability also *in vivo*.

Table IV. Ion concentrations of the Simulated Body Fluid (SBF), human blood plasma ([C] = mmol/dm³) and modified SBF

<i>Ion</i>	<i>SBF</i>	<i>Human blood plasma</i>	<i>P enriched SBF</i>
Na ⁺	142,0	142,0	142,0
K ⁺	5,0	5,0	5,0
Mg ²⁺	1,5	1,5	1,5
Ca ²⁺	2,5	2,5	2,5
Cl ⁻	147,8	103,0	147,8
HCO ₃ ⁻	4,2	427,0	4,2
HPO ₄ ²⁻	1,0	1,0	2,0
SO ₄ ²⁻	0,5	0,5	0,5

Bisphosphonates

In paper I pamidronate was immobilised via a peptide binding through the amine group onto the activated carboxyl group in fibrinogen matrix. The screws were additionally soaked in ibandronate, which was physically adsorbed to the surface. The amount of pamidronate was in the range 170-530 ng/cm² and ibandronate 10-380 ng/cm² (134).

In paper II alendronate was adsorbed (3.1-4.3 µg/cm²) to sol-gel titania coated screws which were preincubated in SBF and formed then a thin layer of CaP.

In papers II and IV alendronate was also administered subcutaneously to the positive control group.

Strontium ranelate

In paper IV Strontium ranelate containing rat diet was purchased from Dietex International Ltd., England. The average rat food portion delivered approximately 800mg/kg/day of strontium ranelate (corresponding to 273 mg/kg/day Sr^{2+}), starting from post-operative day 0.

Blood plasma, buffers and antibodies

In paper III, Complement activation on different titanium surfaces were measured with in situ ellipsometry (see the caption: Methods). Heparinized human blood plasma was obtained from blood donors, and centrifuged to get plasma which was free from red blood cell debris and cell fragments (for details, see paper III). VB++ buffer was in house prepared (details in paper III), and used antibodies were IgG, anti-human C3c, rabbit anti-human IgG, rabbit anti-human fibrinogen and rabbit anti-human high molecular weight kininogen.

SUMMARY OF METHODS

Table V. Summary of the analyses used in this thesis. For more details, see the separate papers

<i>Method</i>	<i>Paper I</i>	<i>Paper II</i>	<i>Paper III</i>	<i>Paper IV</i>
Ellipsometry	x	x	x	
AFM		x	x	
SEM	x	x	x	x
Liquid scintillation counter	x	x		
Ultra violet ozone			x	
Animal model	x	x		x
Pull-out test	x	x		x
MicroCT				x
Histomorphometry				x

Surface characterisations

Null-Ellipsometry

Null Ellipsometry was used to measure fibrinogen film thicknesses and estimate amounts of immobilised bisphosphonate in these films (paper I), thickness of sol-gel derived TiO₂ coatings (paper II), and protein deposition from human blood plasma on model silica surfaces (paper III). Ellipsometry measures changes in laser beam intensity and polarization upon reflection. Monochromatic light is emitted at $\lambda=632.8$ nm by a He-Ne laser and the beam is linearly polarized and falls onto the sample. After reflection the beam passes an analyzer and falls into the detector. The method is applicable for measurements in air or in liquid. The exact polarization change is determined by the sample properties (thickness, complex refractive index or dielectric function tensor). Ellipsometry exploits changes after the reflection of the monochromatic light in intensity and phase information and the polarization state, and can achieve ångström resolution. The technique is suitable for the determination of thicknesses of thin organic and inorganic films, with thicknesses from approximately one

nanometer up to approximately 2000 nm. The samples should then possess well-defined, optically homogenous and isotropic layers. In this thesis silica wafers with extremely homogenous optical constants were used as a base substrate onto which inorganic and organic molecules were adsorbed or immobilised. To measure organic film thicknesses (paper I) and to quantify amount of adsorbed protein (paper III) on optically non-homogenous titania, a glass cuvette was used. The measurements can then be performed in liquid in one spot. The organic layer thicknesses were determined from changes in two measured ellipsometric angles Ψ and Δ , according to the McCrackin evaluation algorithm (135). The refractive index of the organic molecules was set to $n_f = 1.465$ (136). Commonly iterated protein refractive index values range between 1.36-1.6.

AFM

Surface topographies of sol-gel derived TiO₂ coatings were examined on model silica by atomic force microscopy (AFM 3100, Digital Instruments, USA). AFM provides a true three-dimensional surface profile. It is a high-resolution type of scanning probe microscope, with demonstrated vertical resolution of fraction of a nanometer. Contact mode AFM measurements require a physical contact between the scanning probe (tip) and the surface. The AFM principle used in the present thesis was Tapping modeTm. The cantilever is then attached to a piezo crystal that oscillates in the vertical direction in the range of 100-400 kHz. When the tip is brought into proximity of the sample surface, forces between the sample and the tip lead to cantilever deflection and the oscillation is damped. The oscillation amplitude is monitored by a photo diode array and is set to a predetermined value to be held during the surface scanning. Close proximity of tip to surface decreases the amplitude whereas a larger distance increases it. The equipment has a lateral resolution of 2 nm and a vertical resolution at atomic level. The measurement area in the present works was 1 x 1 μ m, consisting 256 scans with a scan rate of 0.400 Hz. The raw data obtained from the topographical AFM equipment in Paper II were analyzed with Nanoscope 5.30r3sr3 software, to obtain the pore-size distribution of the coatings. The distance distributions between the peaks (describing lateral surface topography and the approximately pore-size distribution) were calculated from the AFM line profile.

SEM

Scanning electron microscopy (SEM) was used to image calcium-phosphate precipitations after SBF incubations on implant surfaces (Figure 6). SEM images the sample by scanning it

with a high-energy beam of electrons. The electron beam (typically ranging from few hundred eV to 40 keV) passes through pairs of scanning coils or pairs of deflector plates in the electron column, which deflect the beam in the x and y directions so that it scans in a raster fashion over a rectangular area of the sample surface. The electrons interact with the sample; they electrons loose energy by repeated random scattering and absorption within a teardrop-shaped volume of the specimen known as the interaction volume, which extends from less than 100 nm to around 5 μm into the surface. The energy exchange between the electron beam and the sample vary depending on the surface topography. While the shadows are created by a surface slope away from the detector, higher intensity is resulted by a surface slope toward the detector. The image is then digitally captured and displayed on a computer monitor. Images were taken with magnification varying from x 80 to x k25, with EHT 1-5 kV using signalway “Inlens”.

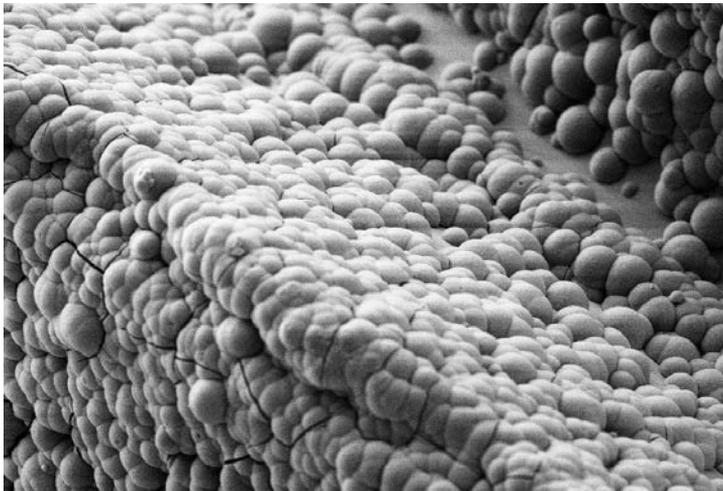


Figure 6. SEM image of calcium crystal formation on screw surface

Heavier elements are able to be detected with backscattered electrons, as they give rise to a heavier intensity than lighter elements. A backscatter detector used together with Energy-dispersive X-ray spectroscopy (SEM-EDX) produce an element contrast map that allow identification of elements. SEM-EDX was used to determine the chemical composition of the substrates.

Liquid scintillation counter

The release rate of immobilised ^{14}C alendronate from fibrinogen and sol-gel titania matrices were measured with a liquid scintillation counter, that measures radiation from beta-emitting nuclides. Samples are then dissolved in “scintillation cocktail” and beta-emitting nuclides transfer energy to molecules in cocktail. These molecules transfer in turn their energy to fluoride in the cocktail and the excited fluoride dissipate the energy by light emission. Each sample was measured during 300 s and the emitted light was detected by a computerized detector system as counts/minute. A standard curve was used for calibration.

Animal model

All the animal experiments described in this thesis were approved by the regional ethics board. National guidelines for experimental animals were carefully followed. A total of 160 male Sprague-Dawley (SD) rats were used for the studies. All animals were adult rats, weighting from 280 g – 411 g. The surgery was performed under aseptic conditions and anesthesia, and all animals received standard postoperative anti-pain medication. All animals were allowed to move freely immediately after operation and were inspected daily (for details, see paper I, II and IV).

In studies I and II, bisphosphonate was bound to the surface of implant and administered locally.

In studies II and IV, bisphosphonates were administered subcutaneously 3 times/ week. In study IV, strontium ranelate was administered orally, and the strontium intake and weight of the rats were controlled daily from post-operative day 0 until the intake was stabilized. Thereafter the strontium intake and weight was controlled 3 days/week.

Mechanical measurements

In papers I, II and IV the screws were pulled out in a computerized material testing machine (Figure 7). The test was carried out at the speed of 0.2 mm/s and was stopped when the maximal force was dropped by 90 % from maximum. The maximal force was counted as a pull out force, and the energy until removal was calculated from the area beneath the load deformation curve. The stiffness was measured as the slope of the curve.



Figure 7. Setup for Pull out measurement

Micro-computed tomography (μ CT)

Micro-computed tomography was used in paper IV to study the 3-dimensional bone microarchitecture around implants in the growth zone of rat proximal tibia. μ CT is a high resolution technique, which uses x-rays to obtain cross-sectional reconstructions of a three dimensional object. The object is rotated at incremental angles through an arc of 180 or 360 degrees, and then a low-energy conical beam from the X-ray tube passes through the object. The radiation is recorded by X-ray sensitive detectors, and a computer performs the direct reconstruction of two-dimensional projection data into a three-dimensional array of points. Micro-CT has become a useful tool for imaging of biomedical objects such as bone, or the bone-implant interface. Details for measurements are described in paper IV.

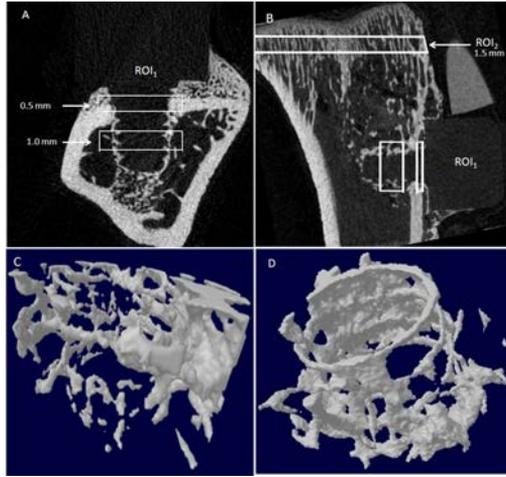


Figure 8. Illustrative μ CT picture of a screw insertion in rat tibia and region of interest (ROI) coaxial with the screw (A and B). 3 dimensional picture of ROI around screw (C and D)

Shortly, cylindrical regions of interest were drawn coaxial with the screw. The cortical portion of the screw was defined as a 0.5 mm cylindrical section from the cortical periosteal surface through the marrow. The marrow portion of the screw was defined to start from the deep end of the screw 1 mm towards the cortical bone (Figure 8). Bone architecture on the proximal tibia that was not in the vicinity of the screw was measured beneath the growth zone. Parameters of relative bone volume (BV/TV %), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (TB.Sp) were performed in CTAn software (Skyscan).

Histomorphometrical analysis

In paper IV the histomorphometrical analyses were done around a PMMA screw to study the bone implant interface. The implants with surrounding tissue were embedded in a light curing resin (Technovit 7200 VLC, Germany) and grounded to 15-20 μm thick sections with a grinding-cutting technique. The samples were stained with hematoxylin-Eosin, and the histological examinations performed with a light microscope. Histomorphometrical evaluations were performed with Leica QWin Standard v3.0 software around implants, as described in more detail in paper IV. Briefly, a blinded investigator tried to isolate bisphosphonate or strontium ranelate treated groups from other groups. Thereafter, a region of interest (ROI) was defined as a triangle between the screw threads. 3 first threads counted from the screw head on both sides of screw were used for quantitative bone area density measurements.

Statistical analysis

Comparisons in paper I and III were performed with independent t-tests. Tukey's test was chosen to evaluate differences in paper II and IV, when comparing more than 2 groups with each other. The data were considered significantly different when $p \leq 0.05$.

SUMMARY OF RESULTS

Paper I

Bisphosphonate coating on titanium screws increases mechanical fixation in rat tibia after 2 weeks

Hypothesis: Immobilised bisphosphonate on titanium screws enhance the implant fixation after 2 weeks.

Results: Alendronate (300 nm/cm^2) immobilised to titanium screws via a crosslinked fibrinogen 60% of immobilized bisphosphonate was released within 8 hours. The administered amount increased successfully the mechanical fixation in rat tibia after 2 weeks by more than 30 % when measured as a pull out force and by almost 50 % when measured as a energy uptake until removal (Figure 9).

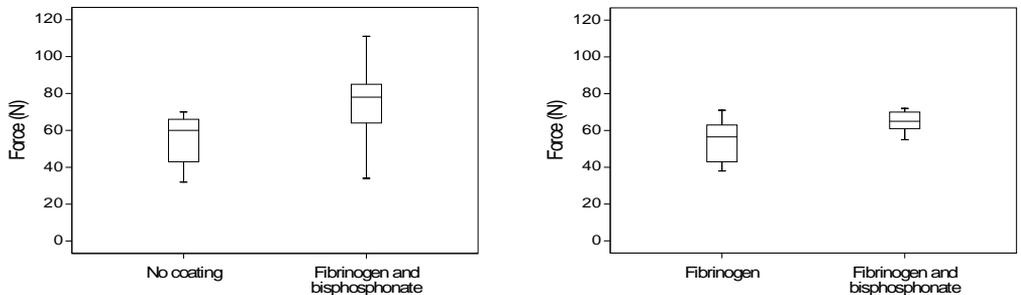


Figure 9. Pullout force of titanium screws at 2 weeks in rat tibia, comparisons of screws with no coating, crosslinked fibrinogen only and crosslinked fibrinogen together with immobilized bisphosphonate.

Paper II

Sol-gel derived titania coating with immobilised bisphosphonate enhances screw fixation in rat tibia

Hypothesis: Bisphosphonate that is immobilised to sol-gel derived titania on stainless steel screws enhance the implant mechanical fixation, similar to systemically administered bisphosphonate.

Results: Stainless steel screws with sol-gel derived titania coatings were soaked first in SBF and then in an alendronate containing solution. The amount of surface bound alendronate was controlled by variation in SBF immersion time. Screws with surface immobilised alendronate gave rise to a significantly increased screw pullout force at 4 weeks of implantation in rat tibia (Figure 10). Sol-gel derived titania and SBF preparation methods show thus promise for a relatively easy control of deposited amounts and release of bisphosphonates.

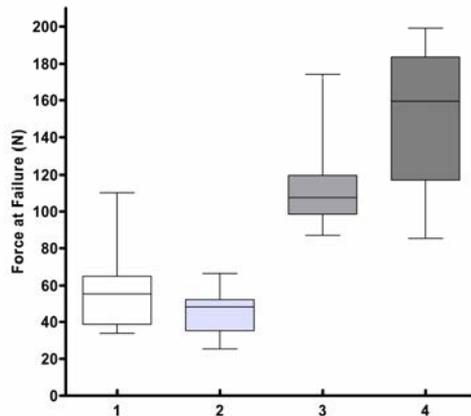


Figure 10. Pullout force at 4 weeks in rat tibia for the following types of screws (1) uncoated SS, (2) Sol-gel derived $\text{TiO}_2 + \text{CaP}$ coating on SS, (3) Sol-gel derived $\text{TiO}_2 + \text{CaP}$ coating on SS combined with systemic alendronate administration, (4) Sol-gel derived $\text{TiO}_2 + \text{CaP}$ coating on SS with surface immobilised alendronate

Paper III

The effect of heat- or ultraviolet treatment of titanium on complement deposition from human blood plasma

Hypothesis: Different titanium surface treatments, such as annealing and UVO-exposure decrease complement activation compared to untreated spontaneously formed amorphous titania.

Results:

PVD prepared titanium surfaces that were coated with sol-gel derived titania, moderately heat treated or UVO-treated decreased complement deposition from blood plasma *in vitro* (Figures 11 and 12). The explanation to this effect may be improved titanium dioxide stoichiometry, increased TiO₂ crystallinity and thereby decreased oxide solubility, and altered OH-binding to oxide.

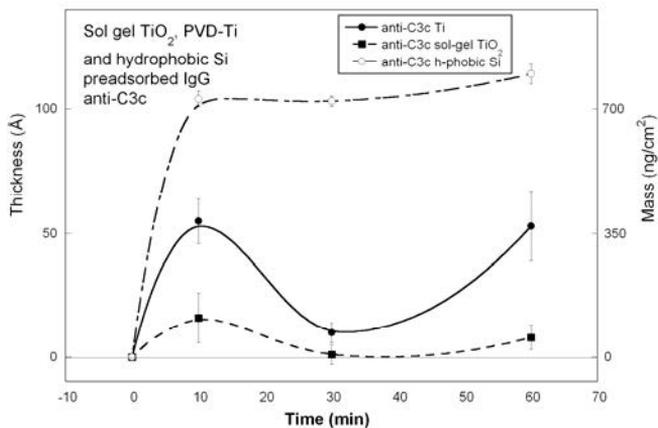


Figure 11. Anti-C3c deposition to blood plasma incubated non-annealed PVD- and annealed sol-gel Ti with pre-adsorbed IgG. Considerably less antibodies bound after *in vitro* blood plasma incubations to sol-gel derived titanium oxide and UVO treated titanium than PVD-prepared titanium oxide without any treatment

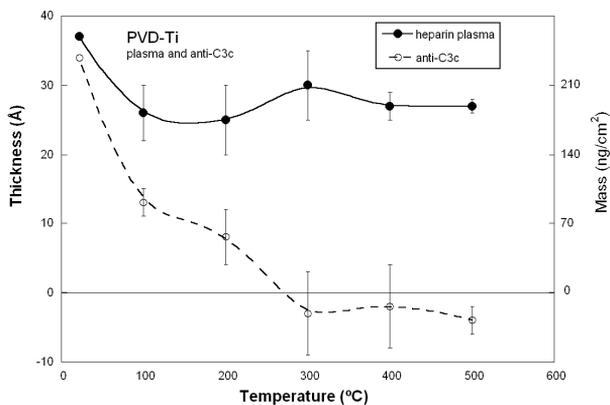


Figure 12. Heparin plasma and anti-C3c deposition to PVD-prepared titanium oxide. The antibody binding after in vitro blood plasma incubation decreased upon pre-heating at 100-500°C of the surface

Paper IV

Weak effect of Strontium ranelate in early bone screw fixation

Hypothesis: The short term metal implant fixation is improved upon delivery of strontium ranelate.

Results: Strontium ranelate did not improve significantly the pullout force or microarchitecture around stainless steel screws after 4 or 8 weeks of implantation (Figures 13 and 14). Systemically administered alendronate, however, enhanced the pullout force by 91% after 4 weeks and by 98% after 8 weeks. Significantly denser bone microarchitecture was then observed in the growth zone of proximal tibia, but not around the screws of strontium ranelate treated animals. Strontium showed, however, a small positive effect after 8 weeks of implantation and a speculation is that Sr is beneficial upon a prolonged treatment time.

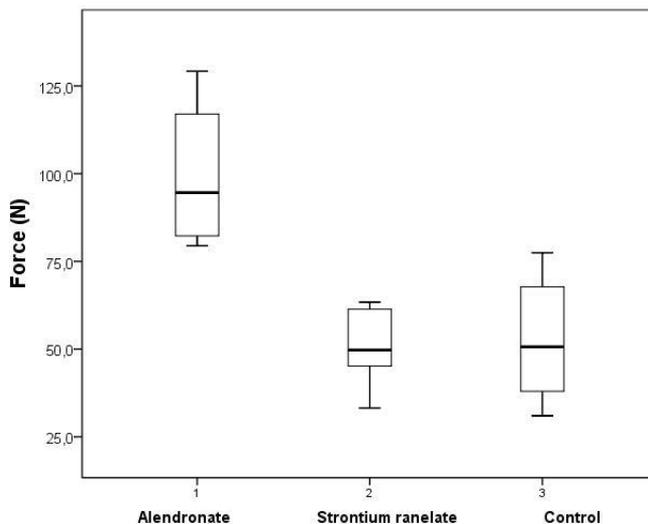


Figure 13. Pullout force after 4 weeks of implantation in rat tibia.

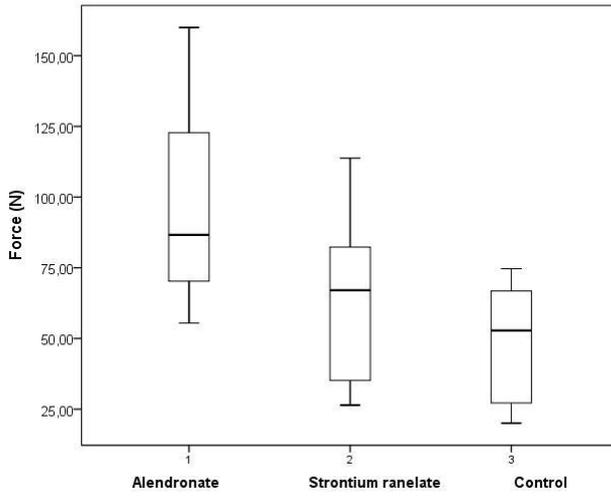


Figure 14. Pullout force after 8 weeks of implantation in rat tibia.

COMMENTS AND CONTRIBUTION TO THE FIELD

The main purpose of my thesis was to improve metal implant integration to bone by preparation of thin drug loaded fibrinogen- and sol-gel derived TiO₂ layers (studies I and II). As a secondary goal, the complement activation on heat- or UVO- treated titanium was studied (study III). Yet another purpose was to explore the potential of a new drug, Sr-ranelate to improve the early implant fixation (study IV). I will summarize and analyse the most important results, and discuss the possible impact of the results for bone anchored biomaterials.

TiO₂ as a biomaterial

Titanium and titanium-based alloys are commonly accepted as stable and corrosion resistant materials inside humans, and is therefore widely used in dental and orthopaedic applications. There exists a lot of literature dealing with surface modifications of titanium to enhance the biological performance. Changes in oxide stoichiometry, surface morphology, crystal structure, crystallite size, and roughness of TiO₂ all seem to enhance the bioactivity *in vitro* (137-142). Recently, researchers suggested that oxygen concentration in the oxide film increases the CaP particle precipitation upon immersion in SBF. Furthermore, the crystal structure of titanium surface oxide depends on preparation methods. In recent years, TiO₂ based biomaterial research have gained much attention from bulk titanium surface modification towards nano particle- and tube coatings.

Surface chemistry /structure and immune complement

Papers II and III focus on sol-gel derived titania coatings for biomaterial applications. Sol-gel derived titania has previously shown good biocompatibility and, in fact, indicated bioactivity in soft and hard tissue applications (21, 67, 143, 144). Recently, it is shown that implant nanotopography modulate cell activity (22, 61) and nano roughness and nano features affect osteoblast activity, migration, proliferation and differentiation, and protein adsorption.

Furthermore, it is also known, that nano roughness can reduce complement activation. However, the mechanisms behind complement activation are still not well understood, although nucleophiles such as $-OH$ and $-NH_2$ groups are suspected to activate and negatively charged surfaces such as $-COOH$ down regulate activity (see Figure 3). In this thesis we observed that besides the nano topography, water or hydroxyl surface chemistry showed an even stronger effect on complement activation. How these findings affect the early implant healing remains to explore and are currently under study.

Effect of surface treatments

In studies I and II we immobilised the well-known osteoporosis drug, bisphosphonate to screw surfaces. We could clearly show that local delivery of alendronate or pamidronate increase the pull-out force after a few weeks of implantation, indicating harder implant fixation already after 2 weeks. We observed no negative effects of the pure fibrinogen matrix coating without bisphosphonate. The amount of released drug was lower in study I than II, although the effect was significant even then. In turn, in study II the amount of released drug could easily be varied by variation of the SBF incubation time and thereby the CaP surface area onto which BP was adsorbed. Furthermore, the type and efficacy of the bisphosphonate can be varied with the method presented in study II while the coating method in study I requires a bisphosphonate containing $-NH_2$ group (N-bisphosphonate). The short term effects, say 2-8 weeks in rat, of bisphosphonate loaded implants is thus significant. The long term effects are yet not studied.

There is an extensive interest for surface modifications of titanium to enhance its biocompatibility and bioactivity, e.g. by pickling and etching techniques, UV-irradiation, nano- and micro oxide particle immobilizations, blasting, immobilisation of nanosized apatite, protein coatings, etc. Especially improved and persistent bone anchorage at shortest possible time is wished because clinically movements of bone anchored implants occur mainly during the early healing phase. Bioactive surfaces may integrate more rapidly with living bone, and do in those cases not form soft tissue around implants (26, 44, 145). The current research trend is moving from osteoconductive biomaterials towards osteoinductive biomaterials, with capability to activate genes that stimulate early regeneration of bone tissue, e.g. via Wnt-pathways and stem cell differentiation (28, 29). To further enhance bioactivity

of TiO₂ coatings presented in this thesis (studies II and III), ions or molecules, such as Zn²⁺, Ca²⁺, MgO, or Sr₂ could be added to a surface oxide or matrix networks. Such elements reportedly change the biomimetic apatite layer composition in SBF and enhance the hard tissue healing by increasing the number of CaP nucleation sites (146-148).

Locally delivered bisphosphonate and rat model

In dentistry, traditional titanium screws inserted in the jaw bone need unloading during several weeks to months to secure osseointegration. In this thesis we show that immobilisation of BP significantly improved the mechanical fixation already after 2 weeks in rat tibia. The main disadvantage of the rat model is probably the difference (compared to humans) in bone structure and turn over, e.g. the rat does not have a Haversian system. The rat shows also higher metabolic activity that result in a faster cellular metabolic activity. In BP experiments, this may result in a faster bisphosphonate “recycling” due to a faster resorption. However, to the author’s best knowledge, no major differences between humans and rats have been reported in the inflammatory system, and it may therefore be assumed that early bone healing shows only minor species differences. Although there is large overall difference in bone healing between human and rat, this indicates that the time needed for osseointegration in humans may be reduced when bisphosphonate coatings are used. However, further studies with bigger animals, rabbits or dog, together with studies on long term bisphosphonate effects needs to be performed. Incorporation of other biological active molecules, such as enzymes, proteins and growth factors may also enhance the osteoproduative power of coatings, and should be investigated.

During 2003 a condition named “osteonecrosis of the jaw” was observed in patients receiving bisphosphonate, zoledronate mainly for the treatment of cancer. The jaw problems were manifested as lack of healing and infection of exposed jaw bone (149-152). The pathophysiology of osteonecrosis is still unclear, but it is suggested that a high concentrations of BP mediated suppression of remodeling plays a significant role (149, 153). Recently this disease was linked to the use of chronic, systemically administered bisphosphonate therapy. It is suggested that a high concentration of bisphosphonates in the jaw bone reduces the resorption of the infected bone, leading to a lesion with an impaired healing capacity. Locally delivered BP is suggested to affect, on the other hand only bone around the implant site, and

if needed, it can be removed together the implant (154). In addition, the amount of locally delivered bisphosphonates is minute (single local dose and < 1 microgram) and given during a short period of time, compared to systematically administered long time therapy. Thus, we believe that locally administered bisphosphonate in dental application does not increase the risk of osteonecrosis of the jaw. This remains, however, to be proven.

Effect of strontium on early implant healing

In study IV, the effect of systemically administered strontium ranelate was compared to the effect of a systemically administered bisphosphonate. This study was performed to find out a potential beneficial effect of strontium ranelate on short time (4-8 weeks) of implant healing. We were, however, unable to observe a significant improvement by Sr after 4 and 8 weeks of implantation. In contrast, systemically administered bisphosphonate enhanced significantly the screw pullout force already after 4 weeks, and the relative bone volume and bone area density in the close vicinity of the screw compared to control or strontium groups.

In literature, several studies describe strontium effects on fracture or implant healing (114, 115). Maimou et al. studied the effect of strontium ranelate with slightly lower strontium ranelate dose than used here, 625mg/kg/day, and were able to show a 34% enhanced pullout force after 8 weeks in rat (124). However, this effect did not reach the same level as the systemic BP treatment in our study.

We observed a significant effect of strontium on architecture on trabecular bone outside the vicinity of the implant site. We are, however, unable to report the reasons behind this. Strontium obviously has some positive effect on bone healing, although the biomechanical effect on short periods is not strong compared to bisphosphonate.

Pharmacological substrates on implants

There is a clear goal to modify bone anchored implant surfaces: to decrease complications such as infections, increase the implant survival and minimize the healing time and with best possible biomechanical outcome. If these goals could be reached, it would be possible to treat even less suitable patient cases with a surgical procedure and likely enhance the life quality for many patients. Furthermore, illnesses such as diabetes or osteoporosis also increase the

risk of implant failure. As the risk for late loosening of implant likely is coupled to early fixation (155), even minor improvements in early implant healing might provide an enhanced long-term outcome. One means to obtain this may be to immobilize pharmacological agents onto the implant surface for a local implant improvement.

There exist several pharmacological agents that were reported to stimulate osteoblast activation and bone generation. Bisphosphonates bound to metallic implants via surface deposited hydroxyapatite, calcium ions or through a poly(D,L)-lactide matrix increased bone formation around implants. Parathyroid hormone (PTH) was observed to increase osteoblast number and affect positively proliferation and differentiation of osteoprogenitors cells (156-159). However, in order to obtain an anabolic effect it should be given intermittently and thus is yet not applicable for controlled drug delivery through coating technologies.

Bone morphometric proteins (BMPs), especially BMP-2 and MP-7, induce osteoblast differentiation, and according to several studies accelerate fracture healing (160-163). Furthermore, some studies have observed that locally delivered simvastatin stimulates BMP and thus improve fracture healing (164, 165). In one study researchers reported that BMP-2 significantly enhanced bone mineralization upon implantation in rat calvarial bone in hydrogel matrix (166).

Recently, a rat study reported positive effects of a novel pharmacological agent, anti-sclerostin that affected the Wnt-signalling pathway and the GSK3beta complex (167). Sclerostin is a product of the SOST gene, and mutation in this gene results in high bone mass in humans (168). Sclerostin is reported to antagonize Wnt signaling at GSK3beta-level, and is therefore a negative regulator for bone formation. Systemically administered sclerostin-neutralizing antibody was thus reported to successfully promote bone healing in a similar implantation model similar to that in study IV (169).

CONCLUSIONS

1. Bisphosphonate that was immobilised via a thin fibrinogen matrix to titanium implant surfaces improved the implant fixation in rat tibia after 2 weeks.
2. A sol-gel made titania coating could also be used as an anchor for local delivery of bisphosphonate. The implant pull-out force increased significantly compared to untreated control without and with systemically administered bisphosphonate.
3. Titanium surfaces that were treated with a mild heat annealing or UVO irradiation changed the surface oxide hydroxylation and decreased immune complement deposition from blood plasma. Likely also the oxide crystallinity increased and the solubility decreased. These findings may have impact in blood contacting applications and for basic treatments of titanium implants.
4. A relatively small effect on bone microarchitecture was observed of systemically administered strontium in rat. However, in our study the biomechanical effect does not reach that of bisphosphonate coated surfaces or systemic bisphosphonate delivery and we suggest that strontium is not suitable for the improvement of early implant fixation.

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