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**Tübingen**

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# Osteoblasts Response to Anodized Commercially Pure Titanium *in vitro*

**Inaugural-Dissertation**
**Zur Erlangung des Doktorgrades**
**Der Zahnheilkunde**

**der Medizinischen Fakultät**
**der Eberhard-Karls-Universität**
**zu Tübingen**

**Vorgelegt von**

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**Zhejiang /China**

**2005**

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*To my families*

# Content

# 1. Introduction

Unalloyed and alloyed titanium has been widely used to construct dental implants because of its good biocompatibility. Rapid achievement of a stable osseointegration between implant and bone tissues of the host is the main aim in implant development. Because surface properties and/or chemical composition play a critical role in achieving successful osseointegration, many efforts have been employed to modify titanium surfaces and to improve its biocompatibility.

Anodic oxidation is an electrochemical method, and it is easy to produce various oxide layers on titanium surfaces by adjusting the anodizing conditions, such as anodizing electrolytes, temperature, anodizing voltage, and so on. However, there are many kinds of combinations of the above parameters, so it is difficult to find out the best combination. Some efforts focused on *in vivo* experiments to investigate bone tissue response to anodized titanium implants, while few articles investigated the basic responses of osteoblasts to these surfaces. Osteoblasts play a critical role at the interface between implants and bone tissue, so it is necessary to study cell behavior of osteoblasts cultured directly on anodized titanium surfaces.

The aim of this study was to evaluate human osteoblast responses to anodic oxides of titanium *in vitro*. Two kinds of anodizing electrolytes with different composition and a series of anodizing voltages were adopted. In the present study, cell cultures of the osteoblast-like cell line SaOS-2, derived from human osteoblastic sarcoma, were performed on anodized titanium surfaces, and cytotoxicity, cell attachment and spreading, cell morphology, cell proliferation and differentiation were assessed.

# 2. Literature Review

## 2.1 Titanium and titanium oxides

Titanium was discovered some 200 years ago in England, and began to be used practically in 1948 when its commercial production started in the United States. Titanium is a lightweight and strong material with a tensile strength comparable to carbon steels, and because the Young's modulus of titanium is only a half of carbon steel, titanium is soft and readily formed. Titanium is classified in two categories: commercially pure titanium (Ti) which is used in the chemical process industries and titanium alloys having such additives as aluminum (Al) and vanadium (V) and which are used for jet aircraft engines, airframes and other components. Further more, according to the content of oxygen, commercially pure titanium was classified into four grades, with grade 4 having the most (0.4%) and grade 1 the least (0.18%) [1]. Because of this good biocompatibility, unalloyed and alloyed titanium has been also widely used in medical engineering for many years and in various applications are varied, such as joint replacement parts, bone fixation materials, dental implants, heart pacemaker housings, artificial heart valves etc.

Since the 1960s dental implants have been used as an artificial anchoring of dentistry in the maxilla and mandible. Titanium or its alloys were commonly used to make up implants because of their optimal physical characteristics and biocompatibility. Good osseointegration should be essentially formed at the interface between implant surface and living bone during healing procedure after implantation surgery [2]. Surface properties of the implant may play a very important role in immediate reactions on the implant surface after exposure to the tissue and influence the initial processes of osseointegration, which are

conceivably important for the clinical success of the implantation. During the past decades, many surface modifications, such as coating, abrasion, blasting, acid etching, oxidation, or combinations of these techniques, were proposed to improve the biocompatibility of the implant surface by altering surface topographies, physical characteristics and chemical properties of titanium [3].

Titanium forms a thin oxide layer approximately 2 to 10 nm thick spontaneously in air, which provides corrosion resistance [4,5,6]. Titanium interacts with biologic fluids through its stable oxide layer, which plays the main role in its exceptional biocompatibility [7,8]. Both thickness and chemical composition of titanium oxide layers may play an important role in adsorption of proteins from biologic fluids and attracting cells to its surface. By using thermal or electrochemical oxidation treatments, much thicker oxides can be produced [9,10]. In most cases, the main chemical composition of titanium oxides is $TiO_2$, however, electrochemically prepared oxides may also contain some impurities due to anion incorporation from the electrolytes used, such as Cl, S, Si, P and Na [9,10]. When exposed to air or to biologic fluids, the titanium oxide layer is easily contaminated by hydrocarbons or other elements, for the $TiO_2$-terminated surface tends to bind molecules or atoms from the surroundings as a monomolecular layer [11].

## 2.2 Titanium surface modifications

In order to improve the biocompatibility of commercially pure titanium surfaces, many methods have been employed, which can be classified into three categories: 1) physical techniques, which mostly only make changes on physical characteristics without alteration of chemical composition of titanium surfaces, 2) chemical treatments, and 3) combination of the two methods above.

### 2.2.1 Physical techniques

Some physical modifications of the titanium surface only affect its physical characteristics, such as roughness, microtopography, or wettability, and the alterations of all these characteristics may affect the osteoblasts response to modified titanium surfaces directly or indirectly. Machined, sandblasted, and titanium plasma-sprayed titanium have been already tested *in vitro* by many authors [12,13,14], and these methods have been applied by some manufacturers to produce commercial implant systems as well [15]. The studies from Mustafa et al. showed that surface roughness of modified titanium increased ($S_a$: the average height deviation from a mean plane, increased from 0.2 μm to 1.38 μm) when the size of the $TiO_2$ particles used for plasma-spray was enlarged (from 63 μm to 300 μm) [13].

In 2002, Shibata et al. used glow discharge plasma (GDP) to modify titanium, and the osteoblast cell culture on titanium with and without GDP modification indicated that GDP promoted cell adhesion and differentiation on Ti by increasing the adsorption of proteins [16].

There are also some treatments, which use physical methods to modify the

chemical composition of the titanium surfaces, such as ion implantation, physical vapor deposition nitriding, and plasma ion nitriding [17,18,19,20]. Thermal oxidization can form an outer “ceramic” layer of rutile on titanium alloy [21]. Feng et al., in 2003, reported that thermal treatment of titanium in a different atmosphere could alter surface chemical composition, surface roughness, surface energy, and furthermore improve osteoblast responses to modified titanium surfaces [22].

### 2.2.2 Chemical treatments

It is well known that chemical compositions of titanium surfaces are important for protein adsorption from biological fluids and cell response to titanium. Feng et al. compared osteoblastic cells responses to three different titanium surfaces containing calcium, phosphate ions, and carbonate apatite, respectively, and demonstrated that calcium ions on titanium surfaces play a more important role than phosphate ions in influencing initial interactions between cell culture medium, osteoblasts and titanium [23]. It has been reported that titanium surfaces are easily contaminated by some elements from air, such as C and N, and contamination of titanium surfaces can affect its biocompatibility [24]. Some chemical treatments have been used to reduce the contamination of C and N [25]. Acid etching and hydroxyapatite deposition are the most commonly used chemical treatments.

Using $HNO_3$ or a mixture of $HNO_3$ and HF to prepare titanium specimens has also been reported in the literature. However, the concentrations of used acids were different between authors, for instance, Bowers and co-workers, in 1992, used a mixture of 25% $HNO_3$ and 3.5% HF, while a mixture containing 52% $HNO_3$ and 10% HF was used by Degasne et al. in 1999 [14,26].

Other inorganic acids, such as HCl, $H_2SO_4$ and $H_3PO_4$, were also used to modify the titanium surface or reduce its contamination with other elements [23,27]. It has been reported that HCl/acetone treatment is an excellent decontamination method for the surface preparation process of Ti [25]. Viornery et al, in 2002, investigated osteoblast cultures on polished titanium disks modified with phosphonic acid. There was no statistically significant difference concerning cell proliferation and differentiation between phosphonic acid modified titanium and unmodified titanium, however, the synthesis of total amount of proteins and collagen type I was significantly higher on the titanium modified with ethane - 1, 1, 2 - triphosphonic acid than unmodified titanium [28].

Hydroxyapatite is a major component and an essential ingredient of normal bone and teeth, and has been widely used as an artificial refill biomaterial for plastic surgery and dental implant. For dental implant applications, plasma-spray, sol-gel, and sputtering techniques were used to produce hydroxyapatite coatings on titanium [29-33]. However, some drawbacks of hydroxyapatite coatings on titanium produced by plasma-spray have been announced: resorption of coating, poor mechanical properties, high thickness, non-homogeneity, lack of adherence [34]. Pulsed laser ablation was used in 2001 as a new method for deposition of a thin layer of hydroxyapatite on titanium surfaces [35]. In the study, the authors used three different laser fluences: 3, 6, and 9 $J/cm^2$ to deposit the hydoxyapatite layer, and they found that the cell response to the treated surfaces correlated with laser fluences used. Hydroxyapatite coated implants have been produced also by ion beam assisted deposition [36,37,38]. Recently, hydrothermal treatment after anodic oxidation of titanium has been reported to be able to form thin hydroxyapatite coatings [39-42]. Since dissolution of hydroxyapatite occurs when it is immersed in extracellular fluids at low PH, other alternative apatites, such as fluorapatite and fluorhydroxyapatite, were used to form coatings with strong

resistance against degradation [43,44].

Protein adsorption from serum to biomaterial surfaces is considered as the initial step of osseointegration happening between bone and implant. It has been proven that some proteins, like fibronectin or vitronectin, which adsorb onto biomaterials surfaces when they make contact with biological fluids, can improve cell adhesion [26]. It was indicated that RGD peptides regulated the spreading of HOS cells on hydroxyapatite but not on titanium surfaces, and the spreading of osteoblasts mediated by the RGD domain of vitronectin and fibronectin might contribute to the oseoconductive ability of hydroxyapatite [45,46]. The studies from Tossati and co-workers, in 2003, have shown that peptides of RGD (Arg-Gly-Asp) and RDG (Arg-Asp-Gly) type functionalized poly (L-lysine)-grafted-poly (ethylene glycol) (PLL-g-PEG) copolymers grafted onto titanium surface can resist non-specific protein adsorption onto its modified surface. Therefore, peptide-functionalized PEG may elicit specific interactions with integrin-type cell receptors in the presence of full blood plasma [47]. A coating of 91.2% de-acetylated chitosan on titanium decreased its wettability, but increased protein adsorption and cell attachment [48].

### 2.2.3 Combination methods

Combinations of physical and chemical techniques can alter both physical characteristics and chemical compositions of titanium surfaces at the same time, or create a more intensive modification than using only one unique technique. Recently, the SLA (sandblasted with large grit and acid etched) surface has been documented to lead to a rapid and strong implant fixation *in vivo* [49-51].

An electropolishing technique was carried out in an electrolyte consisting of 600 ml methanol, 350 ml *n*-butanol and 60 ml perchloric acid, held at – 30 for 5 min with the voltage of 22.5V. A very smooth mirror-like titanium surface with the oxide thickness of 4-5 nm could be produced with this method [52].

Another commonly used combination method is anodic oxidation, which is a similar procedure to electropolishing, but the electrolyte composition and process parameters, such as temperature, voltage and current, in the electrochemical cell should be changed. This method will be reviewed comprehensive in the last part of this chapter.

## 2.3 Effects of modified titanium surfaces on osteoblasts

Recently, lots of literatures have reported that surface topographies, roughness, wettability and chemical compositions may influence protein adsorption onto the titanium surface and cell behavior on substrates [26,27,42,53,54]. However, the mechanisms of how these surface properties affect cell behavior are not yet clear.

### 2.3.1 Effects of physical characteristics

Physical characteristics mainly include surface roughness, hydrophilicity and topography, which have been considered generally as the most important factors affecting response of osteoblasts. Profilometry was commonly employed to measure roughness of titanium surfaces. It has been found that the adhesion and proliferation of osteoblasts *in vitro* or the extent of bone contact *in vivo* is positively correlated with the increasing of roughness [55,56]. However, some authors found that the correlation between roughness and bone apposition is not a simply linear relationship, as it was found that a significantly increased bone apposition to an implant surface was only achieved inside a specific roughness range, and smoother or rougher surfaces produced lower bone response [57,58]. However, the contrary conclusion was also drawn, i.e. that cell attachment on to rougher titanium surfaces was lower than to smoother titanium surfaces. For example, Mustafa et al. found that cellular attachment of cells derived from human mandibular alveolar bone to titanium surfaces with an average roughness of 1.3 μm was significantly lower than to smoother titanium surfaces with a roughness of 0.2 μm [13]. Surface roughness needs to be considered not only in term of amplitude but also in term of organization [59].

Surface roughness was extensively analyzed at scales above cell size (macro-roughness) or below cell size (micro-roughness), and it was found that when the surface roughness was below cell scale, hOBs (human osteoblasts) appreciated their isotropic smooth aspect, although when topography was considered above cell scale, hOBs appreciated their rough isotropic surface [60]. At the molecular level, it has been proven that surface roughness modulates the local regulatory factors produced by osteoblast-like MG-63 cells [27], i.e., secretion of prostaglandin $E_2$ ($PGE_2$) and transforming growth factor β-1 (TGF-$\beta_1$) was increased when cultured on rough surfaces. Boyan and co-workers have reviewed the mechanism that surface roughness mediates several effects on osteoblasts [61].

Many modification procedures can change the hydrophilicity of the titanium surface, which is expressed by the contact angle between substrate surfaces and used testing liquids. It has been proven that contact angles are not dependent on the used testing media, such as distilled water, 1% NaCl aqueous solution or cellular suspension. However, there is a relationship between contact angle and roughness: the contact angle increased linearly with average roughness when the angles were higher than 45 degrees, and decreased linearly with roughness when the angles were less than 45 degrees [62]. When surface free energy is altered, the hydrophilicity of material surfaces can be improved or decreased. Kasemo, in 1988, has reported that proteins may interact with a hydrophilic surface only indirectly via an intermediate water layer and stay in their normal conformational state; on the other hand, if the surface is hydrophobic, the protein is more likely to form bonds directly with the surface atoms, which might cause conformational changes [11].

Argon plasma-cleaning (PC) treatment was used to investigate whether this treatment can enhance *in vitro* osteoblast attachment to titanium [63]. Three exposure time intervals were chosen, 1 min, 5 min, and 10 min. The results indicated that argon plasma-cleaning treatment could dramatically improve surface wettability, however, significant enhancement of cell attachment was observed only on the titanium surfaces plasma treated for 1 min, compared with non-plasma treated surfaces.

Surface topography can be characterized by scanning electron microscope (SEM). Using traditional titanium modification methods, irregular surface topography with pores or spikes will be produced; while using special physical or chemical techniques can create regular surface topographies. Jayaraman et al. compared the influence of two different titanium implant surfaces on attachment of osteoblast-like cells *in vitro*, a sandblasted and acid etched surface and an experimental-engraved surface. It was found that osteoblast-like cells performed better concerning, adhesion and proliferation, on the engraved surfaces than on rough surfaces [64]. In addition, the porous geometries of titanium surfaces may also affect osteoblastic cell behavior to materials [65,66].

### 2.3.2 Effects of chemical alterations on titanium surfaces

Besides topographical properties, chemical composition of the titanium surfaces should also be carefully investigated. X-ray photoemission spectroscopy (XPS or ESCA) and Auger electron spectroscopy (AES) have been widely used to measure the chemical composition of biomaterial surfaces [11,67,68].

Although the chemical compositions of cpTi grade 1 and 4 are more than 99% similar, a differential response of human osteoblast-like cells was found towards these two grades of cpTi. Ahmad and coworkers found that between 4 and 24 h after seeding cells onto titanium specimens, the rate of cell attachment to Grade 1 Ti was significantly different to Grade 4 and glass, which served as control. Significant differences in expression of vinculin, collagen synthesis and calcium content were detected as well between Grade 1 and Grade 4 cpTi [69].

By comparing the initial attachment and subsequent behavior of human osteoblastic cells (SaOS-2) on pure titanium, hydroxyapatite and glass, Okumura et al. found that on hydroxyapatite cells started extension earlier and more quickly than on titanium. They also suggested that earlier osteogenesis may occur on hydroxyapatite than on other materials [70].

Collagen, which acts as one of the important component of extracellular matrix, was covalently linked to the surface of titanium by a surface modification process involving deposition of a thin film from hydrocarbon plasma followed by acrylic acid grafting. Collagen linked titanium implants were implanted in rabbit femur and a significant increase of bone growth and bone-to-implant contact were observed [71].

De Giglio and co-workers investigated cell cultures of neonatal rat calvarial osteoblasts on RGD-grafted (Arg-Gly-Asp) polypyrrole coated titanium substrates, and it was revealed that cell attachment was significantly increased compared unmodified PPy-coated Ti and glass coverslip substrates [72].

## 2.4 Osteoblast responses to modified titanium

### 2.4.1 Osteoblast

Tomes & de Morgen in 1853 first illustrated the existence of a type of cells intimately associated with newly formed bone [73]. In 1864, the term “osteoblast” was first used by Gegenbaur to refer to the “granular corpuscles found in all developing bone as the active agents of osseous growth” [74].

Osteoblasts that originate from osteoprogenitor cells and preosteoblasts play a pivotal role in bone formation. During differentiation of the osteoblastic cell lineage, the preosteoblast expresses transforming growth factor-β (TGF-β), which induces osteoblast cell proliferation [75]. Osteoblasts settle at the surface of the existing matrix and deposit fresh layers of bone onto it. New bone matrix is secreted by osteoblasts, and osteoid is formed, which consists chiefly of type I collagen and the small portion (10-20%) of embedded osteoblasts. While osteoid is rapidly converted into hard bone matrix by the deposition of calcium phosphate crystals, osteoblasts differentiate into mature bone cells, osteocytes. Although the osteocyte continues to secret new bone matrix around itself, it can’t further divide. Besides osteocytes, osteoblasts can also differentiate into the other mature cell type, bone-lining cells. The lining cell is inactive and lacks the ability to secret new bone matrix. However, investigations from Chow and coworkers have shown that these cells can be reactivated into bone-producing osteoblasts [76].

Under the light microscope, osteoblasts whose synthetic activity is high are plump and polyhedral, while the cells with low activity are usually flattened. It has been discovered with the electron microscope that the cytoplasm of osteoblasts

contains large quantities of rough endoplasmic reticulum (ER) forming typical cisterna together with plentiful ribosomes and a well-developed Golgi apparatus, which are necessary cellular organs corresponding to bone matrix secretion [77]. The principal products of the mature osteoblast are type I collagen (90% of the protein in bone), the bone specific vitamin K-dependent proteins, osteocalcin and matrix Gla protein, the phosphorylated glycoproteins including bone sialoproteins I & II, osteopontin and osteonectin, proteoglycans and alkaline phosphatase.

The proliferation and differentiation of osteoblastic cells are regulated by systemic agents and a large number of growth factors and cytokines existing in extracellular matrix (ECM), for instance, the insulin-like growth factors (IGFs), the transforming growth factors (TGF alpha and TGF beta), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), etc. Correspondingly, there are many sorts of receptors for these factors on the cell membrane of osteoblasts, and binding of these factors to their receptors activates signal transduction pathways that finally lead to nuclear responses.

### 2.4.2 Osteoblast responses to titanium and its modifications

#### 2.4.2.1 Cytotoxicity

Safety tests including cytotoxicity assays are required for all products to be used in contact with human beings. Cytotoxicity tests using cell cultures have been accepted as the first step in identifying active compounds and for biosafety testing. Based on the literature from Freshney [78], the choice of cytotoxicity assay will depend on the agent under study, the nature of the response, and the particular target cell. Assays can be divided into two major classes: (1) an

immediate or short-term response such as an alteration in membrane permeability or a perturbation of a particular metabolic pathway, and (2) long-term survival, either absolute, usually measured by the retention of self-renewal capacity, or survival in altered state, e.g., expressing genetic mutation(s) or malignant transformation.

A cellular viability assay, one type of short-term assay, is commonly used to test cytotoxicity of biomaterials. Most viability tests rely on a breakdown in membrane integrity determined by the uptake of a dye to which the cell is normally impermeable; or the release of a dye or isotope normally taken up and retained by viable cells. However, some toxic influences from biomaterials only show their cytotoxicity several hours or even several days later, and short-term toxicity may be reversible. Therefore long-term cytotoxicity assays should be performed to indicate the metabolic or proliferative capacity of cells after rather than during exposure to a toxic influence, or as a supplement to short-term assay.

ISO (the International Organization for Standardization) has established the standard for *in vitro* cytotoxicity tests, i.e., ISO 10993-5. In this standard, three categories of tests are listed: extract test, direct contact test, and indirect contact test. The extract preparation plays a critical role in extract test. In ISO 10993-5, it is required that the ratio between the surface material and the volume of extraction vehicle shall be no more than 6 $cm^2$/ ml and no less than 1.25 $cm^2$/ml. In addition, a positive control and a negative control are necessary to estimate cytotoxicity of biomaterials.

Although it has been demonstrated by many studies that titanium has no cytotoxicity and optimal biocompatibility, and it is a safe implant material when implanted into human body, some reports showed that titanium ions released into

the body fluid from titanium implants during application may cause cellular damage. Generally, titanium resisted corrosion in chloride solutions, but in dynamic, protein-rich, oxygenated living tissues in which nitrogen is largely absent, the dissolution of titanium into the tissues surrounding a titanium implant is promoted [79,80]. Any modification methods will change the physical characteristics or chemical compositions of the titanium surface, and furthermore may affect the dynamic mechanisms for titanium ion release or contaminate titanium oxides with some toxic chemical elements or molecules. Therefore, in experiments designed for biocompatibility testing of biomaterials, cytotoxicity assay should be done first of all.

#### 2.4.2.2 Cell attachment and spreading

Cell adhesion is involved in various natural phenomena such as embryogenesis, maintenance of tissue structure, wound healing, immune response, metastasis, and tissue integration of biomaterials. Since cellular attachment, adhesion and spreading belong to the first phase of cell/material interactions, the quality of this phase will influence proliferation and differentiation of cells on biomaterials surfaces. Cell adhesion may be affected by surface characteristics of materials, such as their physical properties, chemical composition, and microtopography.

In general, there are two kinds of cell adhesion, one is cell-cell adhesion, and the other is cell-ECM (extracellular matrix) adhesion. The latter is always studied to investigate biocompatibility of biomaterials. Although there are many surface adhesion proteins involved in cell adhesion, for instance, integrin, selectin, mucin families, and the immunoglobin-cell adhesion molecule family (Ig-CAM) etc, members of the integrin family are the main cellular receptors for the extracellular matrix. All members of the integrin family are heterodimers composed of two

transmembrane polypeptides, α and β chains, which both contribute to ligand-binding specificity (Fig. 1). 16 α sub-units and 8 β sub-units have been discovered. Each sub-unit has a transmembrane domain and a short cytoplasmic domain. Integrins, which connect to bundles of actin filaments, must interact with the cytoskeleton in order to bind cells to the extracellular matrix, and enable the cytoskeleton and extracellular matrix to communicate across the plasma membrane [81,82]. Postiglione et al., in 2003, found that a higher expression of αβ2, α5, αVβ3, and β1 on titanium plasma-sprayed titanium compared with sandblasted titanium and titanium with smooth surfaces [12].

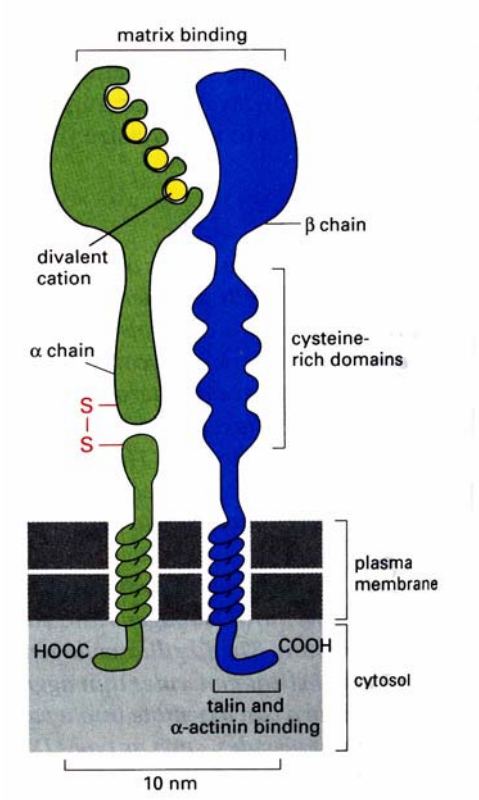



Fig. 1. The subunit structure of an integrin cell-surface matrix receptor. (From Alberts B, Bray D, et al. Molecular biology of the cell, 3rd edition, Garland Publishing, Inc. P 996)

It has been reported in Inoue and co-worker's review [83] that there are three types of adhesion structures with different separation between attached cells and substratum. The first type of cell-substratum contact is to the extracellular matrix

(ECM) with a gap of 100 nm or more between the cell membrane and the substratum. The second type was named closed contact (CC), a large labile structure, where cells are separated from the substratum by approximately 30 nm. The tightest adhesion units with the separation of 10 to 15 nm between cultured cells and substrate surfaces are named “focal contacts”, also called “focal adhesions”, where integrins cluster together to transduce transmembrane signals and link actin filaments to the extracellular matrix. On the internal side of the cell membrane, focal contacts are composed of some associated structural proteins, α-actinin, talin, vinculin, paxillin and tensin. These proteins mediate the signal communication between ECM and cytoskeleton to affect cell behavior. Focal contacts play a critical part in fixing cultured cells on the substrate surfaces, as well as in cell migration on the surfaces. When cells move forward, new focal contacts will be synthesized and old focal contacts must be released. In general, cells with a low motility form strong focal adhesions while motile cells form less of these adhesive structures. In lots of cell culture models, immuno-histochemical focal contacts staining by fluorescence dyes was used to describe cell shapes, even double staining with two or more dyes was involved to show both, focal contacts and cytoskeleton, at the same time. Schneider G and Burridge K, in 1994, found that precoating glass coverslips or titanium disks with serum or fibronectin enhanced cell spreading and resulted in the rapid formation of focal contacts and their associated stress fibers, and the effect on the samples precoated with fibronectin was better than on those coated with serum [84].

Cell adhesion to the material surface was considered as one of the most important indexes of the biocompatibility of materials, because osteoblasts contact the surface of implanted biomaterials in very short time when biomaterials are implanted into the host and this process plays a critical role in the formation of

osseointegration between implants and tissue. The process of adhesion of cells to the substrate involves multiple steps: (1) adsorption of serum proteins to the substrate; (2) interaction between special receptors on the cell membrane which combine with the proteins adsorbed onto the substrate; and (3) spreading of cells on the substrate [85].

During the whole adhesion and spreading process, one very important cell behavior is cytoskeleton reorganization. The cytoskeleton is a complex network of protein filaments that extends throughout the cytoplasm. It is a highly dynamic structure that reorganizes continuously as the cell changes shape, divides, and responds to its environment. The cytoskeleton composes of three types of protein filaments — actin filaments, microtubules, and intermediate filaments. Actin filaments are two-stranded helical polymers of the protein actin, with a diameter of 5-9 nm, and they are most highly concentrated in the cortex, just beneath the plasma membrane. The three types of filaments are connected to one another, and coordinated to carry out functions.

The cortical actin filament network generally determines the shape and mechanical properties of the plasma membrane, and it is organized into three general types of arrays (Fig 2). Parallel bundles are located in microspikes and filopodia, in which the filaments are oriented with the same polarity and are often closely spaced (10-20 nm apart). In contractile bundles, as found in stress fibers and in the contractile ring, filaments are arranged with opposite polarities, and the distance between filaments is always 30-60 nm. The third type of arrays is a gel-like network, in which the filaments are arranged in a relatively loose, open array with many orthogonal interconnections. At one end stress fibers insert into the plasma membrane at special sites, focal contacts, at the other end they attach to a second focal contact or insert into a meshwork of intermediate filaments that

surrounds the cell nucleus.

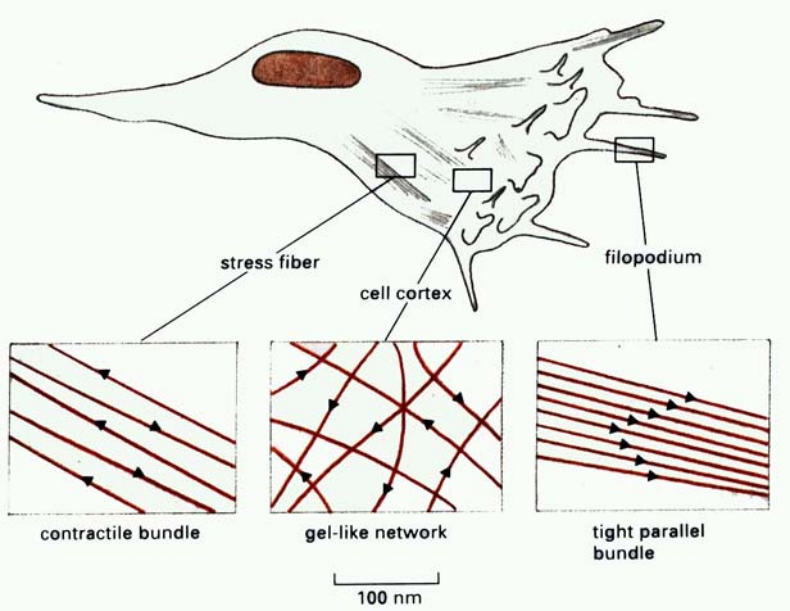



Fig. 2. Three types of cortical arrays of actin filaments. A crarwling cell is shown with three areas enlarged to show the arrangement of actin filaments drawn to scale. Arrowheads point toward the plus end of the filaments. (From Alberts B, Bray D, et al. Molecular biology of the cell, 3rd edition, Garland Publishing, Inc. P 835)

Rajaraman and coworkers [86] classified cell spreading supported by the assembling of cytoskeleton into 4 stages by scanning electron microscopy: 1) rounded cells with a few filopodia; 2) cells with focal cytoplasmic extensions or lamellipodia; 3) circumferential spreading; 4) cells spread fully and flattened into a polygonal shape.

Proteins and other adsorbable macromolecules containing in modified cell culture medium are immediately adsorbed onto the exposed materials' surfaces; 2 to 5 nm layer forms within the first minute of contact. Albumin, prealbumin and IgG were found to be adsorbed onto the $TiO_2$ surface in a short time by immunoelectrophoresis, when the $TiO_2$ powder was equilibrated with serum by shaking. Ellingsen also suggested that calcium ions might act as the intermediate between $TiO_2$ and the macromolecules in serum [87]. These adsorbed proteins and macromolecules play a very important role in the formation of extracellular

matrix and subsequent cell attachment. Fibronectin and vitronectin have shown to be involved in cell attachment and spreading of human osteoblast-like cells, SaOS-2, on titanium surfaces [26,88].

Acid etched, sandblasted and acid etched titanium disks were compared by cell culture of osteoblast-like cells under different medium conditions: only DMEM culture medium, DMEM culture medium supplemented with fetal calf serum, DMEM culture medium supplemented with fibronectin and vitronectin, and DMEM culture medium containing monoclonal anti-integrin ($\beta_1$, $\alpha_v$). The studies revealed that cell adhesion and spreading were significantly decreased by addition of anti $\beta_1$ or $\alpha_v$ integrin monoclonal antibodies to the culture medium. Cells appeared scanty and packed in clusters, when cultured in the absence of FCS, fibronectin and vitronectin; on the contrary, cells extended completely, when cultured in the medium containing FCS, fibronectin and vitronectin [26].

Bowers et al. reported *in vitro* cellular responses of osteoblast-like cells derived from rat calvarial explants to titanium surfaces with different surface morphologies, with the range of roughness of 0.14 - 1.15 μm. Cell attachment assays were performed at 15 min, 30 min, 60 min and 120 min after cell seeding on samples. No significant difference was found at 15 min among all groups with different surface preparations. However, at 30 min, 60 min, and 120 min, the highest percentage of cell attachment was shown on the rough, irregularly patterned sand-blasted surfaces (Ra = 0.87 μm) [14].

Lumbikanonda and co-workers characterized the responses of neonatal rat osteoblast cells to smooth titanium, titanium dioxide-blasted, titanium plasma-sprayed, and hydroxyapatite plasma-sprayed implants, and found that cells spread most quickly on titanium plasma-sprayed implants at the initial cell

culture stage. By means of scanning electron microscopy, attached cells were classified according to stage of attachment, and it was found that cells cultured on the titanium dioxide blasted surface showed no adaption to surface irregularities, while fully spread cells on the smooth titanium implants were closely adherent to the surface [15].

The experiments from Degasne and co-workers, in 1999, indicated that a high surface roughness was a critical element for cell adhesion [26]. The results of these studies showed that rough surfaces could improve cell adhesion on to biomaterial surfaces, which agreed with some other authors [14,89].

However, other authors had other opinions. For instance, Mustafa et al. compared cellular attachment to $TiO_2$-blasted titanium implant material with an average roughness of 0.72 μm, 1.3 μm and 1.38 μm and to turned titanium surfaces with the roughness of 0.2 μm as control. It was found that cellular attachment to the blasted titanium surface 1.3 μm rough was significantly lower than to turned titanium, while there was no significant difference between turned surfaces and blasted surfaces with 0.72 μm or 1.38 μm [13]. Rosa AL and Beloti MM, in 2003 investigated the effect of titanium surface roughness on the response of human bone marrow cells concerning: cell attachment, proliferation, and differentiation, and found that cell attachment was not affected by surface roughness [90].

#### 2.4.2.3 Cell proliferation and differentiation

To grow into multicellular organs and perform special functions, cell proliferation and differentiation must be conducted. The term ‘cell cycle’ has been

used to describe the behavior of cells as they grow and divide. To facilitate understanding of the cell cycle, the whole cycle was divided into several phases and sub-phases (Fig 3). Four phases, $G_1$ phase (first gap phase), S phase (synthetic phase), $G_2$ phase (second gap phase), and M phase (mitosis), respectively, were adopted to demonstrate the cell cycle. The $G_1$ phase is typically the longest and most variable cell cycle phase, and regulated by two cell cycle control checkpoints, the restriction point and the $G_1$ DNA damage checkpoint. During the $G_1$ phase the cell detects the size and physiological state of itself and the condition of surrounding extracellular matrix (ECM). Cells can monitor the external environment for nutrient availability and recognize signals to proliferate coming from other cells and/or the ECM. When cells think that the internal and external environments are not suitable to reproduce themselves, they will trigger the restriction point and arrest the cell cycle at an equivalent point in the $G_1$ phase. By improving adsorption of proteins essential for cell adhesion and secretion of ECM on the biomaterials surfaces, modification of biomaterial surfaces can promote cell proliferation and improve their biocompatibility indirectly.

Besides the four phases of cell cycle mentioned above, there is still a special phase, $G_0$ phase. When cells are in $G_0$ phase, they stop continuing to divide, but are still capable of re-entering the cell cycle, i.e., in this stage, cells can perform their physiological functions and from this state they can be trigged into the proliferative phase by an appropriate stimulus. Cell proliferation means that cells are dividing, and the most reliable way to determine proliferation is counting the number of cells in a culture dish. When cells enter the $G_0$ state, there is no increase in cell number, i.e. no real cell proliferation occurs. However, under certain nutritional conditions and particularly with transformed cells, one can have stationary cell cultures and yet a considerable amount of cell DNA synthesis. In

such a case, the number of cells does not increase, but an autoradiography will show a substantial fraction of cells when labeled with [$^3$H]-thymidine. So a cell DNA synthesis test, for instance, autoradiography of cells labeled with [$^3$H]-thymidine, is thought under certain conditions to be not reliable to estimate cellular proliferation [91].

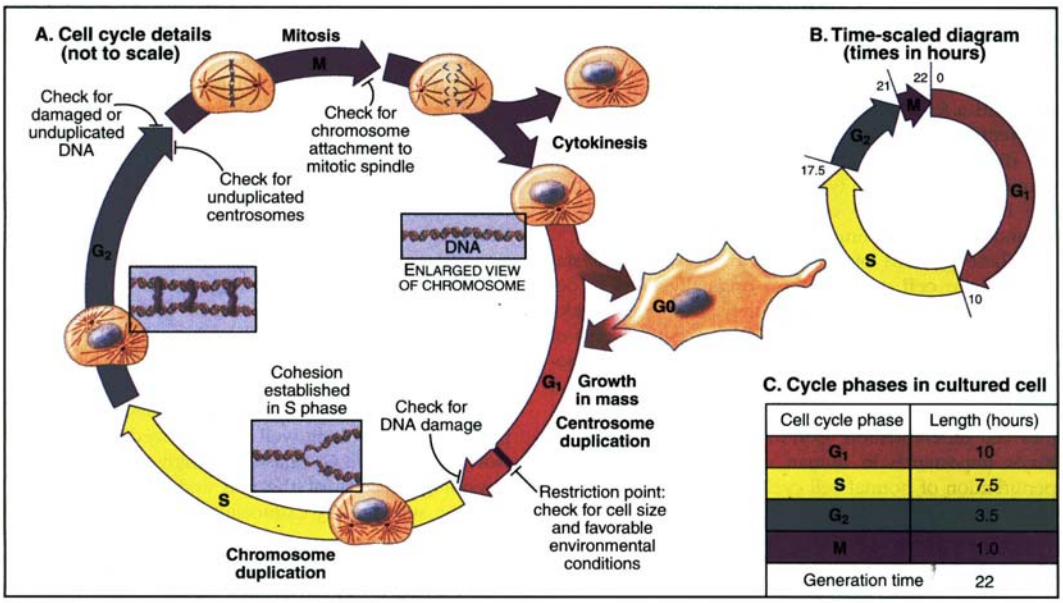



Fig. 3. Introduction to cell cycle phases. A, Diagrams of cellular morphology and chromosome structure across the cell cycle. B, Time scale of cell cycle phases. C, Length of cell cycle phases in cultured cells. (From Cell Biology 1st ed., Thomas D. Pollard, William C. Earnshaw; illustrated by Graham T. Johnson. ISBN 0-7216-3997-6. P 674.)

Differentiation, which is usually used to define the process leading to the expression of phenotypic properties characteristic of the functionally mature cell *in vivo*, is the other important cell functional process during embryonic development or wound healing. Differentiation is generally incompatible with proliferation, that is, as differentiation progresses, cell division is reduced and eventually lost, and vice versa. After implants are inserted into bone tissue, during osseointegration preosteoblasts should differentiate into osteoblasts and osteoblasts should differentiate into osteocytes to form bone tissue around inserted implants. Cell proliferation and differentiation can be promoted by

modifications of the titanium surface.

Stanford and coworkers [92] prepared commercially pure titanium specimens with 600-grit polished, 1-μm polished, and 50-μm Al oxide sand blasted surfaces. Different sterilization treatments were employed to sterilize prepared cp Ti samples, including ultraviolet light, ethylene oxide, argon plasma-cleaning or routine clinical autoclaving. Osteocalcin, collagen expression, alkaline phosphatase activity and alizarin red calcium assay of rat primary osteoblast-like cells were investigated. The results indicated that osteocalcin and alkaline phosphatase activity, but not collagen expression, were significantly affected by surface roughness when these surfaces were treated by argon plasma-cleaning. On a per-cell basis, levels of the marks of cellular differentiation were highest on the smooth 1-μm polished surface and lowest on the roughest surfaces for the plasma-cleaned cpTi.

It is generally accepted that several responses of osteoblastic cells can be promoted by roughened, textured and porous surfaces [22]. Degasne and coworkes [26] found that cell proliferation was increased on rough but not on smooth titanium surfaces. However, other authors revealed the contrary results. Martin et al. [93], compared five different Ti surfaces ranked from smoothest to roughest: electropolished disks (EP), disks pretreated (PT) with hydrofluoric acid-nitric acid and washed (PT), PT disks subsequently fine sandblasted and etched with HCl and $H_2SO_4$ (FA), PT disks subsequently coarse sandblasted and etched with HCl and $H_2SO_4$, and PT disks subsequently Ti plasma-sprayed (TPS). The results of cell proliferation demonstrated that significantly more cells were on EP surfaces than on plastic used as control, while significantly fewer cells were found on TPS surfaces. Postiglione et al. [12], found that human osteoblast-like cells, Soas-2, proliferated on smooth titanium surface significantly faster than on

sandblasted or titanium plasma-sprayed titanium surfaces. On the contrary, the increase of cellular differentiation, indicated by alkaline phosphatase activity, was detected only on sandblasted and titanium plasma-sprayed titanium surfaces. Other authors reported as well that cell proliferation was inversely related to surface roughness [94].

Macroporous $TiO_2$ films, which consist of monodisperse, three-dimensional, spherical, interconnected pores with the size of 0.5, 16, and 50 μm, were prepared and human bone-derived cells (HBDC) were cultured on these surfaces [66]. Higher [$^3$H] thymidine incorporation by the HBDC was observed when they were grown on 0.5- and 16-μm pores compared to the 50-μm pores. However, there was no significant difference of cell proliferation among all three kinds of pore sizes.

The opposite effect of surface roughness on cell proliferation of osteoblast-like cells may attribute to different cell lines used in cell culture or to the differences of roughness ranges. Nevertheless, it is proven that cell proliferation and differentiation can be affected by roughness.

## 2.5 Anodic oxidation on commercial pure titanium

Recently, anodic oxidation, an electrochemical process, on commercial pure titanium has been used to improve the biocompatibility of titanium [39-41,52,80,95-97]. Anodic oxidation is a combination of physical and chemical processes for increasing oxide thickness and altering properties of the titanium surface. The general principle of this technique is the application of an electrical charge to the specimen in an electrolyte solution. The characteristics of the resultant anodic oxide film, such as surface roughness, microstructure and composition, may be influenced by the following factors: used anodic charge voltage and the components and concentrations of electrolyte solutions [96,97].

Larsson et al. used 1 M acetic acid as an electrolyte to anodize titanium implants [52]. Ishizawa and Ogino found that sodium β-glycerophosphate (β-GP) and calcium acetate (CA) were suitable for the electrolytes to form an anodic titanium oxide film containing Ca and P (AOFCP) on commercially pure titanium [39-41]. And the formed oxide films in these electrolytic solutions have a Ca/P ratio equivalent to hydroxyapatite (HA, Ca/P ratio is 1.67). However, no calcium phosphate peak was detected by X-ray diffraction (XRD), and the AOFCP consisted of anatase and only a little rutile. The thickness of the AOFCP produced at 350V was about 10 μm. Further more, the formed AOFCP had a high adhesive strength to titanium after soaking in a simulated body fluid for 300 days.

Zhu et al. produced a titanium oxide film enriched with Ca and P in alternative electrolytes of calcium glycerophosphate (Ca-GP) and calcium acetate (CA) by galvanostatic mode [98]. Under different conditions, i.e., different concentrations of electrolyte, current density and sparking voltages, the range of roughness (Ra) of the formed film was 0.37 – 0.98 μm, Ca/p 0.46 – 1.69, and the thickness was

also increased up to 5-7 μm. In contrast to the oxide films formed in the electrolyte of β-glycerophosphate sodium and calcium acetate, for which it has been reported that some microcracks were observed on the formed film [95], no microcrack was found on all the oxide films anodized in the electrolyte of Ca-GP and CA.

Other alternative electrolytes, such as sulphuric acid and phosphoric acid with or without calcium compounds, were used as well [42,96,97].

The optimal biocompatibility of titanium is due to the most part to oxide layers spontaneously or passively formed on titanium surface. The oxide layers contact directly the cell culture medium *in vitro* or surrounding living tissues *in vivo*, and physical or chemical reactions will happen on these interfaces. It has been reported that metallic ions were released from metallic implants into the surrounding tissue *in vivo*, which may cause acute inflammation without evidence of bacterial infection, allergic reactions and malignant disease [72,99-102]. The corrosion-resistance of titanium oxide films can be greatly improved by anodic oxidation [103]. Anodic oxidation can apparently increase the thickness and stability of oxide layers on titanium, and consequently improve the resistance against release of titanium ions. Kanematu et al., in 1990, produced an oxide-anodized titanium alloy ($TiO_2$/Ti-6Al-4V) with a 138 nm thick layer of titanium oxide compared to the basic titanium alloy with a 1-1.5 nm thick oxide layer, and with decreased titanium ion dissolution [80]. Larsson et al. anodized titanium in 1 M acetic acid at room temperature and using two different voltages, 10V and 80V. Because of the low anodization voltages, thin oxide films were formed 21 nm and 180 nm thick, respectively [52]. However, other authors produced much thicker oxide films with alternative anodizing methods and electrolytes [39-41,95-97], and the thickness of oxide layers can be up to 10 μm.

Since good interaction between implant and bone tissue and enhanced osseointegration are essential for a successful implant, substantial efforts were made to test the bone tissue responses to anodized titanium *in vivo* [52,96,104]. Larsson et al. inserted machined titanium implants, electropolished titanium implants and the anodized implants prepared in 1 M acetic acid with 10V and 80V voltages into proximal tibial metaphysis of adult New Zealand white rabbits, and the machined implants were used as controls. After 7 weeks, the results demonstrated that the highest bone contact was found for the implants with a thick oxide (80V anodization) and the lowest values for the electropolished implants [52].

Sul YT and co-workers [96] prepared implants with 0.2-1 μm thick oxide films surfaces with an average roughness of 0.96-1.03 μm, and the implants were inserted into rabbit tibiae for six weeks. The results showed that implants with an oxide thickness of approximately 600, 800 and 1000 nm demonstrated significantly higher removal torque values than the implants with thinner oxide films, approximately 17 and 200 nm. However, no significant difference between implants with oxide thickness of 17 and 200 nm was detected. Therefore, oxide thickness of implants may play a critical role in bone tissue response to implants. However, it is not fully understood whether these oxide properties influence the bone tissue response separately or synergistically.

Son et al. [104] investigated the bone response to anodized titanium or titanium treated by anodization followed by hydrothermal treatments *in vivo*, and used untreated titanium as control. The removal torque strength was significantly higher for anodized implants than for the untreated implants at 6 weeks after implantation in a rabbit model, although there was no significant difference concerning bone contact on all implants.

Although surface properties of anodic oxides on titanium have been reported extensively in the literature [39-41,95-97], and some studies evaluated their biocompatibility *in vivo*, there are little data about the behavior of osteoblastic cells on anodized titanium surfaces *in vitro*. In the present study, the responses of osteoblast-like cells, SaOS-2, to anodized titanium with different surface properties were observed and compared.

# 3. Aims of the present study

To improve the biocompatibility of alloyed or unalloyed titanium or to achieve a better osseointegration between titanium implant and the bone tissue of the host, many efforts have been undertaken to modify physical and/or chemical characteristics of titanium surfaces. Anodic oxidation has been also used to modify titanium surfaces. Many kinds of anodizing conditions, including anodizing voltage and electrolytes, have been proposed and a couple of *in vivo* investigations have been also reported. However, the effects of anodized titanium surfaces on osteoblastic cells are seldom described yet.

The aims of the current study are as follows:

1. Before a surface modification is further studied, cytotoxicity must be tested. In the present study, the cytototoxicity of titanium specimens after anodization in two electrolytes with different chemical composition will be investigated.

2. Cell behavior on biomaterial surfaces can be described with respect to cell adhesion, spreading, proliferation and differentiation. The responses of osteoblast-like cells to anodized titanium with different surface properties will be determined.

3. By modification of the process parameters for anodic oxidation, a series of surfaces with similar properties will be produced. By comparison of the osteoblastic responses to the different surfaces, an optimization of the anodization process parameter will be achieved.

# 4. Materials and Methods

## 4.1 Anodized titanium specimens

All cp Ti and anodized titanium specimens and the data of their surface properties were friendly provided by Dr. Zhu Xiaolong from department of medical materials and techniques, Center for Dentistry and Oral Medicine, Tuebingen University [118].

### 4.1.1 Specimens preparation

Rectangular specimens 20×10×1 mm in size were cut from a cp titanium plate (ASTM B265 GR. 2) (TITANIA Products, Essen, Germany). Specimens were polished to sandpaper No.1200, etched by mixed $HF/HNO_3$ solution, and cleaned by ethanol and deionised water, then air-dried. Afterwards, pretreated specimens were anodized respectively in two kinds of electrolytes: electrolyte 1: 0.2 M $H_3PO_4$ solution, and electrolyte 2: a mixture of 0.03 M calcium glycerol phosphate (Ca-GP) and 0.15 M calcium acetate (CA). A DC power supply at galvanostatic mode with current density 70 $A/m^2$ was used. In electrolyte 1, 200V, 300V, and 350V anodizing voltages were used; 140V, 200V, 260V, and 300V were adopted in electrolyte 2. Specimens were rinsed with deionised water and dried with nitrogen gas immediately after being anodized. With different voltages of anodic oxidation, the specimens were divided into 8 groups as follows:

Group 1: pretreated Ti as a control (G-1);

Group 2: pretreated Ti and anodized in 0.2 M $H_3PO_4$ at 200V (G-2);

Group 3: pretreated Ti and anodized in 0.2 M $H_3PO_4$ at 300V (G-3);

Group 4: pretreated Ti and anodized in 0.2 M $H_3PO_4$ at 350V (G-4);

Group 5: pretreated Ti and anodized in 0.03 M Ca-GP and 0.15 M CA at 140V (G-5);

Group 6: pretreated Ti and anodized in 0.03 M Ca-GP and 0.15 M CA at 200V (G-6);

Group 7: pretreated Ti and anodized in 0.03 M Ca-GP and 0.15 M CA at 260V (G-7);

Group 8: pretreated Ti and anodized in 0.03 M Ca-GP and 0.15 M CA at 300V (G-8).

Before cell cultures were performed, specimens were ultrasonically cleaned and sterilized in 70% ethanol for 15 min, washed in deionised water, and air dried under a laminar flow hood.

### 4.1.2 Surface characterization

#### 4.1.2.1 Surface topography

The topographies of the prepared groups were analysed by SEM in Fig. 4a-h. Parallel grooves oriented along the polishing direction were displayed on the surface of pretreated cpTi (G-1). When anodic oxidation was performed, grooves produced by polishing were removed gradually as the voltage rose. Because of the lower anodizing voltage, G-2, G-5, and G-6, anodized at 200V, 140V, and 200V respectively, indicated incomplete island-shaped films on the surface and the trace of polishing was only partially removed. The increase of the anodizing voltage resulted in the gradual formation of the anodic oxide film, i.e. from islands to the whole surface, and more and larger irregular micro-pores

generated by sparking during anodic oxidation. When anodizing voltages were over 200V, a pore geometry was formed on the titanium surfaces. But there was difference of the pore geometry between the anodic oxides formed in two different electrolytes. G-4 demonstrated the pore geometry formed in 0.2 M $H_3PO_4$, while G-7 and G-8 displayed the pore geometry anodized in 0.03 M Ca-GP and 0.15 M CA. The size of pores on the surfaces of group G-4 (up to ca. 0.5 μm in diameter) was distinctly smaller than that (up to ca. 2 μm in diameter) on the surfaces of group G-7 and G-8. The general effect of anodic oxidation on the topography alteration was evolved from anisotropy to isotropy as the increasing of anodizing voltage.

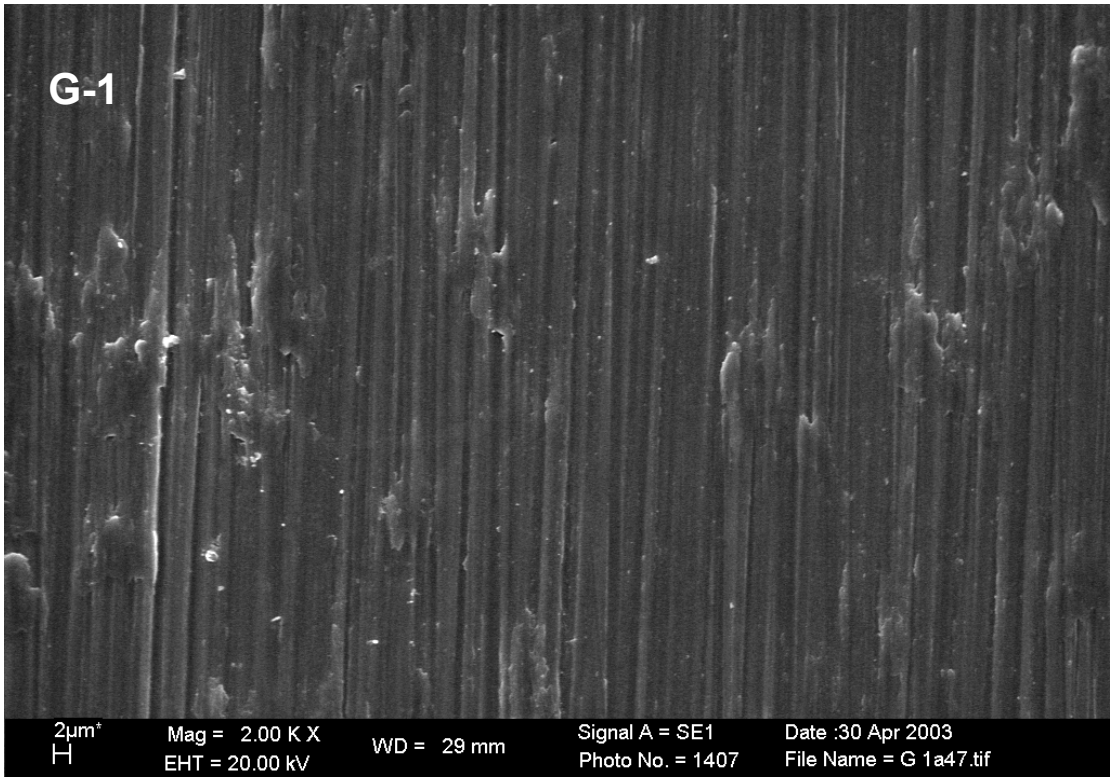



Fig. 4a. **S**urface of pretreated titanium (G-1).

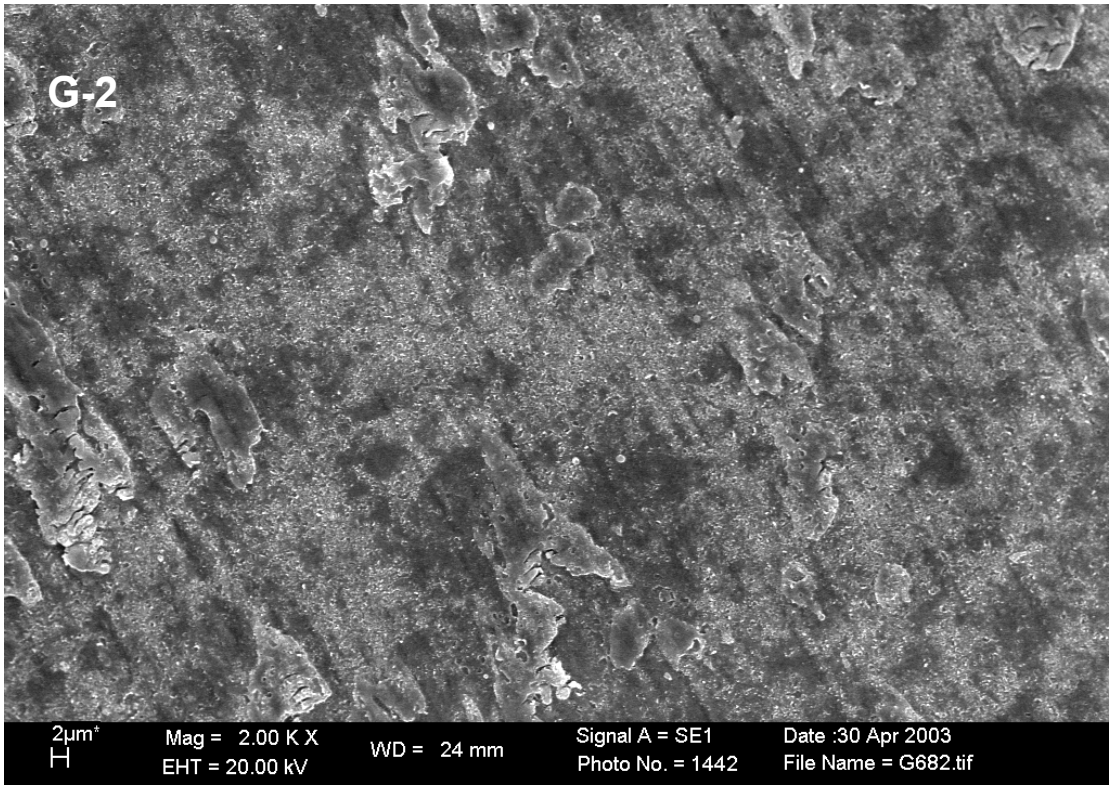



Fig. 4b. Surface of anodized titanium: 70 A/m$^2$, at 200V in 0.2 M $H_3PO_4$; (G-2).

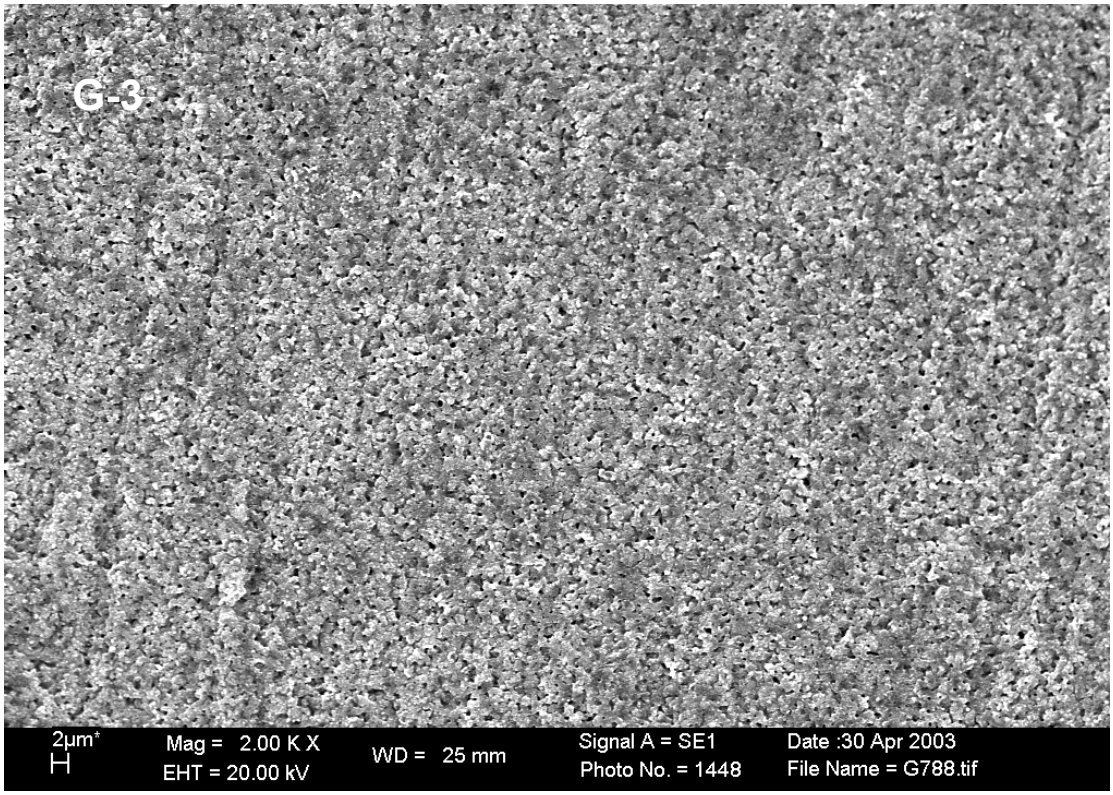



Fig. 4c. Surface of anodized titanium: 70 A/m$^2$, at 300V in 0.2 M $H_3PO_4$; (G-3).

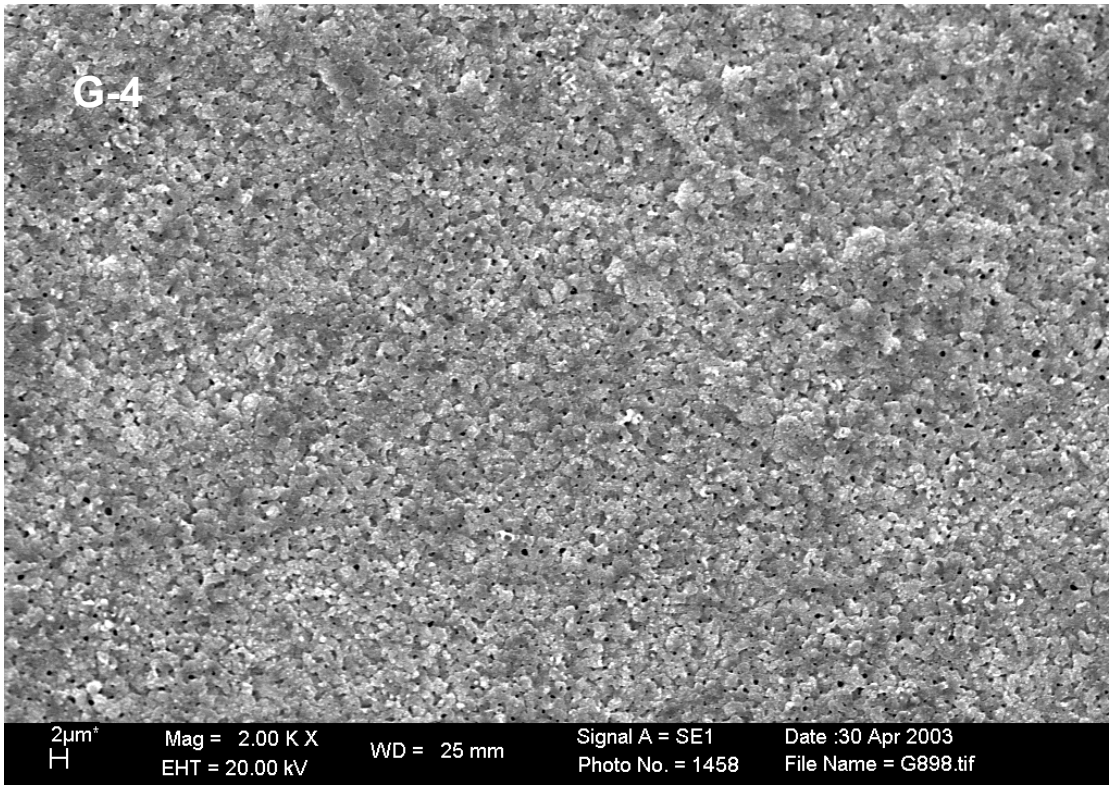



Fig. 4d. Surface of anodized titanium: 70 A/m$^2$, at 350V in 0.2 M $H_3PO_4$; (G-4).

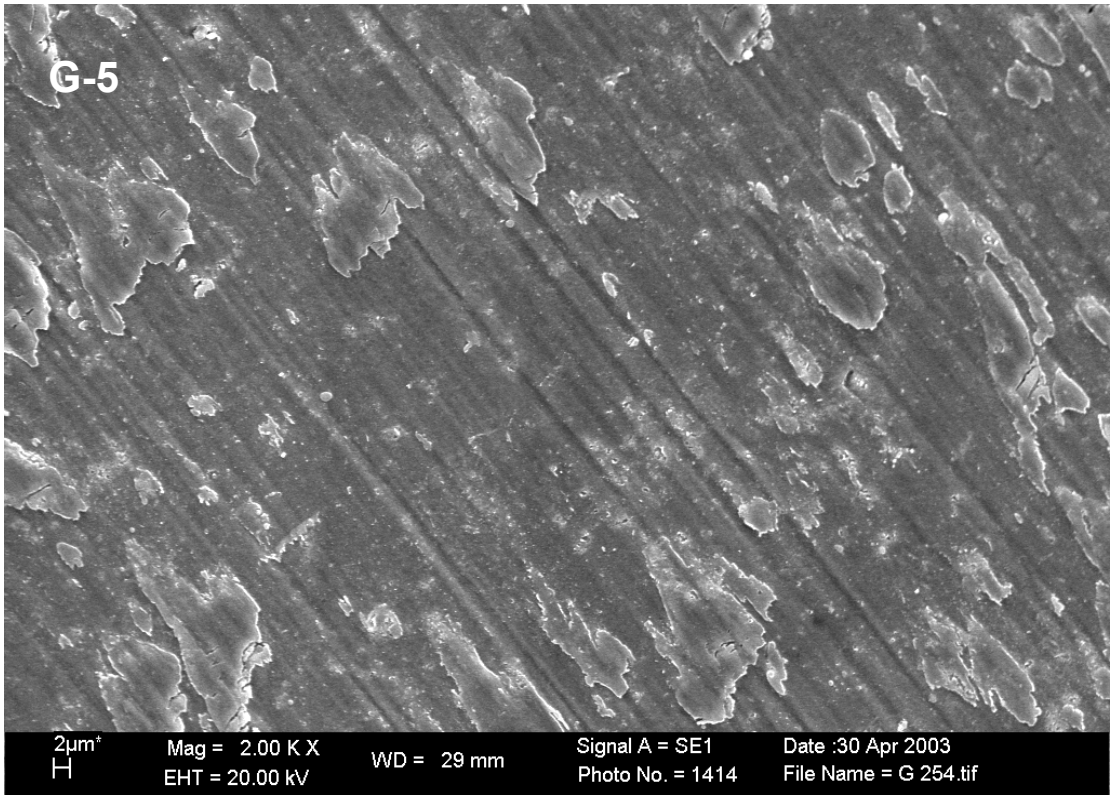



Fig. 4e. Surface of anodized titanium: 70 A/m$^2$, at 140V in 0.03 M Ca-GP and 0.15 M CA; (G-5)

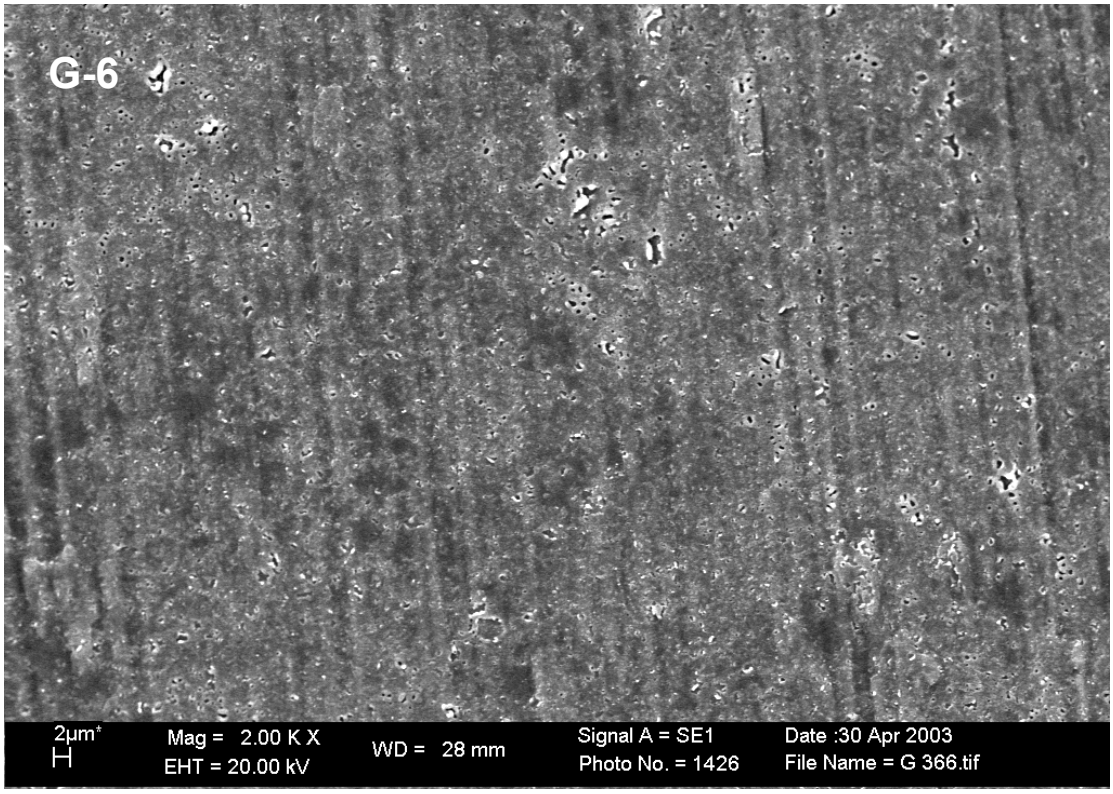



Fig. 4f. Surface of anodized titanium: 70 A/m$^2$, at 200V in 0.03 M Ca-GP and 0.15 M CA; (G-6)

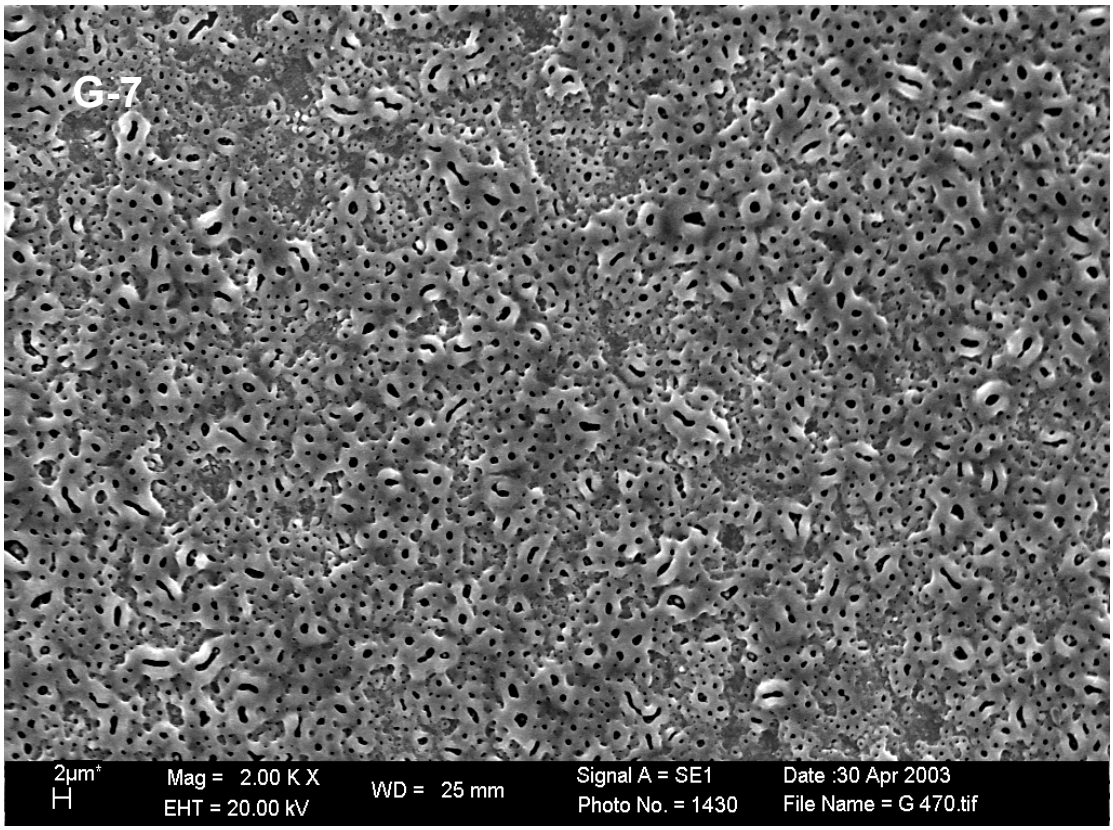



Fig. 4g. Surface of anodized titanium: 70 A/m$^2$, at 260V in 0.03 M Ca-GP and 0.15 M CA; (G-7)

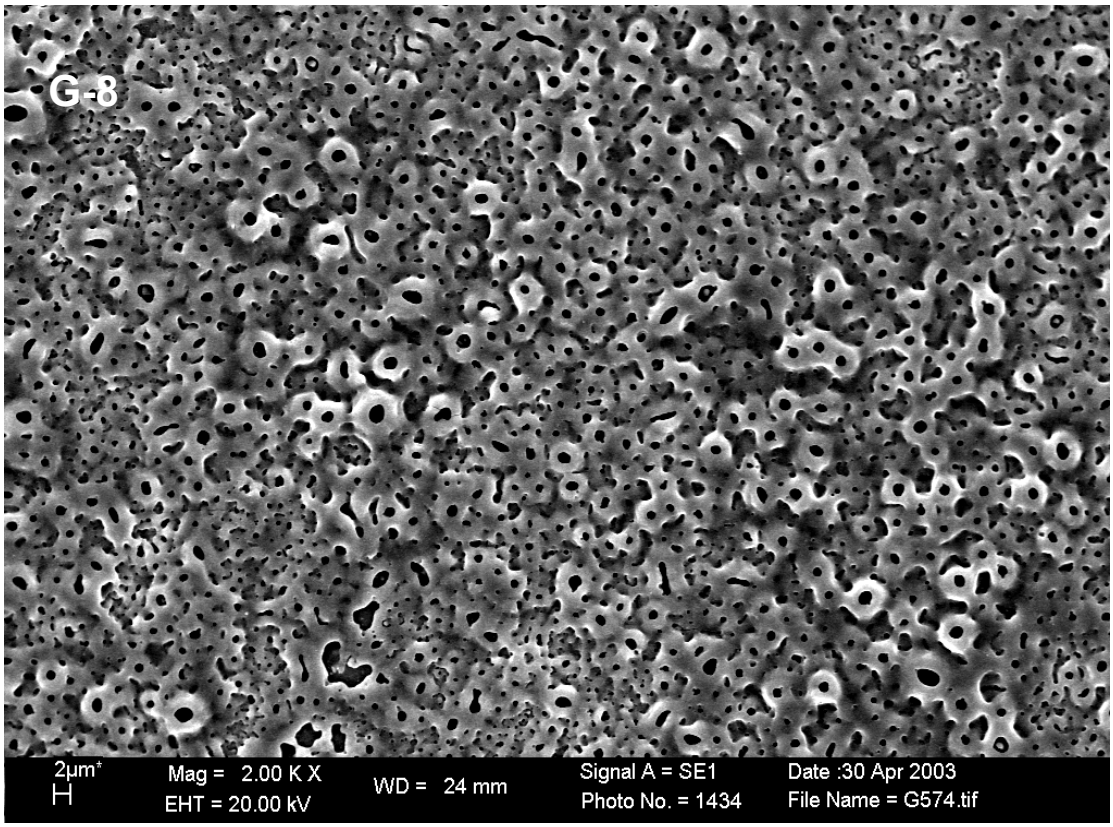



Fig. 4h. Surface of anodized titanium: 70 A/m$^2$, at 300V in 0.03 M Ca-GP and 0.15 M CA; (G-8)

### 4.1.2.2 Surface roughness

The surface roughness of specimens was measured using a surface profilometer (Perthometer S6P, Perthen Instruments, Mahr, Goettingen, Germany). The arithmetical mean of surface roughness of every measurement within the total distance (roughness average =Ra) was assessed. As shown in Fig. 5 and table 1, statistical analyses indicated significant difference in roughness between anodized surfaces and cp Ti. The pretreated cpTi has the smoothest surface with an average roughness $R_a$ of 0.17 µm. All anodized groups have rougher surfaces than the control group, with a range of the $R_a$ values of 0.2 µm to 0.4 µm. Surface roughness on anodic oxides formed in 0.2 M $H_3PO_4$ decreased with an increase in anodizing voltage; while in 0.03 M

Ca-GP and 0.15 M CA, $R_a$ values were enhanced with increasing voltage (Fig. 5, Table 2).

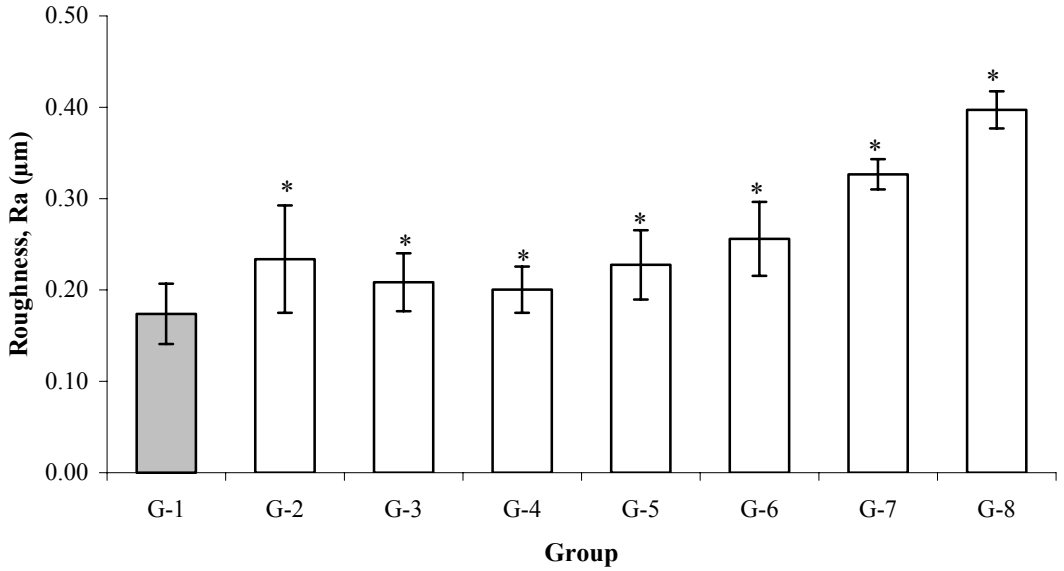



Fig. 5. Average roughness ($R_a$) of the anodized surfaces of titanium, G-1 as a control, *p <0.05.

**Table 1. Average roughness of all groups, Mean±SD (µm)**

| cpTi | G - 2 | G - 3 | G - 4 | G - 5 | G - 6 | G - 7 | G - 8 |
|---|---|---|---|---|---|---|---|
| 0.17 ± 0.03 | 0.23 ± 0.06 | 0.21 ± 0.03 | 0.20 ± 0.03 | 0.23 ± 0.04 | 0.26 ± 0.04 | 0.33 ± 0.02 | 0.40 ± 0.02 |

**Table 2. Comparisons of average roughness by Tukey-Kramer HSD (Abs (Dif)-LSD)**

| | G-2 | G-3 | G-4 | | G-5 | G-6 | G-7 | G-8 |
|---|---|---|---|---|---|---|---|---|
| G-2 | -0.02411 | 0.00428$^+$ | 0.01105$^+$ | G-5 | -0.02078 | 0.00749$^+$ | 0.07818$^+$ | 0.14853$^+$ |
| G-3 | 0.00428$^+$ | -0.02411 | -0.01734 | G-6 | 0.00749$^+$ | -0.02078 | 0.04991$^+$ | 0.12025$^+$ |
| G-4 | 0.01105$^+$ | -0.01734 | -0.02411 | G-7 | 0.07818$^+$ | 0.04991$^+$ | -0.02078 | 0.04956$^+$ |
| | | | | G-8 | 0.14853$^+$ | 0.12025$^+$ | 0.04956$^+$ | -0.02078 |

$^+$Positive values show pairs of means that are significantly different (Alpha = 0.05).

#### 4.1.2.3 Wettability

The wettability of a liquid is defined as the contact angle between a droplet of the liquid in thermal equilibrium on a horizontal surface (Fig 6). The shape of the droplet depends on the type of surface and the used liquid. In present study, contact angles were measured on DSA 10Mk 2 (Kruess), and the deionized water with drop volumes of 5 μl was used. Images were recorded with a video system, and the contact angle values were calculated with the drop shape analysis system (DSA 1).

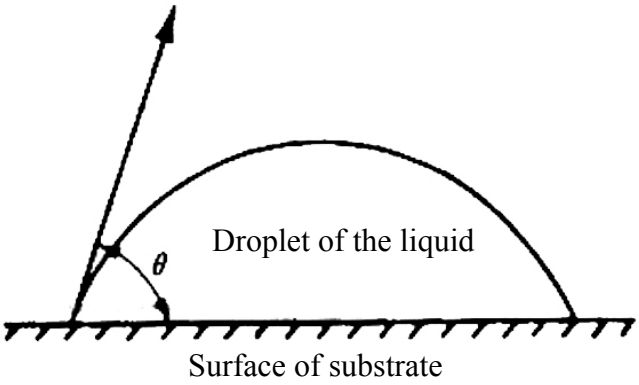



Fig. 6. Diagram of contact angle (θ).

From contact angle measurements (Fig. 7), the range of contact angle for either the control or the anodic oxides was from 60º to 90º, and except for group G-6 there were significant differences between other groups and control group G-1. By Tukey-Kramer HSD test, among the groups anodized in electrolyte 2 the contact angle of G-5 was the lowest, and the contact angles were degressive from G-6 to G-8 was revealed from the current results, hence the anodizing voltage may affect contact angle.

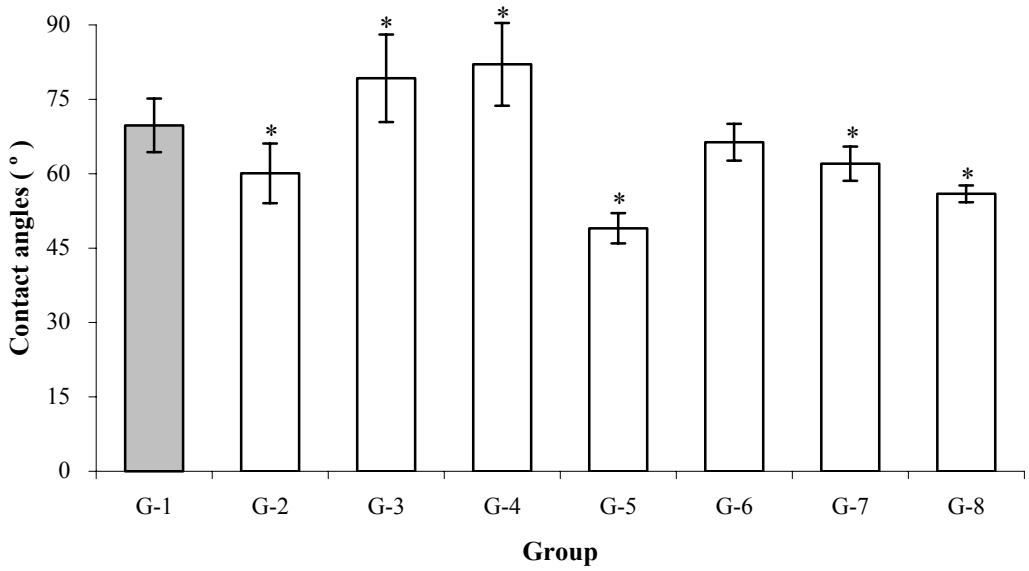



Fig. 7. Wettability of the anodized surfaces of titanium, G-1 as a control, *p <0.05.

**Table 3. Contact angle measurements of all groups, Mean±SD (µm)**

| CpTi | G - 2 | G - 3 | G - 4 | G - 5 | G - 6 | G - 7 | G - 8 |
|---|---|---|---|---|---|---|---|
| 69.75 ± 5.40 | 60.07 ± 6.02 | 79.24 ± 8.82 | 82.06 ± 8.34 | 48.99 ± 3.05 | 66.36 ± 3.71 | 62.02 ± 3.46 | 55.94 ± 1.69 |

**Table 4. Comparisons of contact angle by Tukey-Kramer HSD (Abs (Dif)-LSD)**

| | G-2 | G-3 | G-4 | | G-5 | G-6 | G-7 | G-8 |
|---|---|---|---|---|---|---|---|---|
| G-2 | -8.674 | 10.496$^{+}$ | 13.316$^{+}$ | G-5 | -3.707 | 13.663$^{+}$ | 9.323$^{+}$ | 3.243$^{+}$ |
| G-3 | 10.496$^{+}$ | -8.674 | -5.854 | G-6 | 13.663$^{+}$ | -3.707 | 0.633$^{+}$ | 6.713$^{+}$ |
| G-4 | 13.316$^{+}$ | -5.854 | -8.674 | G-7 | 9.323$^{+}$ | 0.633$^{+}$ | -3.707 | 2.373$^{+}$ |
| | | | | G-8 | 3.243$^{+}$ | 6.713$^{+}$ | 2.373$^{+}$ | -3.707 |

$^{+}$ Positive values show pairs of means that are significantly different (Alpha = 0.05).

#### 4.1.2.4 Chemical compositions

The chemical composition of commercially pure titanium (Group 1, control), as provided by the manufacturer, consists of 0.007% N, 0.014% C, 0.002% H, 0.06% Fe, 0.150% O, residuals <0.3% in total and Ti of balance. The chemical composition of anodized titanium specimens, measured by XPS analyses, is shown in Table 1. The main difference between the samples produced in the two electrolytes consisted in the content of P and Ca, because of the composition of the electrolytes. When 0.2 M $H_3PO_4$ solution was used as electrolyte, the P content in the anodic oxides was irrespective of the anodizing voltage; while in the mixture of 0.03 M calcium glycerophosphate (Ca-GP) and 0.15 M calcium acetate (CA), the both Ca content and Ca/P ratios of the anodic oxides were increased as anodizing voltage increased.

**Table 5 Chemical compositions of anodic oxides of titanium by XPS (at.%)**

| Group | Ti | O | C | P | Ca | N | Si | *Other | Ca/P |
|---|---|---|---|---|---|---|---|---|---|
| G-2 | 10 | 56 | 18 | 10 | | 1 | <0.1 | 5 | |
| G-3 | 7 | 53 | 23 | 10 | | 2 | 1 | 4 | |
| G-4 | 7 | 47 | 31 | 11 | | 2 | <0.1 | < 2 | |
| G-5 | 15 | 55 | 24 | 3 | <1 | 2 | <0.1 | 1 | <0.33 |
| G-6 | 14 | 54 | 23 | 5 | 2 | <1 | <0.1 | 2 | 0.40 |
| G-7 | 13 | 55 | 20 | 6 | 4 | <0.1 | <0.1 | < 2 | 0.67 |
| G-8 | 12 | 54 | 20 | 6 | 6 | <0.1 | <0.1 | < 2 | 1.00 |

*not clearly identified

## 4.2 Osteoblast cell culture

### 4.2.1 Osteoblast-like cell line

The SaOS-2 human osteoblast-like cell line, derived from a human osteosarcoma, was obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures). It is a highly differentiated, stable and non-transfected cell line. Cells were cultured in modified McCoy's 5A medium (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) containing 10% fetal calf serum (FCS; PAA Laboratories GmbH, Linz, Austria), 1% penicillin (10,000 units)/streptomycin (10 mg/ml, GIBCO, Scotland, UK) and 1% 200 mM L-glutamine (PAA Laboratories GmbH, Linz, Austria) and maintained in humidified atmosphere with 5% $CO_2$ at 37ºC. The culture medium was renewed twice a week. When cells reached confluence, old culture medium was discarded and washed with 5 ml phosphate-buffered saline ($PBS^-$ Dulbecco's, Scotland, UK) without magnesium and calcium, then 2 ml Trypsin-EDTA (0.5g/L Trypsin and 0.2g/L EDTA, GIBCO, Scotland, UK) was used to detach cells from the bottom of the culture flasks and 1/3 of total cells were transferred into a new tissue culture flask.

### 4.2.2 Cytotoxicity assay

Cytotoxicity assay was performed by an extract method, which is described in ISO 10993-5 and has been used by other authors [31]. Extracts of all specimens were obtained from contact of samples with modified McCoy's 5 A cell culture medium for 72 h at 37ºC (according to ISO 10993-5).The ratio between sample surface and the volume of the extraction vehicle was 3 $cm^2$/ml. SaOS-2

osteoblast-like cells at a concentration of 5000 cells/well were seeded in a 96-well multiwell plate in 150 µl modified McCoy's 5A medium per well, and incubated in humidified atmosphere with 5% CO2 at 37ºC. After 24 h incubation, the medium was discarded and replaced by the extracts derived from the different samples. Each extract was tested in 3 concentrations: 1) undiluted (150µl extract); 2) 1:2 diluted with medium (50µl extract + 100µl medium); 3) 1:14 diluted (10µl extract + 140µl medium). Each concentration was tested in quadruplicate. Cell culture medium without extract was used as the negative control, and toxic PVC-Film (Rehau GmbH, Germany) extracted in the same manner as the samples was used as positive control. After additional 24 h of culture, cytotoxicity was assayed by XTT-Test (cell proliferation Kit, Roche Diagnostics GmbH, Mannheim, Germany).

The assay is based on the cleavage of the yellow tetrazolium salt XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (Roche Diagnostics GmbH, Mannheim, Germany) to form an orange formazan dye by metabolically active cells. Therefore, this conversion only occurs in viable cells. The formed formazan dye is soluble in aqueous solutions and is directly quantified using an ELISA reader (SLT, Labinstrument Deutschland GmbH, Germany).

### 4.2.3 Cell attachment and spreading

Cell attachment and spreading was investigated by vinculin staining to show focal contacts and actin filaments were probed with FITC-conjugated phalloidin to reveal the arrangements of the cytoskeleton [105].

Specimens of anodized titanium and control group (pretreated commercially pure titanium) were put into 6-well plates, and each specimen located in one well. SaOS-2 cells in a density of $2.0\times10^4$ cells/$cm^2$, suspended in 5ml cell culture medium, were seeded onto the surfaces of specimens, and incubated in an incubator with humidified atmosphere with 5% $CO_2$ at 37ºC for 1 and 2 hours. After 1 or 2 hours, respectively, the specimens were rinsed with $PBS^-$, fixed with 2% paraformaldehyde in $PBS^-$ for 10 minutes, permeabilized with 1% Triton X-100 in $PBS^-$ for 5 minutes, and then blocked with 5% goat serum in the dark for 15 min. Vinculin, one kind of focal contact adhesion related protein, was stained with mouse anti-human-vinculin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) as primary antibody and TRICT- (tetramethylrhodamine isothiocyanate-) conjugated anti-mouse-IgG (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) as secondary antibody. The primary antibody was diluted 1:200 in the sterilized $PBS^-$ solution containing 1% bovine serum albumin (BSA, Sigma-Aldrich Chemie GmbH, Germany) and 5% goat serum (PAA Laboratories GmbH, Austria), and the secondary antibody was diluted 1:64 in the same solution as well. After incubating with 5% goat serum, 100 µl diluted primary antibody was added carefully onto every specimen and maintained in dark environment for 40 min, then followed by the same volume of diluted secondary antibody for 40 min. For staining of actin filaments, fixed cells were incubated with 2µg/ml FITC-conjugated phalloidin for 10 min. After finishing of all staining steps, specimens were fixed in 2% paraformaldehyde again for 10 min. It should be noticed that all these steps are conducted at room temperature, and $PBS^-$ was used to rinse specimens after each step.

After fluorescent staining, the specimens were mounted with fluorescence protection mounting medium (Polyvinyl alcohol mounting medium with DABCO; Fluka Chemie GmbH, Buchs, Switzerland) and evaluated in an epifluorescent

microscope (Nikon-OPTIPHOT-2, Nikon Co., Japan). Cell spreading was assessed by microscopical examination of all samples by one trained examinator. As shown in Fig 8, all attached cells were divided into a) not spread cells: cells were still spherical in appearance, no protrusions or lamellipodia were produced; b) and c) partially spread cells: at this stage, cells began to spread lateral, at one side or more, but the extensions of plasma membrane were not completely confluent; d) fully spread cells: sufficient extension of plasma membrane or flattening of the cells was obviously observed. The number of total attached cells and percentage of spread cells among attached cells were calculated from 5 different random square areas (0.8 x 0.8 mm) of each specimen. The number of attached cells on the surfaces of the control after 1h incubation was set as 100%, and the data of the other groups were compared with it. Pictures showing focal contacts and actin filaments were made using a Nikon Coolpix E950 digital Camera (Nikon-OPTIPHOT-2, Nikon Co., Japan).

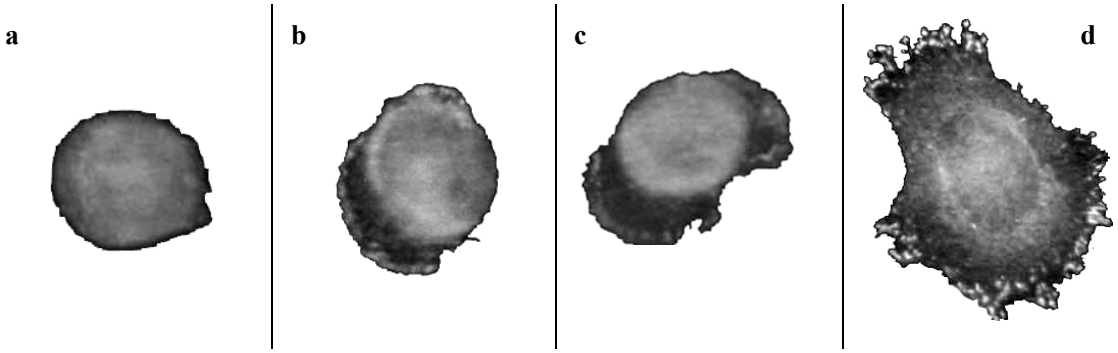



Fig. 8. As shown in these examples from the experiments, all attached cells were divided into a) not spread cells: cells were still spherical in appearance, no protrusions or lamellipodia were produced; b) and c) partially spread cells: at this stage, cells began to spread lateral, at one or more sides, but the extended plasma membrane were not completely confluent; d) fully spread cells: sufficient extension of plasma membrane or flattening of the cells was obviously observed.

### 4.2.4 Cell proliferation (determination of cell numbers)

Each group of specimens used for cell proliferation tests was divided into 3 subgroups and measured after cell culturing for 1, 2 and 4 days, respectively. SaOS-2 cells were seeded on specimen surfaces with the initial cell density of $2.0 \times 10^4$ cells/cm$^2$ in 6-well multiwell plates. At every harvest time point, old cell culture medium was discarded, cells were washed with PBS$^-$ solution, detached from specimen surfaces by incubation with Trypsin/EDTA (0.5g/L Trypsin and 0.2g/L EDTA, GIBCO, Scotland, UK) for 5 min at 37°C, and the detaching procedure was stopped with cell culture medium containing 10% FCS. The suspension with detached cells was collected into a test tube. The above process was repeated once more. Preliminary tests revealed that no residual cells were found on surfaces of specimens after two trypsin treatments. Released cells were counted with a hemocytometer, and for every specimen counting was repeated three times.

### 4.2.5 Alkaline phosphatase activity

After cell proliferation test, cells were collected by centrifugation with 1100 rpm at room temperature for 3 min (Minifuge T, Heraeus, Germany). The cell pellets were lysed in 400 µl 0.5% Triton, and alkaline phosphatase (ALP) activity was measured using p-nitrophenylphosphate (Sigma Diagnostics, Inc, St. Louis, USA) as substrate. A standard solution from p-nitrophenol (Sigma Diagnostics, Inc, ST. Louis, USA) substrates was transferred to colour product by addition of 0.1 N NaOH, diluted into a series of standard concentrations with distilled water (Ampuwa, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany) and used as references for measurement. Absorbance was measured using an ELISA reader with the wavelength of 405 nm [106]. The value of ALP activity

was normalized by the total cell number obtained from proliferation tests, and ALP activity was defined as nMol/min/$10^4$ cells.

### 4.2.6 Statistical analysis

All data were analysed by JMP Version 5.01 statistical analysis program (SAS Institute Inc., Cary, NC). Significant differences between all the anodizing groups and the control were determined using Dunnett's test, which guards against the high alpha size (type I) error rate across the hypothesis tests. Tukey-Kramer HSD (honestly significant difference) test was used to perform multiple comparisons between the groups anodized in the same electrolyte. The Tukey-Kramer HSD test provides a conservative calculation of statistical significance in the analysis of intergroup comparisons that minimizes the risk of type I error by increasing the quantile multiplied into the standard error values to create the least significant difference. Unless otherwise indicated, all data presented were mean ± S.D. and the significant level of 0.05 (alpha) was accepted.

# 5. Results

## 5.1 Cytotoxicity

Figure 9 shows the cytotoxicity of extracts from all sample groups, expressed as decrease in metabolic activity of cells. The results showed that there was no significant difference between negative control, cp Ti and anodized titanium, while the concentration-dependent decrease in metabolic activity of the positive control (PVC) demonstrates the sensitivity of the assay. Thus, anodized titanium surfaces with different contents of P or Ca and P do not reduce the viability and metabolism of osteoblasts.

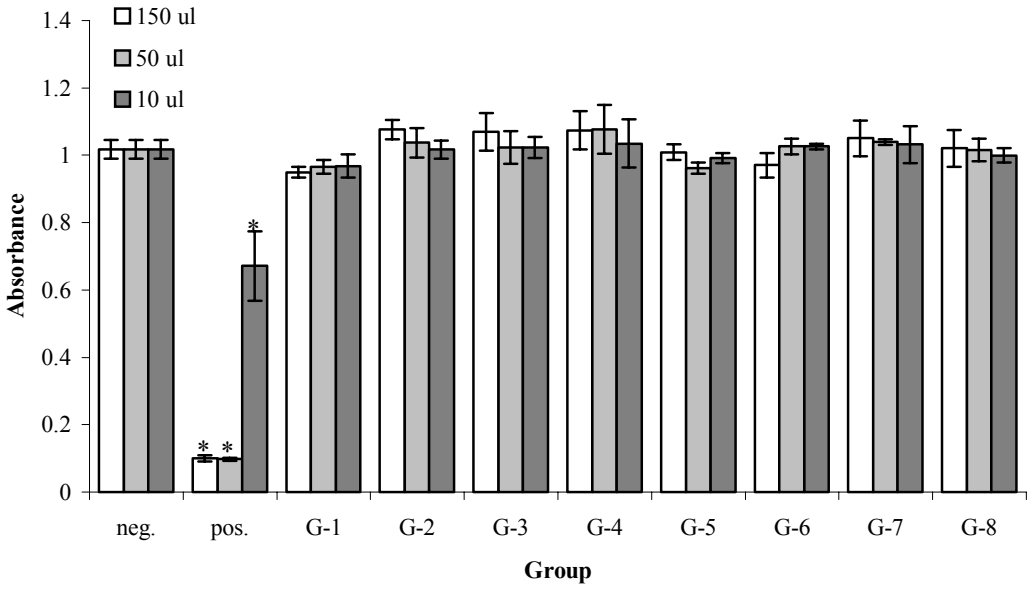



Fig. 9. Cytotoxicity of extracts of cp Ti and anodized titanium determined with SaOS-2 osteoblasts at different extract dilutions by XTT-assay. Pure cell culture medium was used as a negative control and an extract from toxic PVC as a positive control, There was a significant statistical difference between the positive control and all the groups and no statistical difference between the negative control and all the groups, *p <0.05.

## 5.2 Cell attachment and spreading

At both time points (1 and 2 hour), statistical analysis showed that the number of cells attached on nearly all the anodized oxides was significantly higher than on the surface of cp titanium (Fig 10, Table 6). Except for G-8, no statistical difference was indicated for cell attachment each respective surface between the 1 and 2 hour attachment periods. As shown in Table 7 and Table 8, cell attachment on the anodized surfaces produced in the same electrolyte did not significantly differ. The only exception is the G-8 group, which showed significantly more cell adhesion than groups G-5 and G-6 in the 2 hours test.

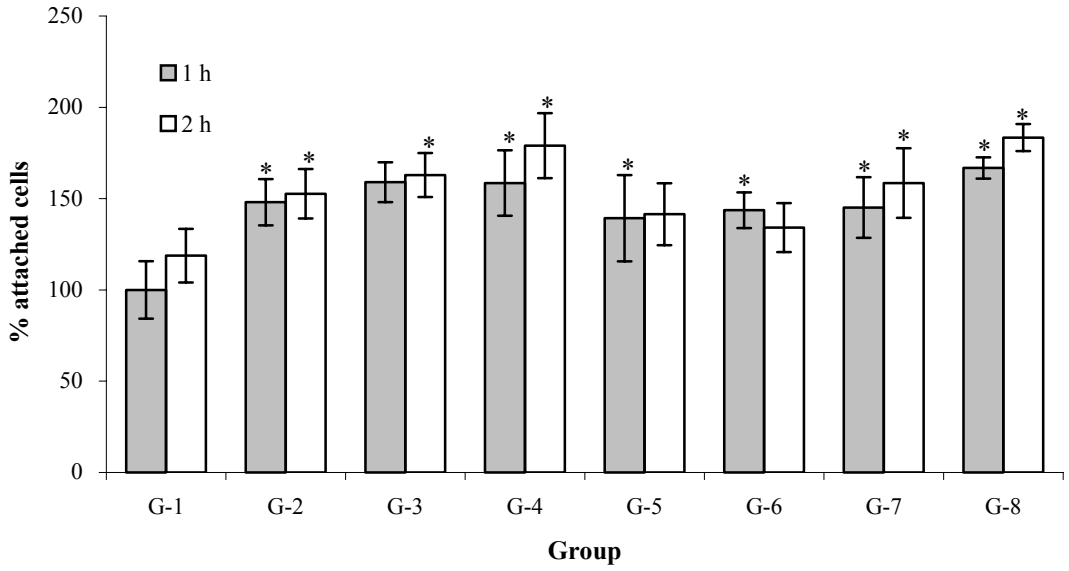



Fig. 10. Percentage of attached cells on anodic oxides normalized by number of attached cells cultured on the surfaces of pretreated Ti for 1h, *p <0.05.

Table 6 The mean value of the attached cells cultured for 1h on G-1 (titanium control) was set as 100%, and all other groups were calibrated with this reference. Mean ± SD.

| Group | 1 h | 2 h |
|---|---|---|
| G - 1 | 100.00 ± 15.74 | 118.78 ± 14.65 |
| G - 2 | 148.05 ± 12.66 | 152.68 ± 13.54 |
| G - 3 | 159.02 ± 10.96 | 162.93 ± 12.03 |
| G - 4 | 158.54 ± 17.94 | 179.02 ± 17.81 |
| G - 5 | 139.27 ± 23.62 | 141.46 ± 17.01 |
| G - 6 | 143.66 ± 9.81 | 134.15 ± 13.44 |
| G - 7 | 145.12 ± 16.65 | 158.54 ± 19.05 |
| G - 8 | 166.83 ± 5.87 | 183.41 ± 7.40 |

Table 7 Comparisons of cell attachment by Tukey-Kramer HSD (Abs (Dif)-LSD) (1 h)

| | G-2 | G-3 | G-4 | | G-5 | G-6 | G-7 | G-8 |
|---|---|---|---|---|---|---|---|---|
| G-2 | -19.606 | -10.606 | -11.006 | G-5 | -23.059 | -19.459 | -18.259 | -0.459 |
| G-3 | -10.606 | -19.606 | -19.206 | G-6 | -19.459 | -23.059 | -21.859 | -4.059 |
| G-4 | -11.006 | -19.206 | -19.606 | G-7 | -18.259 | -21.859 | -23.059 | -5.259 |
| | | | | G-8 | -0.459 | -4.059 | -5.259 | -23.059 |

[+] Positive values show pairs of means that are significantly different (Alpha = 0.05).

Table 8 Comparisons of cell attachment by Tukey-Kramer HSD (Abs (Dif)-LSD) (2 h)

| | G-2 | G-3 | G-4 | | G-5 | G-6 | G-7 | G-8 |
|---|---|---|---|---|---|---|---|---|
| G-2 | -24.743 | -14.499 | 1.599[+] | G-5 | -26.956 | -19.638 | -9.884 | 14.994[+] |
| G-3 | -14.499 | -24.743 | -8.645 | G-6 | -19.638 | -26.956 | -2.566 | 22.312[+] |
| G-4 | 1.599[+] | -8.645 | -24.743 | G-7 | -9.884 | -2.566 | -26.956 | -2.078 |
| | | | | G-8 | 14.994[+] | 22.312[+] | -2.078 | -26.956 |

[+] Positive values show pairs of means that are significantly different (Alpha = 0.05).

In Fig 11 and Table 9-11, the percentage of fully spread cells, related to total attached cells on the respective surface, is shown. No significant difference of spreading between cp Ti and anodized surfaces was found at 1 hour, while at 2 hours, G-2 and G-8 displayed a noticeably lower degree of cell spreading than cp Ti.

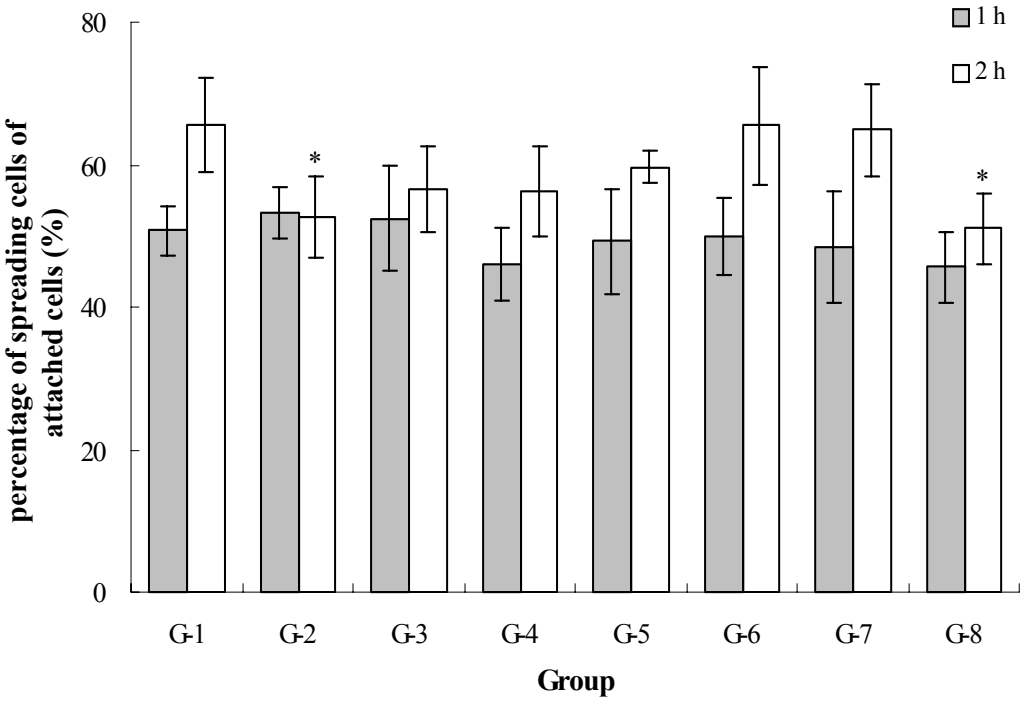



Fig. 11. Percentage of fully spread cells in relation to total attached cells on the surfaces of pretreated Ti and anodized titanium, *p <0.05.

Table 9 Percentage of the fully spread cells in relation to total attached cells after 1 or 2 hours cell culture (%). Mean ± SD.

| Group | 1 h | 2 h |
|---|---|---|
| G - 1 | 50.68 ± 3.55 | 65.52 ± 6.69 |
| G - 2 | 53.20 ± 3.51 | 52.62 ± 5.66 |
| G - 3 | 52.35 ± 7.38 | 56.56 ± 5.96 |
| G - 4 | 46.13 ± 5.08 | 56.27 ± 6.24 |
| G - 5 | 49.25 ± 7.43 | 59.67 ± 2.24 |
| G - 6 | 49.92 ± 5.36 | 65.43 ± 8.38 |
| G - 7 | 48.44 ± 7.89 | 64.88 ± 6.43 |
| G - 8 | 45.71 ± 4.96 | 51.03 ± 4.94 |

Table 10 Comparisons of cell spreading by Tukey-Kramer HSD (Abs (Dif)-LSD) (1 h)

| | G-2 | G-3 | G-4 | | G-5 | G-6 | G-7 | G-8 |
|---|---|---|---|---|---|---|---|---|
| G-2 | -9.3756 | -8.5236 | -2.3016 | G-5 | -11.828 | -11.156 | -11.010 | -8.286 |
| G-3 | -8.5236 | -9.3756 | -3.1536 | G-6 | -11.156 | -11.828 | -10.338 | -7.614 |
| G-4 | -2.3016 | -3.1536 | -9.3756 | G-7 | -11.010 | -10.338 | -11.828 | -9.104 |
| | | | | G-8 | -8.286 | -7.614 | -9.104 | -11.828 |

[+] Positive values show pairs of means that are significantly different (Alpha = 0.05).

Table 11 Comparisons of cell spreading by Tukey-Kramer HSD (Abs (Dif)-LSD) (2 h)

| | G-2 | G-3 | G-4 | | G-5 | G-6 | G-7 | G-8 |
|---|---|---|---|---|---|---|---|---|
| G-2 | -10.055 | -6.113 | -6.407 | G-5 | -10.741 | -4.979 | -5.531 | -2.101 |
| G-3 | -6.113 | -10.055 | -9.761 | G-6 | -4.979 | -10.741 | -10.189 | 3.661$^{+}$ |
| G-4 | -6.407 | -9.761 | -10.055 | G-7 | -5.531 | -10.189 | -10.741 | 3.109$^{+}$ |
| | | | | G-8 | -2.101 | 3.661$^{+}$ | 3.109$^{+}$ | -10.741 |

$^{+}$ Positive values show pairs of means that are significantly different (Alpha = 0.05).

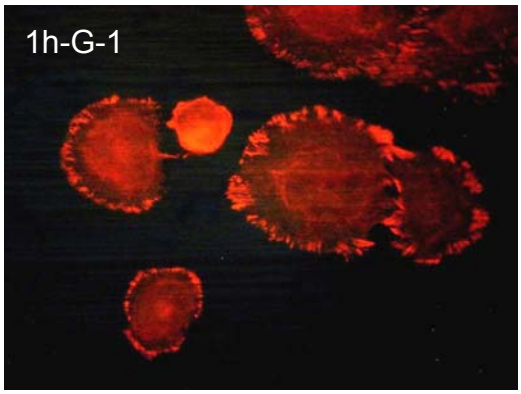
## 5.3 **Cellular morphology**

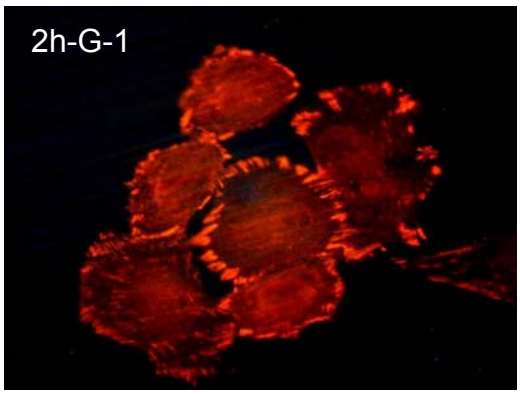
Fig. 12 reveals cellular morphology of osteoblast-like cells SaOS-2 on various surfaces at 1 and 2 hours under fluorescent microscopy with 400 fold magnification. Three kinds of spread cell morphologies were shown, that is, a polygonal shape with many filopodia; a polarized shape, i.e. elongated in the opposite directions, mostly occurring in cells on the oxides formed in 0.2 M $H_3PO_4$; and a round one as appeared on the cp Ti.

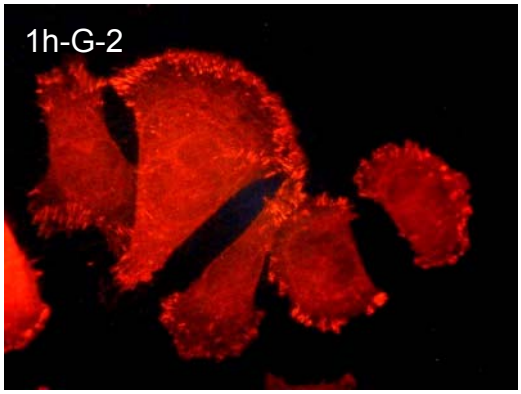
At 1 hour, most of spreading cells on cp Ti and G-5 were round and extended only partially, while the cells on the other anodized titanium surfaces appeared irregular and polygonal. In addition, cells adhered to G-4, G-7 and G-8 became even more irregular and their filopodia extended longer than those of cells on the other anodized surfaces. At 2 hours, cell morphology on cp Ti was not yet markedly changed and only few cells showed irregular extensions, though the number of spread cells on the cp Ti surface was significantly increased. On anodized titanium surfaces, cells extended more intensively after 2 hours of cell culture, while polygonal cells increased compared to 1 hour attachment and much more filopodia were formed.

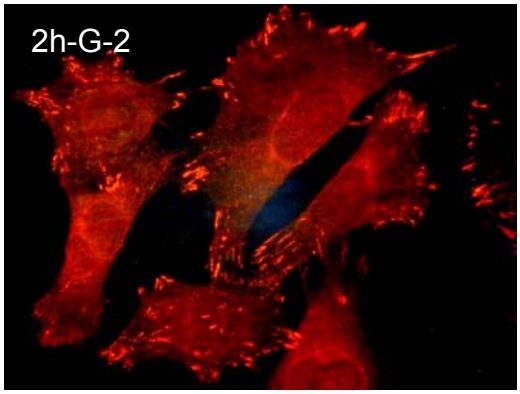
Cellular morphology on the surfaces anodized in the same electrolyte showed a dependence of spreading from the anodizing voltage. Among G-2, G-3 and G-4, cell spreading on G-2 was most similar to cp Ti. However, cells on G-4 showed more irregularities than on G-2 and G-3. As shown from the pictures of surfaces anodized in the other electrolyte, the same phenomenon was also observed in this electrolyte solution.

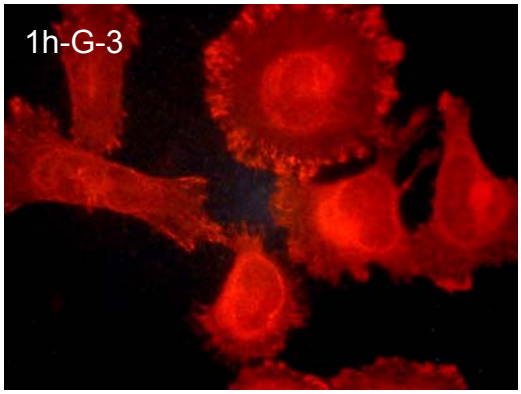
Because vinculin was stained with fluorescent dyes, focal contacts could be displayed clearly. After 1 and 2 hours, lots of focal contacts were revealed on cp Ti, while cells on G-4, G-7 and G-8 developed the fewest number of contacts. The formation and development of focal contacts are probably affected by surface morphology. More irregular pores on these groups may lead to the observed decreased formation of focal contacts.

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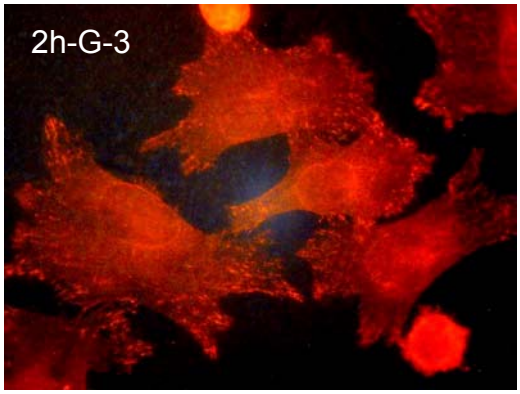
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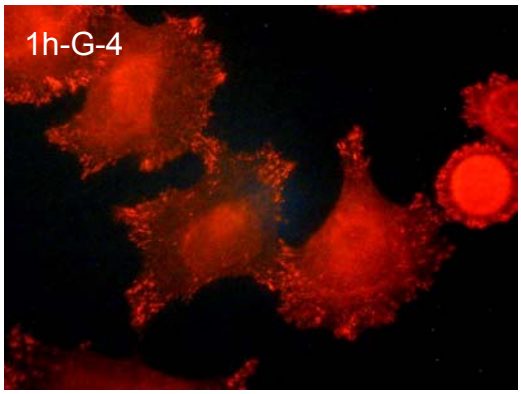
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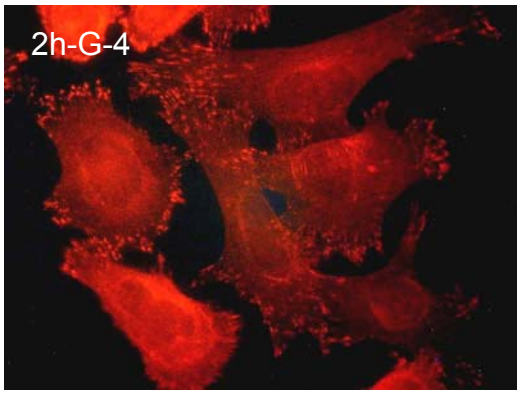
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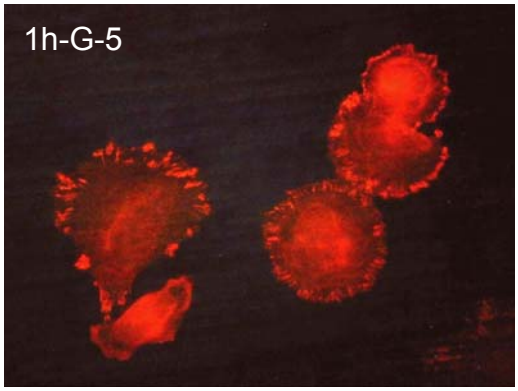
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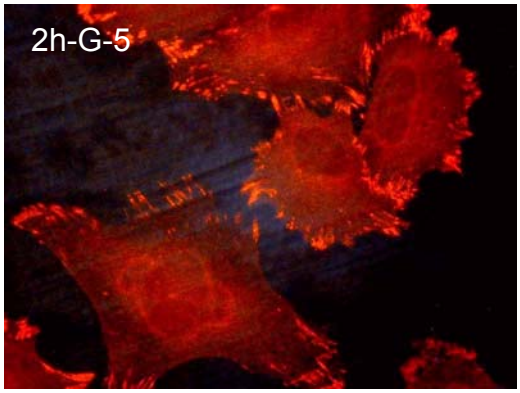
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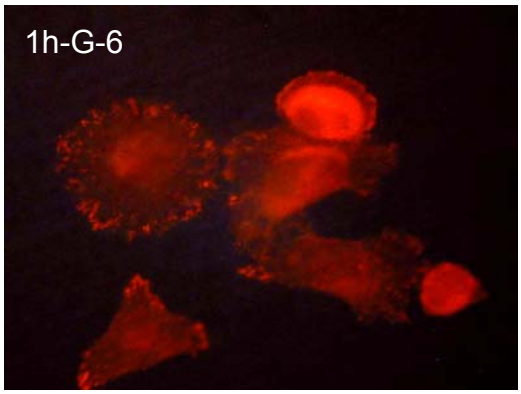
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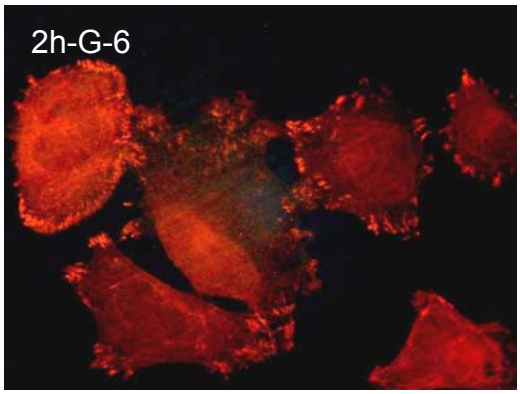
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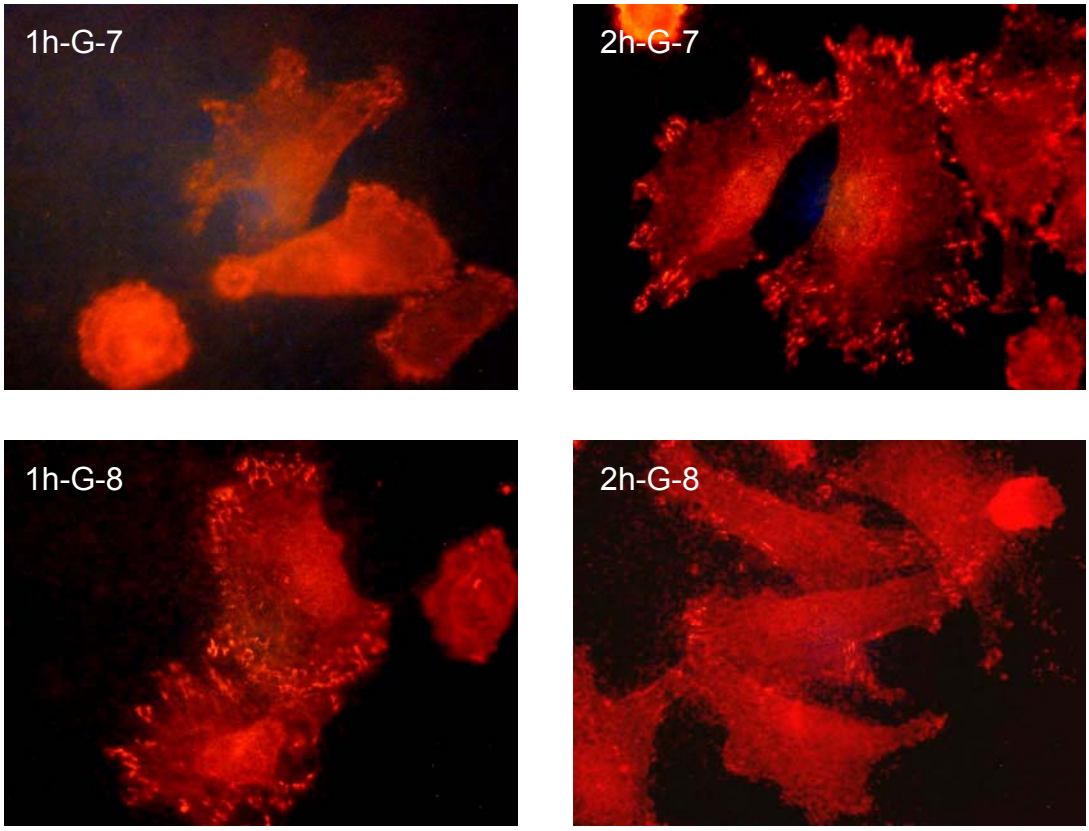



Fig. 12. Focal contacts with anti-vinculin staining were observed under fluorescence microscopy with 400 fold magnification: photos 1h-G-1 to 8 showed the result after 1 hour of cell culture; while photos 2h-G-1 to 8 indicated 2 hours of cell culture.

## 5.4 Cytoskeleton organization

With the multiple exposure technique, actin cytoskeleton and focal contacts can be demonstrated in a same picture (Fig. 13-1, 2). The photographs show green long bundles of stress fibres, assembled by actin filaments, while the orange drops indicate focal contacts. It can be observed clearly from the pictures that stress fibres terminated on focal contacts. Cellular morphology is obviously affected by the distribution of stress fibres: when stress fibres are circumferential around the nucleus or radiate from nucleus to edge, the cells display a round shape; when stress fibres are across the whole cell, cellular morphology shows polarity or a polygonal form.

As shown in Fig.14, after 1 hour of cell culture, the actin cytoskeleton in most of the cells on the surface of cp Ti was more circumferentially organized, showed no stress fibers, and less lamellipodia were formed. The lamellipodia stain was more intense along the perimeters of the cells, probably corresponding to the active polymerization or elongation of actin filament. The circumferential orientation of actin cytoskeleton was also visible on the surface of G-5. On the contrary especially, on the surfaces of G-2, G-4 and G-8, actin filaments were assembled to form stress fibers besides the circumferential actin cytoskeleton, and finger-like filopodia of the plasma membrane were developed. After 2 hours, on all surfaces much more stress fibres with intense staining could be observed than at 1 hour, hence the organisation of stress fibres is obviously time-dependent and not yet finished at the initial cell culture period (1 h).

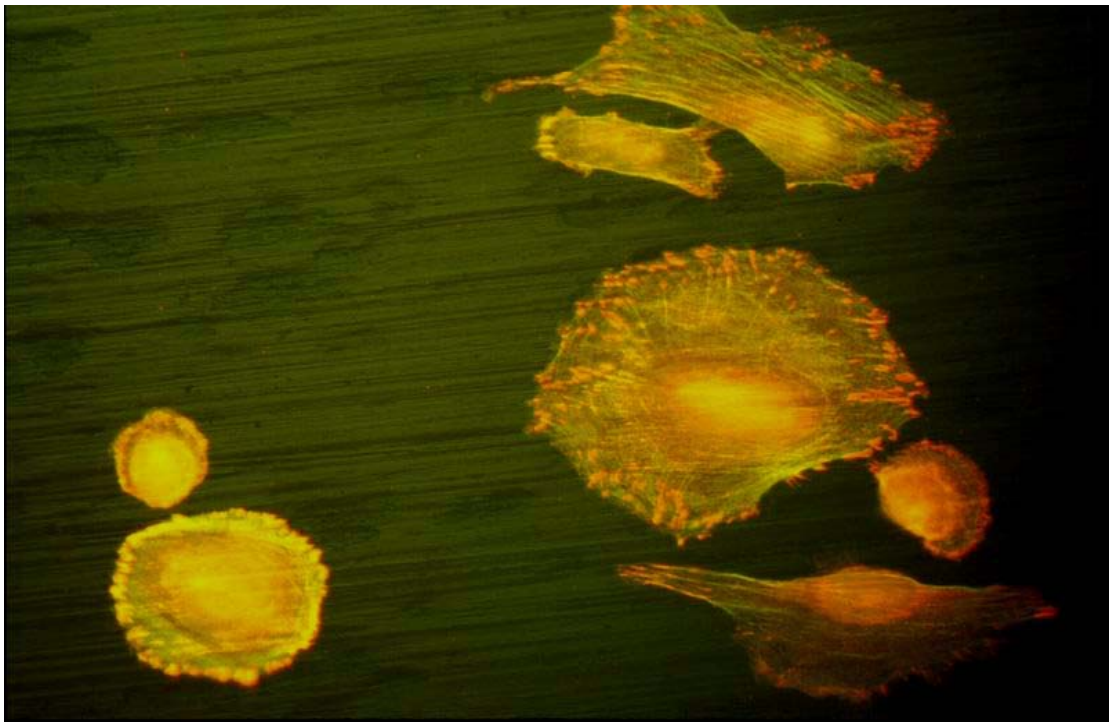


Fig. 13-1 Cytoskeleton stained with phalloidin (Green) and focal contacts (Red) after 2 hours cell culture on cp Ti surface, 400 fold magnification.

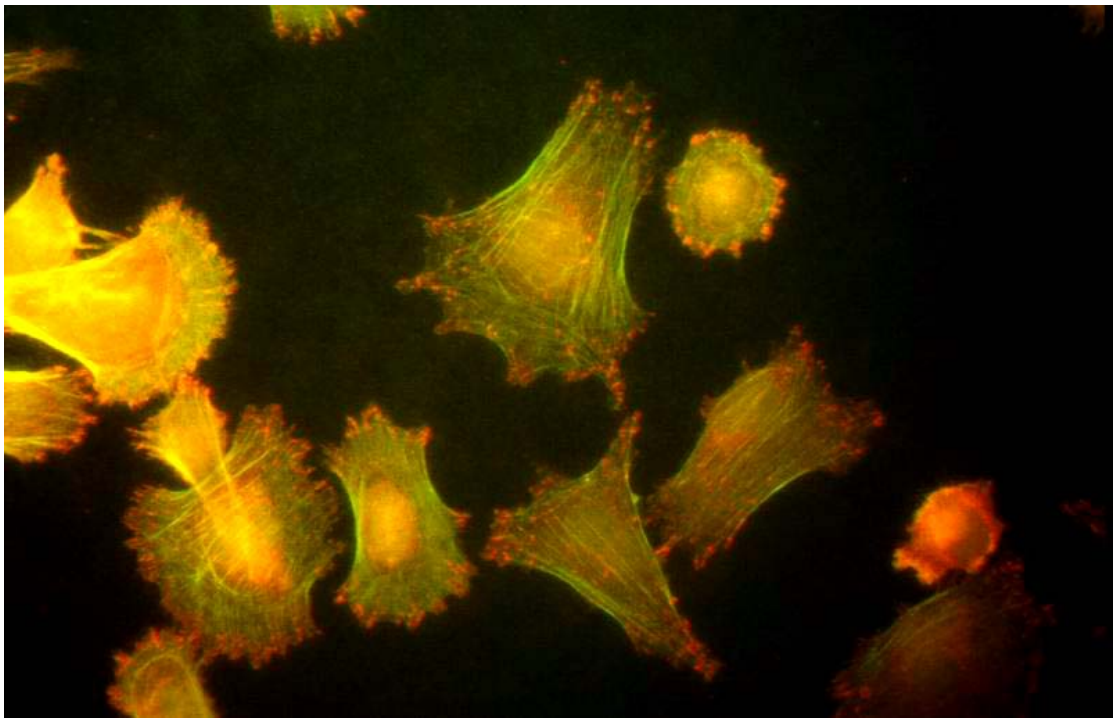
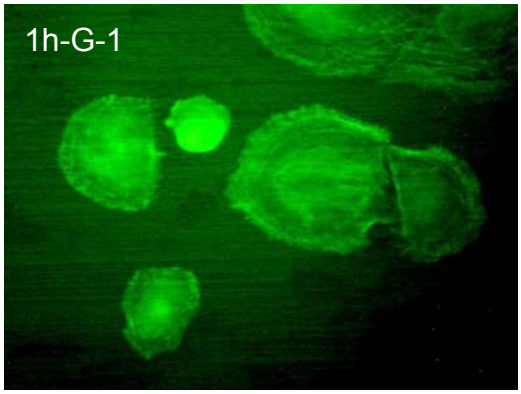


Fig. 13-2 Cytoskeleton stained with phalloidin (Green) and focal contacts (Red) after 2 hours cell culture on G-4 surface, 400 fold magnification.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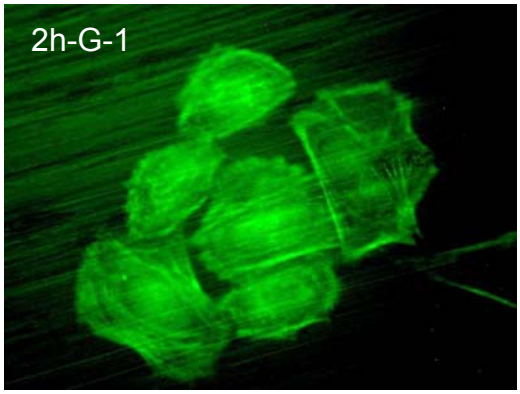
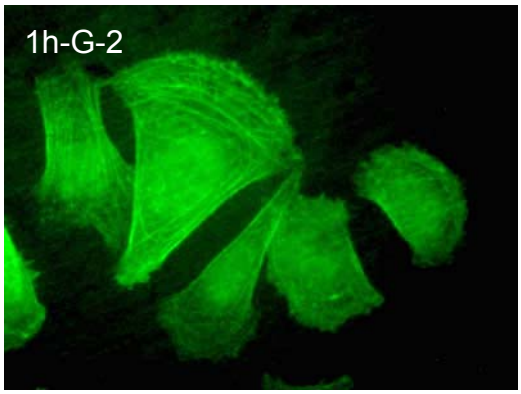

2h-G-1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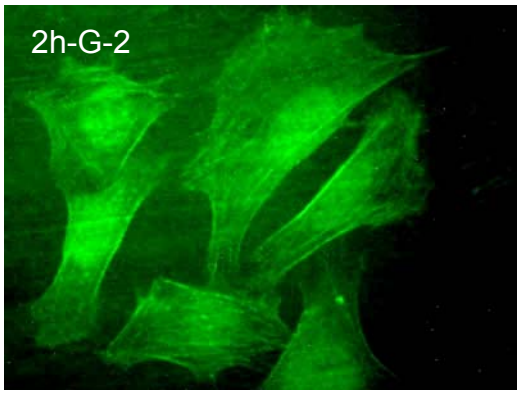
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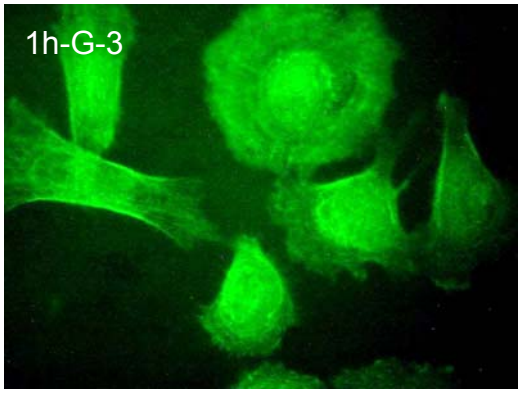
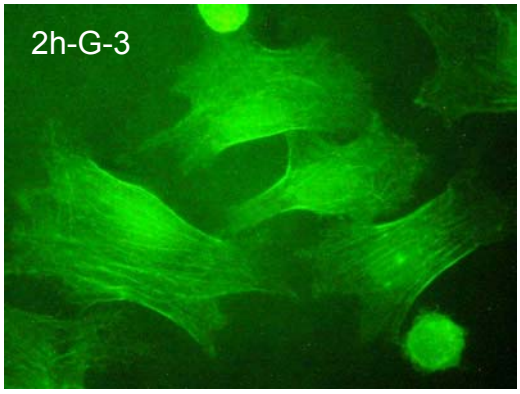

1h-G-3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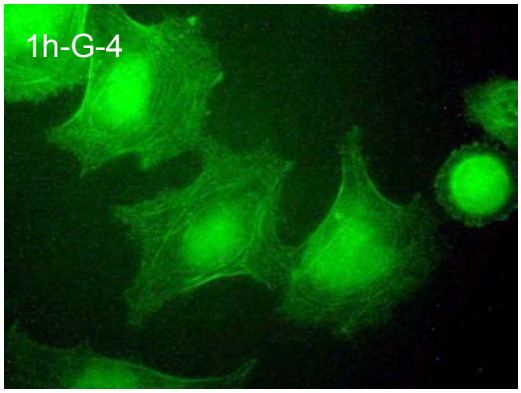
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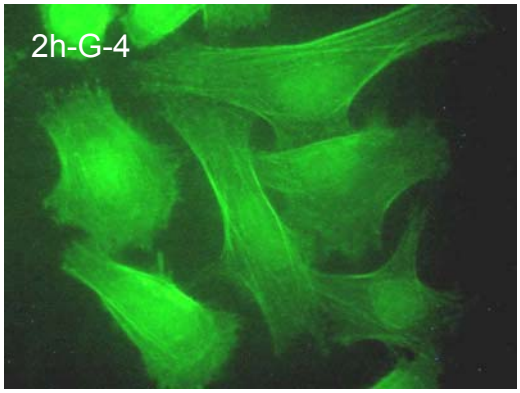
2h-G-4

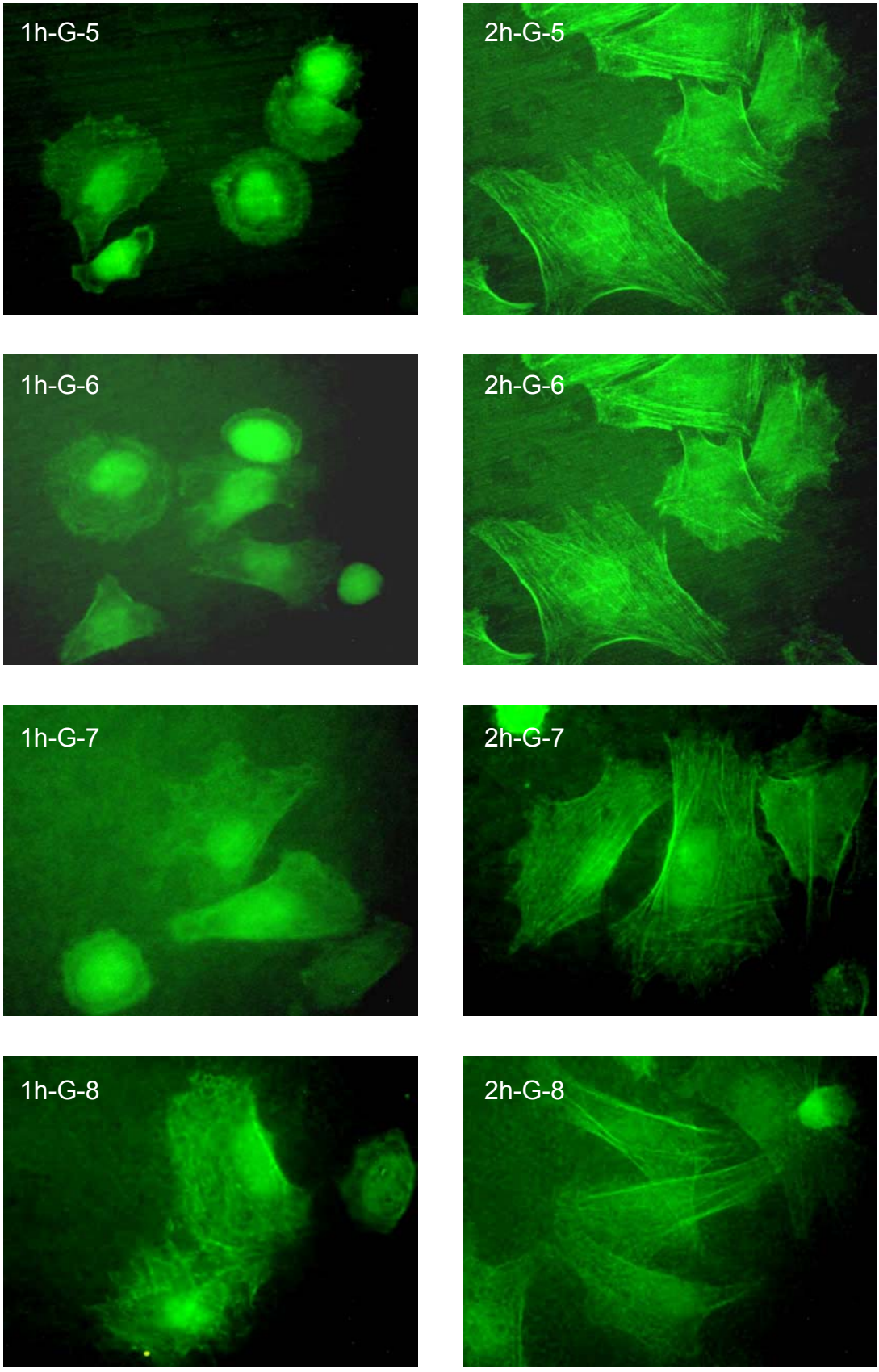


Fig. 14. Photos 1h-G-1 to 8 show actin cytoskeleton stained with phalloidin after 1 hour of cell culture; while photos 2h-G-1 to 8 demonstrated 2 hours of cell culture. With 400 fold magnification.

## 5.5 Cell proliferation (determination of cell numbers)

As shown in Fig 15, there was no significant difference between cp Ti and anodized Ti surfaces concerning cell proliferation after 1 day of culture. After 2 days, cell number on G-7 was significantly higher than on cp Ti. At day 4, higher cell proliferation was shown on almost all of the anodized surfaces except for G-2 and G-5 compared to that on cp Ti.

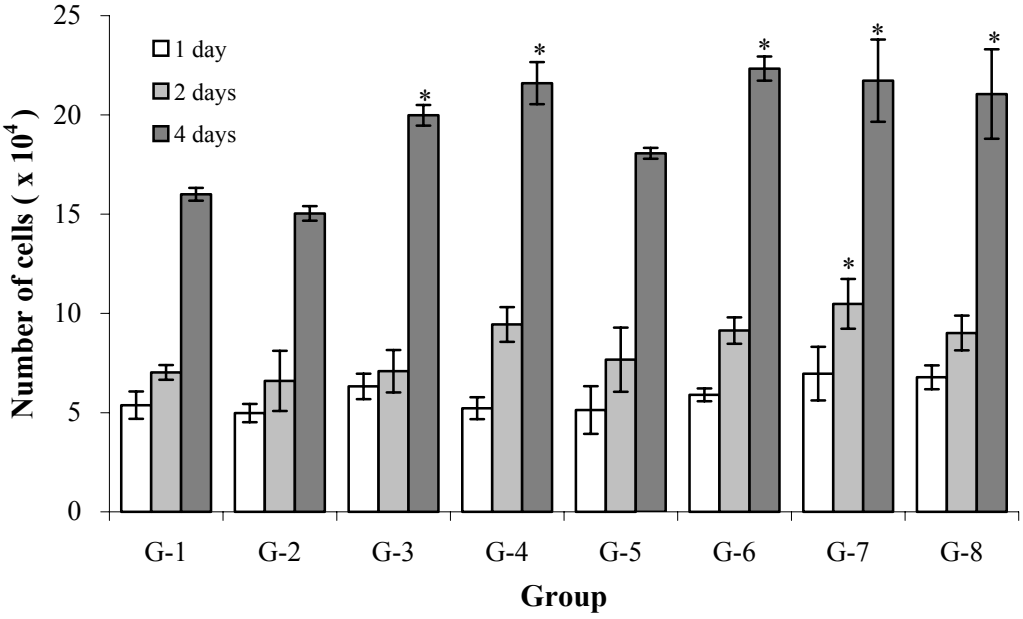


Fig. 15. Proliferation of osteoblast-like SaOS-2 cells on the different surface modifications of titanium after 1, 2 and 4 days cell culture, G-1 as control, *p <0.05.

Table 12. Mean cell counts after 1, 2 or 4 days cell culture (× $10^4$), Mean ± SD.

| | 1 day | 2 days | 4 days |
|---|---|---|---|
| **G-1** | 5.38 ± 0.69 | 7.03 ± 0.37 | 16.00 ± 0.32 |
| **G-2** | 4.98 ± 0.46 | 6.60 ± 1.51 | 15.03 ±0.37 |
| **G-3** | 6.33 ± 0.64 | 7.09 ± 1.07 | 19.98 ± 0.52 |
| **G-4** | 5.23 ± 0.55 | 9.44 ± 0.87 | 21.60 ± 1.06 |
| **G-5** | 5.13 ± 1.20 | 7.67 ± 1.61 | 18.06 ± 0.27 |
| **G-6** | 5.90 ± 0.32 | 9.14 ± 0.66 | 22.34 ± 0.61 |
| **G-7** | 6.97 ± 1.35 | 10.48 ± 1.25 | 21.73 ± 2.07 |
| **G-8** | 6.78 ± 0.60 | 9.01 ± 0.87 | 21.05 ± 2.25 |

Table 13

Comparisons for all pairs in the same electrolyte using Tukey-Kramer HSD (Abs (Dif)-LSD) (Day 1)

| | G-2 | G-3 | G-4 | | G-5 | G-6 | G-7 | G-8 |
|---|---|---|---|---|---|---|---|---|
| G-2 | -1.3935 | -0.0501 | -1.1435 | G-5 | -2.5235 | -1.7601 | -0.6901 | -0.8735 |
| G-3 | -0.0501 | -1.3935 | -0.3001 | G-6 | -1.7601 | -2.5235 | -1.4535 | -1.6368 |
| G-4 | -1.1435 | -0.3001 | -1.3935 | G-7 | -0.6901 | -1.4535 | -2.5235 | -2.3401 |
| | | | | G-8 | -0.8735 | -1.6368 | -2.3401 | -2.5235 |

[+] Positive values show pairs of means that are significantly different (Alpha = 0.05).

Table 14

Comparisons for all pairs in the same electrolyte using Tukey-Kramer HSD (Abs (Dif)-LSD) (Day 2)

| | G-2 | G-3 | G-4 | | G-5 | G-6 | G-7 | G-8 |
|---|---|---|---|---|---|---|---|---|
| G-2 | -2.9679 | -2.4779 | -0.1246 | G-5 | -3.0317 | -1.5651 | -0.2184 | -1.6884 |
| G-3 | -2.4779 | -2.9679 | -0.6146 | G-6 | -1.5651 | -3.0317 | -1.6851 | -2.9084 |
| G-4 | -0.1246 | -0.6146 | -2.9679 | G-7 | -0.2184 | -1.6851 | -3.0317 | -1.5617 |
| | | | | G-8 | -1.6884 | -2.9084 | -1.5617 | -3.0317 |

[+] Positive values show pairs of means that are significantly different (Alpha = 0.05).

Table 15

Comparisons for all pairs in the same electrolyte using Tukey-Kramer HSD (Abs (Dif)-LSD) (Day 4)

| | G-2 | G-3 | G-4 | | G-5 | G-6 | G-7 | G-8 |
|---|---|---|---|---|---|---|---|---|
| G-2 | -1.9786 | 2.7395[+] | 4.5914[+] | G-5 | -4.0985 | 0.1815[+] | -0.4285 | -1.1018 |
| G-3 | 2.7395[+] | -2.4233 | -0.5938 | G-6 | 0.1815[+] | -4.0985 | -3.4885 | -2.8152 |
| G-4 | 4.5914[+] | -0.5938 | -1.9786 | G-7 | -0.4285 | -3.4885 | -4.0985 | -3.4252 |
| | | | | G-8 | -1.1018 | -2.8152 | -3.4252 | -4.0985 |

[+] Positive values show pairs of means that are significantly different (Alpha = 0.05).

## 5.6 Alkaline phosphatase (ALP) activity

No significant difference of ALP activity was found between cp Ti and anodized surfaces after cell culture for 1 or 2 days (Fig.16). At day 4, the ALP activity of cells on G-6 was statistically lower than on cp Ti and other anodized surfaces. With the increase of culturing time, generally the ALP activity decreased. This may be attributed to a preponderance of the proliferation of cells over the production of ALP activity.

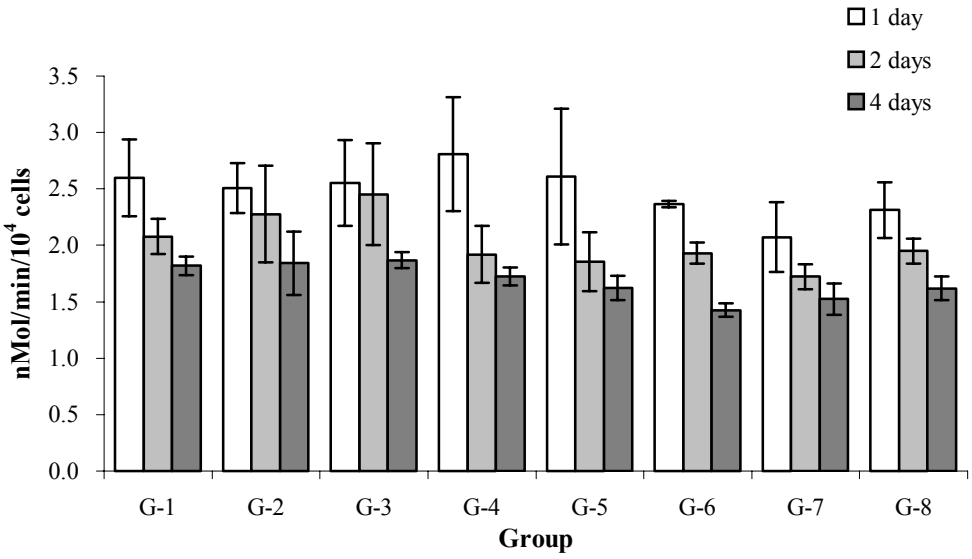


Fig. 16. Alkaline phosphatase (ALP) activity of cells on different surface modifications of titanium after 1, 2 or 4 days. *p <0.05.

# 6. Discussion

## 6.1 Experimental design

In order to improve biocompatibility of implants or facilitate the healing procedure and bone reconstruction around inserted implants, a couple of techniques have been used recently to modify the surfaces of titanium implants. In some current studies, anodic oxidation of pretreated commercially pure titanium was investigated. Although anodized titanium implants have been already investigated *in vivo*, the basic interactions of osteoblastic cells with anodized titanium surfaces were not well known. The main aim of the current study was to reveal the responses of osteoblast-like cells to anodized titanium with different surface properties and to find out the most suitable anodizing conditions.

After an implant is inserted into the bone tissue, osteoblasts secrete new bone matrix and differentiated into osteocytes to reconstruct new bone during the wound healing procedure and subsequent osseointegration between implant and bone tissue. Therefore, osteoblasts are normally chosen to investigate biocompatibility of biomaterials *in vitro*. Both the quantity and quality of cell attachment to implant surfaces at the initial contact stage are regarded as very important for the following cell processes, such as cell spreading, cell migration, cell proliferation and cell differentiation. In this work, cell culture of a human osteoblast-like cell line (SaOS-2) was performed *in vitro* on surfaces of anodized and non-anodized titanium. To investigate basic osteoblast reactions on these surfaces, cytotoxicity of the tested biomaterials, initial cell attachment and spreading, cell morphology, cytoskeleton organization, cell proliferation and

alkaline phosphatase (ALP) activity were determined.

In our studies, we had to estimate the differences between the test groups and the control group, as well as the differences between each two related groups. Therefore, the JMP Version 5.01 statistical analysis program was used to analyse all data. Because Dunnett's test can guard against the high alpha size (type I) error rate across the hypothesis tests, it was adopted to make a statistics between all the anodizing groups and the control. While Tukey-Kramer HSD (honestly significant difference) test, which was used to perform multiple comparisons between the groups anodized in the same electrolyte, provides a conservative calculation of statistical significance in the analysis of intergroup comparisons that minimizes the risk of type I error by increasing the quantile multiplied into the standard error values to create the least significant difference.

## 6.2 Cytotoxicity assay

Cytotoxicity of extracts from specimens were assayed prior the other further biocompatibility tests. The incubation time and the ratio of extracts to medium are important factors in cytotoxicity tests of extracts. Although different ratios of 1 $cm^2$/ml [107] and 2 $cm^2$/ml [31] were performed to prepare material extracts by some authors, according to the ISO 10993-5 the ratio of 3 $cm^2$/ml and incubation for 72 hour at 37□ were adopted in the present study. The results of extracts cytotoxicity test indicated that anodizing procedure didn’t damage osteoblast cellular viability and metabolism compared with the negative control, while positive control with different concentrations displayed significant toxicity. It was therefore concluded that the procedures used for anodic oxidation in this study could be used safely to modify and improve the surface properties of commercially pure titanium.

## 6.3 Cell attachment and spreading

Initial cell adhesion to a biomaterial surface is one of the key processes determining the host response to implanted biomaterials. Osteoblasts contact the surface of implanted biomaterials in relatively short time after biomaterials are implanted into the host. The process of adhesion of cells to the substrate involves multiple steps: (1) adsorption of serum proteins on the substrate; (2) contact of cells with the substrate; (3) attachment of the cells to the substrate; and (4) spreading of the cells on the substrate [85]. Proteins and other adsorbable macromolecules, e.g. from human blood system, which are also present in serum-supplemented cell culture medium, are immediately adsorbed onto the exposed materials' surfaces. These adsorbed proteins and macromolecules play critical roles in guiding the followed cell attachment and spreading. E.g. fibronectin and vitronectin have been shown to be involved in cell attachment and spreading of human osteoblast-like cells, SaOS-2, on titanium surfaces [26].

Adsorption of proteins to biomaterials' surfaces and, subsequently, cell attachment may be altered as the surface properties are modified. In the present attachment tests, significantly more cells attached on nearly all anodized titanium surfaces than on the cp Ti surface after 1 hour or 2 hours of cell culture. This could be caused either by a direct influence of the altered surface properties on the cells, or mediated by an indirect effect, i.e., modification of the adsorbing proteins as mentioned above. On the other hand, there was a tendency, that the number of attached cells on the surfaces anodized in the same kind of electrolyte increased with the enhancement of the anodizing voltage. This can not be due to changes in chemical compositions of the surfaces, as discussed above, since all groups of surfaces treated in the same electrolyte have a comparable surface chemistry (Table 5). It is possible,

that these effects are caused by differences in surface morphology. It has been reported that roughness can affect cell attachment and spreading on biomaterial surfaces. However, so far, contradictory effects have been demonstrated, i.e., some authors found that roughness could increase or ease cell attachment and spreading [3,14,56,59,108]; while the results of others indicated that roughness decreased cell adhesion [13,109]. In the present study, the results of cell attachment shown that for both electrolytes cell attachment increased as anodizing voltage increased. However, the increasing voltage altered roughness of specimens anodized in the both electrolytes in a different way: roughness of the specimens prepared in 0.2 M $H_3PO_4$ was decreased with the enhancement of anodizing voltage, and the inverse was observed for specimens prepared in 0.03 M Ca-GP and 0.15 M CA (Fig 5). Thus, based on the present experiments, it is difficult to draw a conclusion whether roughness alone will enhance cell adhesion or reduce it. However, it was evident that roughness can alter cell adhesion onto anodized titanium surfaces, but other properties of the oxide surface obviously also play a role in the enhancement of cell attachment on the anodized titanium surfaces, cooperating with the effects from roughness.

The next important cellular process following attachment of cells to the biomaterials' surface is cell spreading. During cell spreading, special adhesion structures between cell and substratum are formed, and the cellular skeleton is reorganized to change and maintain the shape of cell. Cell membrane receptors, e.g. integrin family, and many kinds of cytoskeleton proteins (vinculin, talin, paxillin) are involved in this process [110]. Two types of adhesions between cell and culture substrates have been observed, close contacts and focal adhesions, the latter also being named as focal contacts or adhesion plaques. Focal contacts are the areas of tightest adhesion with a distance of 10-15 nm,

whereas close contacts range from 30 to 100 nm [111]. Vinculin and other cytoskeleton proteins locate at the cytoplasmic face of focal contacts, and appear to be involved in linking actin filaments to the membrane at these sites [84]. In the present study, focal contacts were stained by immuno-histochemical methods with mouse anti-human-vinculin antibody and TRICT (tetramethylrhodamine isothiocyanate) conjugated anti-mounse-IgG. There were no significant differences in the percentage of fully spread cells to attached cells between the anodized groups and cp Ti. However, the morphologies of cells were markedly different between the groups. Most of the spreading cells on the cp Ti surface were round and flattened, while cells on the anodized titanium surfaces appeared more irregular and polygonal, especially on the group 4, 7 and 8. Focal contacts on the cp Ti surface showed more intensive staining than on the anodized groups. Also, the contacts were concentrated circular along the rim of cells. The distribution of focal contacts on the surfaces with different roughness has been reported that: on smooth surfaces, focal contacts were distributed uniformly on all the membrane surface which was in contact with the substratum; while on rough surfaces, focal contacts were visible only at the extremities of cell extensions where cell membranes were in contact with the substrate [59,112]. The different spreading behavior of SaOS-2 on the non-anodized and anodized groups may due to the different roughness and topography of oxide films.

It has been observed that MG63 cells migrated into the pits of rough titanium disk surfaces [93]. In the present study, different topographies of the oxides were found between the specimens, which were anodized in two different electrolytes. The increase of the anodizing voltage resulted in the gradual formation of the anodic oxide film, i.e. from partially to completely covering the whole surface, and more and larger irregular micro-pores generated by

sparking during anodic oxidation. The surface topography of samples anodized in 0.2 M $H_3PO_4$ was distinct from that in the other electrolyte, 0.03 M Ca-GP and 0.15 M CA. Different sizes of the pore geometry were formed on the titanium surfaces in electrolyte 1 and in electrolyte 2. Diameter of pores on surfaces of group 4 was ca. 0.5 μm, while the diameter of pores on the group 8 was ca. 2μm. Thus, a hypothesis may be set up that the small size pore geometry formed during anodic oxidation may produce forces resistant to the tendency of cell spreading, when cells extend on the anodized titanium surfaces. Because of the uneven distribution of pores and peaks, the surface may exert different amounts of resistance against the spreading of the cell. This kind of asymmetric extension may further result in more cells with irregular and polygonal shapes on the anodized groups than on cp Ti.

After implants are inserted into the host, osteoblasts should move from bone tissue on to the surface of implants to create new bone tissue and reach osseointegration. It may also promote the osseointegration process, if osteoblasts readily migrate across the implant surface to reach a uniform distribution rapidly. In general, cell migration is dependent on cell-substrate adhesiveness and cells that form strong focal adhesions are less migratory [113-115]. Osteoblast population migration characteristics on substrates modified with immobilized adhesive peptides have been quantitatively estimated by an under-agarose cell migration assay. It was indicated that osteoblast population haptokinesis was significantly decreased on substrates modified with adhesive peptides [116]. In our experiments, the shallow appearance of focal contacts on the more intensively anodized groups may indicate that cell migration on the anodized titanium surface was more facilitated than on the cp Ti surface. Much more filopodia and lamellipodial involved in cell migration were observed on the more intensively anodized titanium than on the

cp Ti. This phenomenon may also be used to support the postulate that anodic oxidation maybe improve cell migration, which is desirable for osteoblasts to migrate onto the newly inserted implant surfaces at the bone-implant interface.

## 6.4 Cell proliferation and differentiation

Degasne I, et al. investigated the effects of roughness on attachment, spreading and proliferation of human osteoblast-like cells (SaOS-2) on titanium surfaces, and the results showed that proliferation of cells in culture on smooth disks was significantly reduced than when cultured on rough disks [26]. Mustafa K, et al. studied the response of cells derived from human mandibular alveolar bone to titanium blasted with $TiO_2$ particles of different grain size (63-300 μm), and concluded that the proliferation and differentiation of cells in that cellular model was enhanced by surface roughness of the titanium implant, but the enhancement was not strictly correlated with the increasing of roughness [13]. This result agreed with other authors [14]. However, Martin JY, et al., reported the contrary results with the using of the other osteoblast-like cell (MG 63) [93]. Our studies revealed a proliferation stimulating effect from rougher surfaces in cultures of the human osteoblast-like cell line SaOS-2. The results of the proliferation experiments corresponded with initial cell attachment on the materials' surfaces. More polar cells attached on the anodized titanium surfaces were observed than on the cp Ti surfaces.

In our studies, proliferation enhanced only after longer cell culture period. After 1 d cell culture, there was no statistic difference between test groups and control group (cp Ti). It was found, that only one group, G-7, significantly differed from cp Ti after 2 day. However, at day 4, higher cell proliferation was shown on almost all of the anodized surfaces except for G-2 and G-5 compared to that on

cp Ti.

Alkline phophatase activity was often used as an early marker of osteoblasts differentiation [13,69,93]. Martin et al. reported that roughness inhibited differentiation of MG63 cells, but some other authors demonstrated the contrary results [93,117]. In the current study, differentiation of human osteoblast-like cells was investigated after 1, 2 and 4 days culture. It was found, that alkline phosphatase activity decreased constantly with culture time. At all time points, no significant difference between anodized groups and cp Ti was found. Thus, no apparent correlation between osteoblasts differentiation and roughness or other surface properties was found. This may be due to the dominance of proliferation during the early cell culture period. Longer cell culture time will be needed to determine the effect of anodic oxidation to cell differentiation.

# 7. Conclusion

In this study, the effects of different procedures for anodic oxidation of titanium surfaces on cell response of osteoblasts were investigated. Grade 2 commercially pure titanium specimens with 20×10×1 mm in size were anodized in two electrolytes with different compositions: 1) 0.2 M $H_3PO_4$ and 2) 0.03 M Ca-GP and 0.15 M CA. In electrolyte 1, 200V, 300V, and 350V anodizing voltage were used; 140V, 200V, 260V, and 300V were adopted in electrolyte 2. Because of the different anodizing conditions, various oxide layers with different surface properties and chemical compositions were formed. Biocompatibility of these anodized titanium surfaces was evaluated with cultures of the osteoblast-like cell line SaOS-2. Metabolic activity, cell attachment and spreading, cell morphology, cell proliferation and differentiation were investigated. According to the results, it can be concluded that:

1. The anodic oxidation techniques adopted in the present study were not toxic;
2. Cell attachment on anodized titanium surfaces was enhanced after 1 and 2 h cell culture;
3. The different expression of focal contacts suggests, that cell migration on anodized surfaces may be also facilitated;
4. Cell proliferation was enhanced on surfaces anodized with higher anodizing voltage, while no significant difference of cell differentiation was found between anodized titanium and cp Ti;
5. Detailed comparison of osteoblast response between experimental groups treated in the same electrolyte revealed a tendency to more favorable cell responses on surfaces anodized with higher voltages at the

longer time intervals tested (2h for cell attachment und 4 days for cell proliferation, respectively).

In summary, the anodic oxidation techniques investigated here could be safely used to modify the titanium surface, and *in vitro* cell attachment and proliferation were enhanced by anodic oxidation.

Although the biocompatibility of anodic oxidation of titanium was investigated from several aspects, there are still some questions, which should be addressed in further studies:

1. Longer-term tests for formation of extra cellular matrix (ECM) and calcification should be conducted.
2. Differentiation between effects caused by morphological or chemical surface properties on responses of osteoblasts should be profoundly investigated.
3. Studies of basic cellular mechanisms involving receptors of the cell membrane and signal transduction pathways should be performed.
4. In the present study, only *in vitro* experiments were conducted to test the responses of osteoblast-like cells to anodized titanium. Before any new biomaterial is used in the human body, *in vivo* experiments should be performed, because of the much more complicated conditions in the body. So, in future, further studies at the molecular level should be done to identify basic cell/biomaterial interactions, and an animal model should also be constructed.

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118. Effects of topography and composition of titanium surface oxides on osteoblast responses. X. Zhu, J. Chen, L. Scheideler, R. Reichl, and J. Geis-Gerstorfer. Biomaterials 25 (2004): 4087-4103.

## Publications

Publications based on the present study:


1. X. Zhu, J. Chen, L. Scheideler, C. Schille, and J. Geis-Gerstorfer. In vitro Osteoblast Responses to Anodic Oxides Containing Ca and P on Titanium. J Dent Res 82 (Spec Iss B) 2003, B-308, 2379. (Abstract)

2. X. Zhu, J. Chen, L. Scheideler, R. Reichl, J. Geis-Gerstorfer. Effects of topography and composition of titanium surface oxides on osteoblast responses. Biomaterials 2004 (25): 4087-4103.

3. X. Zhu, J. Chen, L. Scheideler, T. Altebaeumer, J. Geis-Gerstorfer, D. Kernb. Cellular Reactions of Osteoblasts to Micron- and Submicron-Scale Porous Structures of Titanium Surfaces. Cells Tissues Organs 2004; 178: 13-22.

# Acknowledgments

This work was carried out at the Section of Medical Materials & Technology, Department of Prosthodontics, Clinic of Dentistry and Oral Medicine, University Tuebingen from 2002 – 2004. During the whole phase of my living, studying and working in this beautiful university-city, Tuebingen, I have got so many helps from people who have lent aids to me. I owe my deepest gratitude to all these persons:

I am grateful to my supervisor, Prof. Dr. Juergen Geis-Gerstorfer for the excellent design and guidance of the present study, thank for the support in all the topics of my works and my life. I gratefully acknowledge Prof. Dr. Heiner Weber for the acceptance as a doctor student in his department.

I owe my gratitude to Dr. Zhu Xiaolong for good co-operations. All cp Ti and anodized titanium specimens and the data of their surface properties used in this study were friendly provided by Dr. Zhu. I wish to thank Dr. Lutz Scheideler, for his introducing me to the world of microbiology and correcting for my dissertation. His friendly aids to my language skills and my life in Tuebingen must be appreciated as well.

I appreciate all colleagues in our laboratory, Evi, Conny, Aida, Tanja, Shila and Michael for their selfless aids in improving my experiment and language skills. I thank the whole staff of our department and all guest doctors.

I owe my gratitude to all my friends, special to Prof. Dr. Herwig Brunner, Mr. Norbert Stroh and his families.

I am deeply grateful to my parents, my wife and my brothers for their love and supports of all aspects.

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