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BONE-IMPLANT INTERFACE. EVALUATION OF OSTEOBLASTIC CELLS BEHAVIOUR ON NANOPATTERNED TITANIUM SURFACES: AN IN VITRO ANALYSIS.

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Introduction

The replacement of missing teeth by means of endosseous titanium implants has become an evidence-based treatment modality for both completely and partially edentulous patients, (Jemt, Chai et al. 1996, Lindquist, Carlsson et al. 1996, Buser, Mericske-Stern et al. 1997, Lambrecht, Filippi et al. 2003). The benefit of better mastication provided by implant-supported prostheses improves the quality of life and has an important impact on the overall health of the patient. Short- and long-term success of this implant-supported dental prosthetic treatments depend on a multitude of factors, which are related to the host general and local health's conditions and to the biological and mechanical characteristics of the biomaterial which constitutes the implants. Notably, bone quantity and quality are pivotal factors in determining the eligibility of each patient for the implant therapy. Moreover, surgical technique, type of dental implants adopted - including material, shape and chemical and topographical surface characteristics - and mechanical load conditions at the implant site represent key factors influencing a dental implant treatment plan, (Variola, Brunski et al. 2011).

For these reasons, dental implants are generally limited to patients with ''good bone quality'', leaving out a large segment of the population that has lost supporting jaw bone with age or due to medically compromised conditions, such as chronic diseases or chromosomical and genetical syndromes affecting bone microstructure as well as bone remodelling rates.

Treatment outcomes have become highly predictable in healthy patients, with success rates of 95.9–97.9% reported after 10 years (Priest 1999, Ferrigno, Laureti et al. 2002, Karoussis, Salvi et al. 2003, Rasmusson, Roos et al. 2005, Schwartz-Arad, Kidron et al. 2005, Blanes, Bernard et al. 2007). Endosseous implants preserve the adjacent teeth, offer good functional and aesthetic outcomes, and prevent disuse atrophy of the alveolar

bone (Isidor 2006). However, the therapeutic use of dental implants is often contraindicated (Smith, Berger et al. 1992) in medically compromised patients (MCP).

In patients with chromosomical and/or genetical syndromes, multiple systemic diseases i.e., congenital heart diseases, as well as diabetes mellitus, deficit of the immune or muscle-skeletal systems, etc. are often associated. The prosthetic rehabilitation supported by endosseous dental implants present several contraindications and the elected treatment plan rather includes the use of removable or fixed partial dentures on natural teeth. Nevertheless, in these patients, the lack of single or multiple dental elements is often observed, generally due to congenital agenesis, (*i.e.* in Down's Syndrome patients), (Russell and Kjaer 1995, de Moraes, de Moraes et al. 2007)**,** or to traumatic injuries. Hence, contiguous teeth are found intact and in function. In this contest, a dental implant treatment preserving the integrity of the adjacent teeth is particularly desirable. Endosseous implants guarantee a conservative approach towards residual teeth and an improved function and stability of the oral prostheses. Not less importantly, especially for syndromic patients, who are often young adults, the implant treatment may also contribute to a better psychological and social adaptation of the patients towards the oral prosthetic rehabilitation.

Noteworthy, as well as for the general population, patients suffering for genetical and/or chromosomical syndromes have nowadays an improved life expectancy. Down's Syndrome (or Trisomy 21) represents the most frequent chromosomical syndrome, with an incidence of 1 over 800-1000 born alive. Currently, patients affected by Trisomy 21 have a mean life length till the age of 55-65 years, (Siffel, Correa et al. 2004, 2006, Shin, Besser et al. 2009), thanks to the progress achieved in the diagnoses and in the therapy of the associated diseases.

Thus, it is imperative that oral prosthetic treatments, including dental implants, which guarantee optimal masticatory function and long-term durability, may be offered to a larger segment of the population, regardless its general and/or specific anamnesis.

Chapter 1

Analysis of implant-healing related factors

1.1 Osseointegration and biocompatibility

The treatment concept of implant-supported dental prostheses is based on the biologic phenomenon of osseointegration, which indicates a direct structural and functional connection between living bone and the surface of a load-bearing implant. A prerequisite for a successful osseointegration is the establishment of a direct bone-to-implant contact (BIC) without the interposition of any other tissue (Branemark, Adell et al. 1969, Albrektsson 1983, Davies 1998).

In fact, it is well known that the implant surface status is one of the influential factors for osseointegration, thus significant attention has been devoted towards enhancing its biocompatibility and osteoconductivity, (Albrektsson and Wennerberg 2004, Albrektsson and Wennerberg 2004, Buser, Broggini et al. 2004, Butz, Aita et al. 2006). Pure titanium (Ti) and its alloys are considered the best metallic materials for dental implants, since titanium show a high and proved biocompatibility. Biocompatibility is defined as the natural

ability of a biomaterial to accomplish specific biomedical functions, without stimulation of any pathologic immune or tissutal reaction (Brunski, Puleo et al. 2000, Vetrone, Variola et al. 2009). Biocompatibility of a titanium dental implant is mainly related to the initial bone-implant interaction, that takes place immediately after its surgical insertion. It is represented by a complex series of cascade events, closely related to the specific biomaterial properties, both chemical and physical.

Since the implant surface is the first component that directly contacts the host tissue, its biocompatibility and its properties are determinant for the achievement of a rapid and successful osseointegration. Thus, in the attempt to guide and control the oteogenesis events, the implant surface must be considered. Recently, several surface modifications, whether topographical or chemical have been proposed with the aim of enhancing and accelerate the tissues healing responses. However, the exact mechanism by which the implant surface interacts with the surrounding tissues is not yet fully clarified (Anselme and Bigerelle 2006, Liu and Webster 2007, Vetrone, Variola et al. 2009).

Significant progresses have been made in this research field during last decades, in the attempt to achieve shortened and controlled tissues healing responses by means of implant surface modifications which may exert a favourable effect on the osteogenesis events.

1.2 The role of proteins

The first event taking place at the implant surface is the protein adsorption from tissue fluids. It is well documented that protein adsorption onto metal surfaces occurs immediately following implantation of a biomaterial, (Walivaara, Aronsson et al. 1994, Horbett 2003). Irrespective of the biomaterial and its topography, proteins will be attracted from blood at the implant site; the adsorption process takes place spontaneously in the range of milliseconds (Turbill, Beugeling et al. 1996). Yet a little later, cell extensions, cell

membrane and cell receptors, mainly integrins, recognize and bind specific active sites on the proteins molecules, thus initiating the bone formation process (Rupp, Scheideler et al. 2004, Jimbo, Ono et al. 2011). In fact, it has been suggested that protein adsorption play a key role in the osteoconduction stage of osseointegration (Reddi 1985, Park and Davies 2000, Akagawa, Kubo et al. 2009) and that some proteins significantly enhance initial adhesion, growth and differentiation at the bone-implant interface (Jimbo, Sawase et al. 2007). Proteins deriving from plasma, extracellular matrix and bone are dynamic, as each one constantly changes position and orientation, depending on the molecular weight (Vroman and Adams 1969). A mixture of proteins adsorbs onto titanium surfaces and may undergo to conformational changes, denaturation, and/or replacement by the so-called Vroman effect, suggesting that proteins play different roles in the bone-implant healing response. The resulting adsorbed protein layer is probably never static, but subjected to changes in composition and conformational state during the whole healing period, (Kasemo and Gold 1999). It has been reported that the main salivary proteins that adsorb onto titanium in vitro and in vivo are amylase and albumin (Kohavi, Klinger et al. 1995, Steinberg, Klinger et al. 1995). Sela et al. (Sela, Badihi et al. 2007) found that fibronectin is the most adsorbed protein onto differently treated (smooth, etched and etched plus sandblasted) titanium surfaces as compared to other proteins alone, (such as Albumin, IgG, and fibrinogen), or contained in plasma where competing conditions with different proteins are present. Fibronectin*, a* high molecular weight glycoprotein, is one of the earliest cell-binding proteins produced by osteoblasts and is involved in cell adhesion (Proctor 1987).

Fibronectin is reported (Yang, Cavin et al. 2003) to favourably influence the osteoblast cells migration and attachment at the implant site and to act as bone density regulator, (Bentmann, Kawelke et al. 2010, Miyamoto, Lafrenie et al. 1998). Moreover, fibronectin and fibronectin receptors were preferentially found at the osteoblasts-titanium surface

contact sites, (Meyer, Joos et al. 2004). Similarly, but with opposed mechanism, vitronectin has a positive influence on the bone-implant healing, as it reduces the undesirable attachment of epithelial cells on dental implants whereas type IV collagen adsorption should be avoided, as it may help the epithelial cells to attach and grow onto titanium surfaces, negatively affecting the oteogenesis events, (Park, Kim et al. 1998).

1.3 Implant surface modifications

Clinical success of an implant is related to its surface properties. For this reason, a multitude of surface modifications, have been attempted to increase its osteoconductive properties and enhance the biological responses to implants, (Buser, Broggini et al. 2004, Lemons 2004, Ong, Carnes et al. 2004). Commonly utilized modifications of titanium implants are alterations in chemistry and surface texture (Buser, Broggini et al. 2004, Lemons 2004, Ong, Carnes et al. 2004).

In fact, it has been reported that chemical and physical properties of a biomaterial surface, such as chemical composition, curvature, porosity, roughness, energy, etc. may strongly influence the cellular response surrounding dental implants. (Brunski, Puleo et al. 2000, Engler, Bacakova et al. 2004, Anselme and Bigerelle 2006, Vetrone, Variola et al. 2009). Chemical modifications, aiming to impact the surface chemistry, include acid etching (Nanci, Wuest et al.1998, Sandrini, Giordano et al. 2007, Giordano, Sandrini et al. 2006), plasma treatment, (Itala, Ylanen, et al. 2002, Nitschke, Schmack et al. 2002), etc. Plasma techniques provide simple in situ process for chemically tailoring surfaces without compromising the inherent favourable bulk properties of the biomaterial, (Lin, Cheng et al. 2005). Plasma treatments may provide enhanced surface cleaning and decontamination.

Topographical (physical) modifications include mechanical roughening, *(i.e*. polishing, grinding, machining, (Cassinelli, Morra et al. 2003, Xavier, Carvalho et al. 2003), sandblasting, (Anseleme Bigarelle 2006, Guizzardi, Galli et al. 2004) elettro-erosion and

acid etching, which produce surface patterning at the micro- and nano- scales.

Although micro-patterned surfaces can impact the tissue healing and the micromechanical retention of an implant to the surrounding tissues, they may have a minor direct effect on the molecular and cell activities, (Buser, Schenk, 1991, Abrahamsson, Berglundh et al. 2004).

The most correct approach to modify implant surfaces is combining chemical and topographical surface modifications, considering surface chemistry and micro-architecture as important properties that synergistically influence the tissue response, (Kasemo and Gold 1999).

1.4 Impact of nanotopography on the osteogenesis events and nano-patterning techniques

Currently, nan-topography has received increased attention as it has proven to significantly enhance osseointegration (Wennerberg and Albrektsson, 2010). Several studies have shown that nanostructured implant surfaces modifications may improve osteoblast adhesion and spreading in vitro (Raimondo et al. 2010, Gittens et al. 2011) and bone responses in vivo, (Bjursten et al. 2010; Jimbo et al. 2012). The beneficial effects of these alterations may be in part related to their ability to mimic the nanoscale architectural environment in which cells reside. In fact, cell-substrate interactions are mainly governed by nanometric surface cues as proteins, cell membrane receptors and filipodia are in the order of few tenths of nanometer, (Puleo and Nanci 1999, Whitesides 2003, Lord et al. 2006, Curtis et al. 2004, Kriparamanan et al. 2006). These biological entities regulate cell attachment, migration, proliferation and differentiation, (Curtis and Wilkinson 1999, de Oliveira and Nanci 2004).

More recently, efforts have focused on the attempt of realizing new “intelligent” biomaterials for dental implants, such as bioactive titanium surfaces, which may be able to

specifically control the first event taking place at the implant surface: the protein adsorption from tissue fluids.

Ideally, such modified implant surfaces are able to selectively adsorb beneficial molecules for the osteogenesis events. The objective is thus to promote growth and differentiation of osteoblastic cells and inhibit that of other cell lines, as fibroblastic or epithelial cells, which prevent the ideal osseointegration process at the bone-implant interface. Physical and mechanical principles must be taken into account in the attempt to regulate the newly formed tissues at bone-implant interface; however biological principles presiding over this process can't be excluded.

The application of nanotopography to implant surfaces is currently considered core concept of biomimetic engeneering.

Topographical surface modifications at the nano-scale level aim to match the biological entities and produce an enhanced affinity between the substrate and the cells, thus guiding desired and controlled biological reactions, (Curtis and Wilkinson 1999, de Oliveira and Nanci 2004, Kiparamanan et al. 2006, Curtis et al. 2004, Lord et al. 2006).

Nano-modified titanium surfaces may be produced by several methods, *i.e.* photolithography (Flemming, Murphy et al. 1999) electron beam lithography (Kasemo and Gold 1999) chemical oxidation (Nanci, Wuest et al. 1998, de Oliveira and Nanci 2004, de Oliveira, Zalzal et al. 2007), particle deposition (Lipski, Jaquiery et al. 2007, Kasemo and Gold 1999).

The size ranges of interest include the smallest proteins (~1 nm) and the largest cells (<100μm). The smallest feature size obtainable by conventional photolithography is around 0.3μm, while electron beam lithography can produce features down to 10 nm, depending on processing procedures and materials being patterned. The principle of lithography is to covering the surface with a radiation-sensitive film, (usually a polymer), then expose certain areas of the film to a beam of radiation and finally remove these

exposed and modified areas of the film, leaving a film that serves as a mask for following treatment of the remained uncoated areas. The following treatment may be an etching treatment, or deposit of thin film, etc. Photolithography is one of the most popular and conventional techniques from the onset of micro-nano-fabrication as it is a quick method through which many features can be patterned at once (Singh et al. 2013). It uses radiation sources of different wavelengths: when features of small dimensions are being patterned, such as nanometers, radiation sources with wavelengths similar to those of the desired feature sizes must be used. In this technique, the electron beam hits only desired areas of the polymer film. Then, the modified polymer serves as mask for the surface. For example, micropatterned surfaces characterized by micro-cubes can be created by ion-beam-etching the biomaterial surface following its masking with the patterned polymer film, (*i.e.*, a matrix pattern of 5-μm squares). It is possible to vary the cube dimensions and spacing by changing the pattern definition *(i.e.,* the mask) and etching time. Features on this size scale can influence the adsorption of proteins at the surface, for both topographical as well as chemical reasons. One drawback of electron beam lithography is that it is slow and very difficult to execute on non-planar surfaces, due to focusing problems, (Kasemo and Gold 1999)..

A simple chemical method for surface nanopatterning is represented by the acid treatment which uses a solution of equal volumes of sulphuric acid (H_2SO_4) and 30% aqueous hydrogen peroxide (H_2O_2), as proposed by Nanci et al. (1998). This treatment determines de-oxidation and controlled re-oxidation of titanium surfaces and yields a clean surface without contaminants. It generates a reproducible titanium oxide surface layer characterized by nanopitted topography with increased roughness. The produced nano-pits have a size range of 20-100 nm in diameter, depending on the etching time and the volumetric ratio between the acid and the oxidative agent used (Vetrone et al. 2009).

Several studies have already demonstrated how these nano-modified titanium surfaces accelerate the osteogenic potential in vitro: they provide an improved osteoblast adhesion and spreading and upregulate the early expression of bone-marker proteins in osteogenic cell cultures) (de Oliveira and Nanci 2004, de Oliveira, Zalzal et al. 2007, Richert, Vetrone et al. 2008).

Thus, nanoscale surface roughness may play a decisive role in the osteogenesis events because the size of cell adhesion mediating structures matches those of nanotextured surfaces. Although histological evaluations carried out in several studies have proved that nanometer length scale modification effectively enhanced osseointegration, some other studies did not detect the effects of nano-scale modification; hence, further understanding of these delicate alterations are needed (Svanborg, Hoffman et al. 2011, Coelho, Cardaropoli et al. 2009).

Furthermore, nanotopographical surfaces factors have been reported to contribute to differences in the adsorption of proteins. (Jimbo Coelho et al. 2011a, Jimbo, Sotres et al. 2012, Puckett, Taylor et al. 2010, Mac-Donald, Markovic et al. 1998, MacDonald, Rapuano et al. 2004; Eriksson, Nygren et al. 2004).

In particular, Deligianni et al. (2001) found higher amount of Fibronectin adsorbed to rough titanium surfaces than smooth surfaces and demonstrated that both protein adsorption process and cell attachment and differentiation are roughness sensitive. Accordingly, Sela et al. (2007) found significantly higher rate of proteins adsorption to acid-etched and blasted surfaces as compared to smooth ones and attributed this finding to the increased roughness of the former surfaces. However, the interfacial interactions between the nano-structures and the osteogenic cells have not been clarified to a full extent. As said, it is widely believed that the protein adsorption play a critical role on the initial osteoblasts adhesion by the advantageous interaction between nanosize irregularities of the biomaterial surface and adsorbed cell-adhesion mediating molecules.

Moreover, an increased roughness of the substrate surface in the nano-scale presumably allows higher volumes of adsorbed proteins by the increased available surface (Keller, Schneider et al. 2003, Xavier, Carvalho et al. 2003).

Both protein-surface and cell-surface interactions are influenced by the surface micro- and nano-topography. Curvatures, pits and protrusions that have sizes comparable with those of biological components (proteins ~1-10nm, cells 1-100 μm) will induce different biological interactions.

More recently, surface-coating with osteoconductive proteins, growth factors, peptides, etc., have also been investigated (Yoo et al. 2014, Jimbo, Sotres et al. 2012). The surface releases of ions or more complex organic molecules that penetrate the cells membrane or activate cell receptors may strongly stimulate the cell and ultimately the global tissue response. Hence, it is widely believed that intentionally doping implant surfaces with molecules that have a positive effect on the osteogenesis events, may improve and shorten the osseointegration process (Jimbo, Sotres et al. 2012, Rammelt et al. 2006, Hilbig et al. 2007).

In addition, based on the favourable outcomes showed by surfaces with increased roughness, osteoconductive protein-coating has been associated to nanopatterned dental implants, in the attempt to further enhance the effect of adsorbed protein layer (Schwartz-Filho et al. 2012, Schlegel et al. 2013, Lutz et al. 2013, Liu, Enggiest et al. 2007).

A recent study (Yoo et al. 2014) compared the in vivo outcome of implants coated or not with Bone morphogenetic Protein 2 (BMP-2.). However, the major problem reported was the osteogenic protein release rate (Liu, Enggiest et al. 2007, Kempen, Lu et al. 2008). The most of the adsorbed protein was diffused away or competitively replaced by other proteins present at the implantation site within the first hour after implantation in vivo (Leonard and Vroman 1991), thus limiting the enhancing osteogenesis effect at the earlier period. On the other hand, excessive dosage and consequent not controlled, long-term

release of osteogenetic factors may yield undesirable events, such as recruitment and activation of osteoclast cells (Liu, Enggist et al. 2007, Liu, Huse et al. 2007).

1.5 Experimental hypothesis

When a surface comes in contact with the biological environment, the first event that takes place in the range of milliseconds (Webster et al. 2001) is the protein adsorption. The resulting protein adlayer will act as a framework on which cells can adhere, spread and proliferate. Despite numerous studies conducted in the field, there are still questions about the degree of influence that the protein adsorption has on the initial cellular events, particularly when the surface exhibits topography (Nakanishi et al. 2001, Brynda et al. 2005, Kasemo and Gold 1999). Up to date, it still remains to be elucidated whether the cellular effects are solely mediated by the adsorbed protein layer or whether essential cuing may be provided by the physical surface of the biomaterials. Only few studies have tested the outcome of cells under conditions in which there is no initial protein adsorption, (Brynda et al. 2005).

Despite numerous studies in the field, there are still questions about the degree of influence that the protein adsorption has on the initial cellular events, particularly when the surface exhibits topography (Nakanishi et al. 2001, Brynda et al. 2005, Kasemo and Gold 1999).

To investigate this question, we compared the in vitro outcome of osteogenic cells exposed or not during the initial culture phase to serum proteins derived from foetal bovine serum (FBS), which was added to the culture medium. The osteogenic cells were growth onto polished and nanoporous titanium surfaces and glass as standard culture substrate.

The hypothesis is that physical-chemical properties of titanium surfaces without mediation by proteins are sufficient to sustain osteogenic cell culture.

This may not be crucial to achieve optimal implant osteointegration in healthy patients, but may have an important effect in medically compromised individuals in whom the composition of tissue fluids, with their extracellular matrix proteins content, is altered and the initial healing phases may be impaired. Ideally, a specifically modified implant surface should improve clinical implant success, regardless the medical conditions of the patients.

Chapter 2

In vitro study

2.1 Abstract

Statement of the problem:

Protein adsorption occurs immediately following implantation of a biomaterial, and it is widely assumed that this will have an influence on the subsequent cellular response. Despite numerous studies, there are still questions on the degree of influence that this process has on initial cellular events, particularly when the surface exhibits topography.

To investigate this question, it has been compared the osteoblastic cell growth onto polished and nanoporous titanium substrates and glass as control, by modulating the exposure to serum-derived proteins (FBS) during the initial phase of cell culture.

Materials and methods:

Substrates consisted of: 1) commercial grade 2 titanium disks polished to a mirror finish, (Poli-Ti), 2) polished disks nanotextured by treatment with H_2SO_4/H_2O_2 for 2h, (Nano-Ti), and 3) glass coverslips as control, (GC). In the pre-adsorption phase, substrates were treated for 1h with alpha Minimum Essential Medium (αMEM) alone (M-noFBS) or with αMEM supplemented with 10% foetal bovine serum (M-FBS), as serum-derived proteins.

Mouse calvaria-derived osteoblastic cells (MC3T3-EI) were seeded on these pre-treated substrates and cultured for 3h and 24h, in M-noFBS and M-FBS. After this initial seeding period, the culture medium was replaced with MS and cultures were maintained for 3 and 7 days. Cell number was evaluated using Alamar blue and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assays.

Cell activity was evaluated through the fluorescence optical microscope imaging for: cell division activity by immunolabeling for Ki-67 nuclear protein; cell shape at day 1 and 3 by actin staining; osteogenic cell activity by immunolabeling for Osteopontin (OPN) and staining with alkaline phosphatase (ALP), as bone marker proteins.

SEM imaging was also used to evaluate cellular morphology at 3h, at day 1 and at day 3. In an additional experiment, the same substrates (n=24 for each) were treated under same conditions, as described above and then maintained in culture with a special osteogenic media for 30 days. At day 30 the osteogenic cell activity was determined through the quantitative evaluation of cell mineral production by staining with Alizarin Red. Data were analysed using the ANOVA test and Bonferroni multiple comparison test. Values of $p<0.05$ were considered significant.

Results:

At day 3 and day 7, under all cell culture conditions tested, the presence or absence of serum-derived proteins during the pre-adsorption phase had not a significant effect on cell number, regardless the substrate. Moreover, either after 3 or 7 days, only the presence or absence of FBS during 24h of culture significantly affected the cell number ($p<0.0001$), whereas no effect was detected for its presence or absence during 3h.

At day 3, both titanium surfaces performed better than glass, ($p<0.01$), regardless the presence or absence of FBS during the pre-adsorption or during 3h/24h culture. At day 7 Poli-Ti performed better than Nano-Ti and Glass, ($p<0.0001$), regardless the presence or absence of FBS during the pre-adsorption or during 3h/24h culture.

The growth rate of cells between day 3 and day 7 was not affected by the initial absence of FBS, either for 3h or 24h. In fact, on each substrate, a significant increase in cell number was observed for each condition, as compared to day 3, ($p<0.0001$).

Staining with ALP and immunolabeling for OPN and Ki-67 showed that the osteogenic activity and mitotic activity were ongoing at 72h, irrespective of the substrate.

The morphological analysis through SEM imaging revealed that the absence of FBS for 3h or 24hours had no major influence on cell shape and spreading.

At day 30, the Alizarin Red staining results showed that either in the absence or in the presence of FBS during the pre-adsorption phase or during the first 3h of culture, cells on titanium surfaces had a significantly higher mineralizing capacity than on glass ($p<0.0001$).

Discussion and Conclusions:

The presence or absence of FBS and any resulting protein adsorption is not critical for the initial cell interactions with the tested substrates. This fact is intriguing and suggests that physico-chemical interactions without mediation by proteins are sufficient to sustain the initial phase of culture and guide osteogenic cells toward differentiation. This observations applie to MC3T3 cell line tested, thus the universality of our results should be validated with other cell lines. The challenge is avoiding adsorption of 'undesirables' molecules that may negatively impact on the cueing that cells receive from the surface. This may not be a problem in healthy patients, but may have an important role in medically compromised individuals in whom the composition of tissue fluids is altered. Notably, attention must be paid when translating these results to *in vivo* conditions, since the composition of tissues fluids is complex and dynamic and in vitro studies are not able to fully demonstrate the complex tissue response to biomaterials. Thus, it is strongly recommended that these topics be further investigated through *in vivo studies* on animals and eventually on humans.

2.2 Objective of the study

The objective of the study was to compare the osteoblastic cell growth onto polished and nanoporous titanium and glass as control, by modulating the exposure to serum-derived proteins during the initial phase of cell culture.

The null hypothesis is that the initial protein adsorption onto titanium and glass surfaces has an effect on the early adhesion, spreading and growth of osteogenic cells in vitro and that there is no difference in the cell culture outcomes among the tested substrates.

2.3 In vitro experiments

The in vitro experiments were performed in the Laboratory for the Study of Calcified Tissues and Biomaterials of the Faculty of Dentistry, (Department of Stomatology), of the University of Montreal (QC, Canada).

Chapter 3

Materials and methods

3.1 Substrates

The substrates consisted of commercial grade 2 titanium (Ti) disks (12-mm in diameter and 2 mm in thickness) and round glass coverslips (GC) of same diameter (Fisher Scientific, Nepean, ON, Canada). Glass surface (Glass) was used as standard culture substrate and represented the control group.

Ti-disks were polished to a mirror finish (Poli-Ti) by means of 3 abrasive carpets with decreasing granulometry (Carbon carpet 240/P280 PSA, Diamond Carpet 12" Text Met C PSA; Silica Carpet 12" Microcloth PSA, Buehler, Illinois, USA) mounted on a polishing machine. Poli-Ti disks were then washed in 70% ethanol, rinsed in sterile distilled water (dH_2O) and stored under ultraviolet light.

Half of the disks were subsequently nanotextured (Nano-Ti) by treatment with a mixture of equal volumes of concentrated H_2SO_4 and 30% aqueous H_2O_2 for 2h at room temperature (RT), as described above, (Nanci et al. 1998). In order to assure sterile conditions during the chemical etching, all procedures were performed in a laminar flow cabinet. This oxidative treatment generated a unique surface layer topography characterized by

nanosized tips of approximately 20 nm in diameter and increased roughness. The cleaned, oxidized samples were rinsed with sterile dH_2O, air-dried and stored under ultraviolet light.

Characterization of some Poli- and Nano-Ti disks was verified at JEOL JSM-7400F field emission scanning electron microscope (SEM) operated at 1–2 kV.

3.2 Cell cultures

The experimental protocols started with a pre-adsorption phase. During the pre-adsorption phase the three substrates (GC, Poli-Ti and Nano-Ti) were immersed at 37°C in humidified atmosphere with 5% of CO_2, in 24-well-plates containing 500ml/well of alpha Minimum Essential Medium (αMEM, Gibco) alone (M-NoFBS) or enriched with 10% foetal bovine serum (M-FBS).

Mouse calvaria-derived osteogenic cells (MC3T3-E1) were seeded on these pre-treated substrates with an initial cell density of 30.000 and 60.000 cells/well and cultured in M-noFBS and M-FBS for 3h and 24h, (1h/3h and 1h/24h experiments).

After this initial seeding period, the culture medium was replaced with M-FBS and cell cultures were maintained for 3 and 7 days at 37°C in a humidified atmosphere with 5% CO_2, (Fig.1).

In an additional experiment, GC, Poli-Ti and Nano-Ti substrates, (n=24 for each), were exposed to the pre-adsorption phase, as described above for 1h/3h experiments: immersion for 1h in M-FBS or M-NoFBS; after that, the MC3T3-EI cells were seeded at a cell density of 30.000 cells/well on these pre-treated substrates and cultured for 3h in M-FBS or M-NoFBS; subsequently, cells were maintained in culture with M-FBS for 72h. Then the culture media was replaced with a special osteogenic media, M-FBS supplemented with ascorbic acid (50 μgr/ml) and beta-glycero-phosphate (3mM), which was changed 3 times/week, and cells were maintained in culture for 30 days at 37°C in a

humidified atmosphere with 5% CO_2. At day 30 the osteogenic cell activity was determined through the quantitative evaluation of cell mineral production by staining with Alizarin Red.

3.3 Experimental conditions

To summarize, the FBS/NoFBS experimental conditions of the 1h/3h and 1h/24h experiments were analysed for each tested substrate (GC, Poli-Ti, Nano-Ti) as follows:

- ***a)*** 1h pre-adsorption in M-FBS followed by 3h/24h cell culture in M-NoFBS or M-FBS (FBS-preadsorp);
- ***b)*** 1h pre-adsorption in M-NoFBS followed by 3h/24h cell culture in M-NoFBS or M-FBS, (NoFBS-preadsorp);
- ***c)*** 1h pre-adsorption in M-FBS or M-NoFBS followed by 3h/24h cell culture in M-FBS, (FBS-culture);
- ***d)*** 1h pre-adsorption in M-FBS or M-NoFBS followed by 3h/24h cell culture in M-NoFBS, (NoFBS-culture).

Every experiment was repeated at least twice with a minimum of 6 samples for each substrate and condition.

3.4 Considerations about cells used for in vitro experiments.

MC3T3-E1 cells, as well as other osteoblasts cells models, are developed due to limited availability of primary human osteoblast cells.

Primary cells behaviour is more reflective of the in vivo situation; thus they are more relevant for preclinical and clinical studies. However, they require long isolation procedures and their phenotype and behaviour is sensitive to donor-related factors, (i.e. gender, age, skeletal location, etc.). Moreover, primary cultures with cells isolated from tissues are more difficult to grow and they may loose phenotypic specificity when they are passaged in culture (Variola et al, 2011).

Human osteosarcoma cells may represent a good choice for osteogenic in vitro cultures. In fact, they are available in unlimited number, without the need for time-consuming isolation procedures or ethical approval. They also present the advantage of more reliable reproducibility. Transformed cells lines, in large part reflect the activity of the cell type from which they derive, although they may not necessarily produce similar biological outcomes as primary human osteoblast cells.

On the other hand, primary mouse derived cells are easily available and present the possibility to control the selection of the donor-animals. Moreover, cells can be extracted from all bones in skeleton, although they may present some sign of replicative senescence, particularly when cultured for more than 40 passages. Nevertheless, MC3T3 mouse calvaria-derived osteogenic cells, which are originally with a fibroblast-like phenotype, are capable of differentiating from pre-osteoblasts into mature osteoblasts in appropriate culture environments. Moreover, they exhibit a similar grow rate as human osteoblasts (Czekanska et al.2012).

3.5 Qualitative analysis of cells

Cell activity was evaluated on each substrate and condition through imaging at optical microscope for:

a) Cell shape at day 1 and day 3 by actin staining with Rhodamine-Phalloidin.
b) Cell division activity at day 1 and day 3 by immunolabeling for Ki-67 nuclear protein. This protein is related to proliferative cell activity.
c) Osteogenic cell activity at day 3 by immunolabeling for Osteopontin (OPN) and staining with alkaline phosphatase (ALP). OPN and ALP are bone specific matrix proteins, which are synthesized and secreted during the process of osteoblast differentiation and mineralization (Thorwarth et al. 2005, Liu and Webster 2007).

3.6 Cells immunolabeling and staining procedures

Cells were fixed for 15min at RT using 4% paraformaldehyde in 0.1 Molar (M) sodium phosphate buffer (PB), pH 7.2. After washing in PB, they were processed for immunofluorescence labeling. They were permeabilized with 0.5% Triton X-100 in PB for 10min, followed by blocking of non-specific sites with 5% skim milk in PB for 30min. Subsequently, the samples have been incubated in 0.5% solution of Skim milk in PB containing 1/150 dilution Rhodamin-phalloidin, (Molecular Probes, Invitrogen) to visualize the actin cytoskeleton and primary monoclonal antibody to Ki-67 (dilution 1:200, Bio Markers, Fremont, Canada) or OPN (dilution: 1/800, Bio Markers, Fremont, Canada) to visualize respectively the proliferative and the osteogenic activity of the cells. The samples were then incubated with the corresponding Alexa Fluor 488 (green fluorescence)—conjugated goat secondary antibody (Molecular Probes, Eugene, OR) at a dilution of 1:500. Replacement of the primary antibody with PB was used as control. All antibody incubations were performed for 1h at RT in humidified environment. Between each incubation step, the samples were washed in PB (3x5min). Before mounting for microscope observation, samples were briefly washed with dH_2O. Metal disks were mounted facing up on glass slides, while a glass coverslip was mounted on the surface containing cells using DAPI mounting medium (Prolong antifade 4',6-diamidino-2-phenyl-indole, dihydrochloride, Molecular Probes, Invitrogen). This product allows the visualization of the cell nuclei under fluorescence optical microscope. Control GCs were mounted face down with DAPI mounting medium on glass slides. The samples were then examined by epiluminescence under a conventional fluorescence microscope (Axiophot; Carl Zeiss, Oberkochen, Germany), using Plan-Neofluar objectives (x10/0.25, x20/0.40).

For ALP staining, after fixing the samples with ethanol, cells were permeabilized with 0.2% Tween solution in PB for 10min, then washed with dH_2O and stained with ALP (Sigma

B5655) dissolved in dH_2O. To stop stain development, samples were washed with warm phosphate buffered saline (PBS) and then stained with 0.9% Neutral Red for 10min. Finally, samples were rinsed with dH_2O, dehydrated with 100% ethanol and analysed at the optical microscope (Bright field, 20X).

For Alizarin red staining, after fixing the samples with ethanol, cells were washed with PBS and dH_2O at RT for 5min each. The samples were then stained a 2% solution of Alizarin Red (A-5533, Sigma) in dH_2O at RT for 15min under constant stirring. Subsequently, samples were washed 5 times with dH_2O at RT for 5min each, then washed with PBS and finally with dH_2O at RT for 5min each.

3.7 Morphological analysis

SEM imaging was used to evaluate cellular morphology and spreading for each substrate and condition at 3h, at day 1 and at day 3. For the SEM morphological analysis cells were fixed for 1h at RT in 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA), then osmificated with 1% Osmium (Osmium tetroxide, Electron Microscopy Sciences) at RT for 1h under controlled conditions in a laminar flow cabinet. Samples were then progressively dehydrated at RT in solutions of ethanol at 30% to 100% concentration, for 15min each. Finally, the samples were processed inside a critical point drying machine (BALZERS CPD 030 Critical Point Dryer, BAL-TEC, AG), (Nanci, Zalzal et al. 1996, Irie, Zalzal et al. 1998). Before examination at JEOL JSM-7400F SEM, Glass samples were coated at the carbon-coating machine, whereas Poli-Ti and Nano-Ti samples were examined without coating.

3.8 Quantitative analysis

At day 3 and day 7 a quantitative analysis of the cell number was performed using two different methods: **a)** Alamar blue assay and **b)** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay.

a) The **Alamar Blue assay** uses a non-toxic reagent for continuously monitoring cell viability. Healthy living cells maintain a reducing state within their cytosol. This reducing potential of cells converts the Alamar blue reagent into a detectable fluorescent product. Viable cells continuously convert the blue reagent into a red fluorescent product, thereby generating a quantitative measure of viability. Fluorescence is then monitored into a fluorescence plate reader at the wavelength range of 560-590 nm.

b) The **MTT assay** is used as final readout of a cell population in culture. MTT is a water-soluble tetrazolium dye that produces a yellowish solution when dissolved in culture media or in saline solutions. Only living cells will reduce it to a purple formazan product that is read into the absorbance plate reader (Mosmann 1983). MTT viability test is based on the amount of formazan generated and consequently is directly proportional to the number of viable cells. The MTT assay is an indirect marker for cytotoxicity.

For quantitative analysis of cell proliferative activity, Ki-67 expression at day 1 was measured on 3 randomly selected microscopic images per sample at 20X. Three samples for each substrate were examined. The proportion of immune-reactive cells over total number of cells was calculated at day 1 by means of image analysing software (Image J method) and expressed as mean values±standard deviations for each substrate and condition (von Wilmowsky et al. 2009, de Oliveira and Nanci 2004).

The quantification of the Alizarin red to evaluate the osteogenic cell activity was performed at day 30 through dissolution of the Alizarin Red dye from the mineral production of the cells and subsequent measurement at the spectrophotometer (Stanford, Jacobson et al. 1995).

3.9 Statistical analysis

Numerical data were analysed using the ANOVA test and Bonferroni multiple comparison test to analyse the effect on the osteogenic cells outcome of the following parameters: initial cell density (30.000 and 60.000 cells/well), the substrate (GC, Poli-Ti, Nano-Ti), the presence or absence of serum-derived proteins during the pre-adsorption phase (FBS-/NoFBS- preadsorp) and during the first 3 or 24 hours of culture (FBS-/NoFBS- culture). Values of $p<0.05$ were considered significant.

Chapter 4

Results

4.1 Surface features

Surface characterization by SEM confirmed that the machined and polished titanium surface exhibited a smooth appearance as compared with the nanotextured titanium surface. At high magnification the polished surface did not reveal any topographical feature (Fig.2A). Surface chemical treatment with the H_2SO_4/H_2O_2 oxidative solution created a reproducible nanopitted surface texture characterized by uniformly distributed pores of approximately 20-nm-diameter size (Fig.2B).

Glass surface has been used as reference material, as it represent a commonly used smooth substrate for in vitro cell culturing.

4.2 Cell morphology

4.2.1 Optical microscope

Cell morphology was assessed at the optical miscroscope for:

a) Cell shape by actin staining with Rhodamine-Phalloidin at day 1 and 3.

b) Dividing cells by immunolabeling for ki-67 nuclear protein at day 1 and 3.

c) Osteogenic cell activity by immunolabeling for OPN and staining with ALP at day 3**.**

a) Cell outlines and spreading was examined at fluorescence optical microscope through cell nuclei labelling and actin staining, which highlighted the cell contours and filaments.

At day 1 of 1h/24h experiments, actin staining revealed that cells were attached and partially spread both on metal and glass substrates in all conditions (FBS- and NoFBS-culture, FBS- and NoFBS-preadsorp), except for Glass in NoFBS CULTURE condition. In this case, only few cells were found on the examined samples, thus proper morphological analysis was not possible (Fig.3).

In the FBS culture condition, cell spreading was mostly concentrated in the central area of each sample. Cells predominantly showed a polygonal shape. Some fusiform cells were also observed. Cytoskeleton was properly developed and spread, with uniform and dense pericellular actin network. A lot of cells also emitted thick and long fibrillar extensions, mostly those on metal substrates. Cells on Poli-Ti presented thick focal adhesions at cell edges, not observed instead on Nano-Ti (Fig.4).

For each substrate in NoFBS culture condition, irregular stellate and fusiform cells were often observed. Cell dimensions were smaller and actin extensions were shorter and thinner as compared to those observed in FBS culture condition (Fig.5). Moreover, it was noticed that cells on Poli-Ti presented a considerable number of very short actin filaments (dots) inside the cytoskeleton, not observed on Nano-Ti (Fig. 6).

Irrespective of the substrate, cells presented increased dimensions and longer actin filaments in the FBS-preadsorp as compared to NoFBS-preadsorp condition.

Cells grown both on Ti and GC became progressively more spread all over the samples surface from the day1 to the day3 of culture period.

At day 3 of 1h/3h experiments, for each substrate, cells were similarly spread in FBS-culture (Fig.7) and NoFBS-culture conditions (Fig.8). However, in NoFBS-culture cells grown on Nano-Ti and Poli-Ti were more spread than on Glass (Fig.9).

At day 3 of 1h/24h experiments, in NoFBS-culture condition the cells cytoskeleton was less spread, with some wrinkles, as compared to FBS-culture condition, regardless the substrate (Fig.10, 11).

Cells on Nano-Ti and Poli-Ti presented longer filaments than on Glass in all experiments and conditions. For all substrates and conditions at day 3 cells were grown on multiple layers as compared to day 1, except for Glass in NoFBS-culture where cells were spread on a single or only few layers (for 1h/24h experiments compare Fig.4-5 and Fig.12, 13 at day 1 with Fig.10, 11 at day3).

b) At day 1 of 1h/24h experiments, immunolabeling for Ki-67 nuclear protein showed that a considerable amount of diving cells were present on all substrates in FBS-culture, in NoFBS-preadsorp and in FBS-preadsorp conditions, on Poli- and Nano-Ti substrates in NoFBS-culture condition, whereas it was not possible to identify Ki-67 positive cells on Glass in NoFBS-culture condition (Fig.12, 13).

At day 3 of both 1h/3h and 1h/24h experiments ki-67 positive cells were still evident in all conditions, irrespective of the substrate (Fig. 7-11).

c) At day3 of 1h/3h experiments, staining with ALP and immunolabeling for OPN showed that the osteogenic activity was ongoing in all conditions, irrespective of the substrate (Fig.14, 15). However, when FBS was omitted during the pre-adsorption phase or from the culture medium, (in NoFBS-preadsorp and NoFBS-culture

conditions), expression of ALP and OPN decreased on all substrates. In all cases, Glass surfaces showed the least ALP and OPN positive cells as compared to titanium surfaces.

4.2.2 SEM imaging

SEM imaging of cell morphology was performed:

- **a)** at 3h for 1h/3h experiments;
- **b)** at 1 day for 1h/24h experiments;
- **c)** at 3 days for 1h/3h experiments.

a) On all substrates, the evaluation at SEM **at 3h** showed some differences in cell shape and spreading between FBS and NoFBS conditions.

In FBS-culture, cells presented larger dimension as compared to those on NoFBS-culture condition. Cells generally showed a round shape in FBS-culture condition, although some of them presented irregular shapes (Fig.16). Actin filaments were noticed in considerable number and although thin and veil-like, some could reach the closest cells (Fig.17). In NoFBS-culture condition cells shape was often similar to irregular asterisks. Cytoskeleton was not largely developed on the surfaces and rather concentrated around the peri-nuclear area. Few, thin filipodia were also observed, (Fig.18).

b) At day 1, the absence of FBS had no a major effect on cell shape and spreading, irrespective of the substrate (Fig19). SEM analysis revealed no significant differences in cell shapes between glass control, nanotextured and polished titanium surfaces for each condition. However, cell dimensions were larger as compared to those observed at 3h, irrespective of the substrate and condition. Actin extensions and focal adhesions of the cells appeared more abundant at 1day than at 3h. Even in the

absence of FBS for 24h of culture, cells were able to grow on each substrate after they adhered to the substrates within the first 3h. Moreover, numerous cytoplasmatic interconnections among adjacent cells were observed, regardless the presence or absence of FBS during the pre-adsorption or the culture period (Fig. 20).

The predominant cell shape at day 1 was polygonal (Fig.19), with some cells showing long-limbed or stellate profiles, the latter characterized by numerous thin projections (Fig. 21A). Other cells presented instead a uniform, perinuclear actinc spreading (Fig.21B). At higher magnification, some projections appeared long and thin (Fig. 20D), others were larger or finger-like (Fig.20C, 22A). It was also noticed on both metal surfaces the presence of numerous filipodia along the periphery of the cells (Fig.22B, 23). Although cytoplasmatic projections and filipodia were found on glass control surfaces as well (Fig.24), they were more abundant on nanotextured and polished ones. On Poli-Ti these filipodia had a linear aspect (Fig. 22B), whereas on Nano-Ti they appeared rather corrugates (Fig.23). Their aspect was porous and extremely fine, which allowed the underlying nanotexture to appear through them (Fig. 23C). Moreover, a noteworthy structural cell feature was the release of multiple lateral extensions from the main cytoplasmatic projections (Fig.23). These lateral filipodia seemed to span some of the larger surface topographical features. Their outlines appeared to follow the walls of the nanocavitations created by the etching treatment. Some of the cells irregularities attached to the edges of the nanocavitations created by the etching treatment (Fig.23D).

c) At day 3, cells dimensions were bigger than those observed at day 1, spread on the whole available surface and superimposing to each other, regardless the substrate and condition. Cell nuclei appeared bigger than those observed at 3h and day1. Abundant cytoplasmatic extensions and interconnections among cells were still

evident. Moreover, extracellular accumulation of matrix was noticed on each condition, mainly on Nano-Ti surfaces (Fig.25, 26).

4.3 Quantitative results

4.3.1 Influence of Cell density

The influence of the initial cell density on the cell culture outcomes was analysed for 1h/3h and 1h/24h experiments by means of Alamar Blue test at day 3.

In the 1h/3h experiments a significant interaction was found between cell density and substrates: when a 60.000c/w cell density was used Poli- and Nano-Ti substrates showed significant more cells than GC (Nano-Ti vs GC: $p=0.003$; Poli-Ti vs GC: $p=0.006$); when a 30.000 c/w cell density was used the substrates did not show a significant difference in cell number.

In the 1h/24h experiments, instead, a significant interaction was found between cell density and FBS/NoFBS-culture: for each initial cell density, a significant higher cell number was detected in FBS-culture than in NoFBS-culture condition ($p<0.0001$).

4.3.2 Alamar Blue assay

4.3.2.1 Alamar Blue results for 1h/3h experiments at day 3

At day 3, with both initial cell density of 30.000 or 60.000 cells/well, Alamar Blue results did not allow to discern any difference in cell number between Nano-Ti and Poli-Ti surfaces. However, when using an initial cell density of 60.000 cell/well, glass surfaces exhibited significantly lower cell number than metal surfaces (Nano-Ti vs GC: $p=0.003$; Poli-Ti vs GC: $p=0.006$), regardless the pre-adsorption or the culture conditions (FBS/NoFBS). Differences in cell number among glass and metal surfaces were not detected when an initial cell density of 30.000 c/w was used, regardless the pre-adsorption or the culture condition.

The presence or absence of FBS during the pre-adsorption phase or during the first 3h of culture had no an effect on the cell number, irrespective of the substrate, (Graphic 1).

4.3.2.2 Alamar blue results for 1h/24h experiments at day 3

At day 3, as for 1h/3h experiments, for 1h/24 experiments either with 30.000c/w or 60.000c/w cell density, the presence or absence of FBS during the pre-adsorption phase had no effect on cell number, regardless the substrates. Differently, the absence of FBS during the first 24h of culture significantly affected the number of cells on all substrates, (*$p<0.0001$, Graphic 2).

At day 3, with an initial cell density of 60.000 c/w, when considering the combination of 1h/3h and 1h/24h experiments, it was found that both metal surfaces performed significantly better than glass surfaces, irrespective of the presence of absence of FBS during the pre-adsorption or culture (Graphic 3).

4.3.2.3 Alamar Blue results for 1h/3h experiments at day 7

At day 7, as at day 3, Alamar Blue assay showed that with an initial cell density of 30.000c/w the presence or absence of FBS during the pre-adsorption phase or during the first 3h of culture had no effect. A significant increase in cell number was observed for each condition as compared to day 3, ($p<0.0001$, Graphic 4).
Both titanium substrates performed better than glass, regardless the pre-adsorption or the culture conditions (Nano-Ti>GC: $p=0.023$; Poli-Ti>GC: $p<0.0001$; Poli-Ti>Nano-Ti: $p=0.001$).

4.3.2.4 Alamar Blue results for 1h/24h experiments at day 7

At day 7, with an initial cell density of 30.000 c/w, cell number results were similar to 3-days-outcomes: the presence or absence of FBS during the pre-adsorption phase had no effect on cell number, regardless the substrates; differently, the absence of FBS during the first 24h of culture significantly affected the number of cells on all substrates (Graphic 4). However, each substrate showed a significant increase in cell number from day 3 to 7 ($p<0.0001$), regardless the presence or absence of FBS during the pre-adsorption phase or during the first 3h or 24h of culture (Graphic 4, 5).

Moreover, Poli-Ti performed better than Nano-Ti, regardless the presence or absence of FBS during culture ($p=0.002$), while each titanium surface had a similar cell number as compared to Glass, regardless the presence or absence of FBS during pre-adsorption or during culture.

At day 7, with an initial cell density of 30.000 c/w, when considering the combination of 1h/3h with 1h/24h experiments, Poli-Ti performed better than Nano-Ti and Glass, ($p<0.0001$), whereas no differences in cell number were detected between Nano-Ti and Glass, regardless the presence or absence of FBS during the pre-adsorption or during culture (Graphic 6).

4.3.3 MTT assay

MTT assay was used as final readout of cell population in cell cultures for 1h/3h experiments at day 3 and for 1h/24 experiments at day 3 and 7.

4.3.3.1 MTT results for 1h/3h experiments at day 3

At day 3, MTT results were similar to those obtained with Alamar blue test. MTT test showed for 1h/3h experiments with initial cell density of 60.000c/w, that the presence or absence of FBS during the pre-adsorption or during culture had no effect on the amount of cells counted (Graphic 7, 8). On the other hand, the substrate had a significant effect on the cell number: Nano-Ti and Poli-Ti surfaces presented significantly higher cell number than Glass surfaces (p=0.0001, Graphic 9).

4.3.3.2 MTT results for 1h/24h experiments at day 3

At day 3, for 1h/24 experiments with initial cell density of 60.000 c/w, MTT results slightly differed from those obtained with Alamar blue test. MTT test showed that the presence or absence of FBS during the pre-adsorption or during culture had no effect on the cell number (Graphic 7, 8). Differently, Alamar Blue test had detected a significant effect of FBS/NoFBS during culture.

The substrate significantly influenced the cell outcome, as a higher cell number was observed on Nano-Ti than on GC (p=0.018).

4.3.3.3 MTT results for 1h/24h experiments at day 7

At day 7, with an initial cell density of 30.000 c/w, the presence or absence of FBS during the pre-adsorption phase and during the first 24h of culture had a significant effect on the cell number. Actually, a significantly lower cell number was observed in NoFBS conditions for each substrate (NoFBS- vs FBS-preadsorp: $p<0.006$, Graphic 10; NoFBS- vs FBS-culture: $p<0.0001$, Graphic 11). Differently, Alamar Blue test results did not detect any effect of the FBS-/NoFBS-preadsorp conditions, but of the FBS-/NoFBS-culture conditions.

Moreover, MTT results showed that both titanium substrates performed better than glass, irrespective of the presence or absence of FBS during pre-adsorption ($p<0.0001$). Similarly, Poli-Ti performed better than Glass, regardless the presence or absence of FBS during culture, ($p<0.0001$), whereas Nano-Ti had a higher cell number than glass in the presence of FBS during culture ($p<0.0001$), but similar to Glass in the absence of FBS during culture. Differently from Alamar Blue test results, which not always revealed significant differences in cell number between metal and glass surfaces, MTT test results highlighted the tendency for both metal substrates to perform better than glass (Graphic 12).

4.3.4 Ki-67 results

At day 1, the presence or absence of FBS during the pre-adsorption phase had not an effect on the dividing cell rate obtained, irrespective of the substrate. However, the culture condition (FBS-/NoFBS-culture during 24h) significantly influenced the outcome: for Glass substrate, it was found a significantly lower proportion of dividing cells in NoFBS-culture than on FBS-culture ($p=0,004$), while for metal substrates a similar proportion was found in FBS- and NoFBS-culture conditions. However a similar number of proliferative cells were found among substrates for each condition.

4.3.5 Alizarin red results

At day 30, the Alizarin Red staining results showed that either in the absence or in the presence of FBS during the pre-adsorption phase, cells on Ti-surfaces showed a significant higher mineralizing capacity than on Glass ($p<0.0001$).

Moreover, in the presence or absence of FBS during the first 3h of culture, cells on Nano-Ti surfaces showed a significantly higher mineralizing capacity than on Glass ($p<0.0001$), while cells on Poli-Ti revealed a similar behaviour as those on Glass and

Nano-Ti. Surprisingly, cells on Nano-Ti showed a significant higher mineralizing capacity in NoFBS-preadsorp than in FBS-preadsorp, ($p=0.004$, Graphic 13).

Chapter 5

Discussion

The null hypothesis that the protein adsorption onto titanium and glass surfaces has an effect on the early cellular events was rejected. Both the **pre-adsorption of the substrates** before seeding the cells and the **adsorption for 3h or 24h of culture** with serum-derived proteins did not affect the attachment and growth of MC3T3 cells on the tested substrates (Glass, Poli-Ti and Nano-Ti). Although the proteins adsorption during the first 24h of culture had a significant influence on the cell number, cells fared well in every condition. In fact, a significant increase in cell number was recorded on each substrate from day 3 to day 7, both for 1h/3h and 1h/24h experiments. This demonstrates a recovery ability of MC3T3 cells on each substrate, even when cultured in not ideal conditions.

It may be hypothesized that this trend could be maintained over longer periods of culture, to the point where a similar amount of cells would be found for culture conditions with and without FBS during the first 24h.

It has been previously reported that treatments such as acid etch and blast may cause significant changes in topographic characteristics and roughness values of the modified

biomaterial, but minor alterations in the chemical composition of the surfaces (Sela et al. 2007). Hence, the authors concluded that the observed differences in adsorption of proteins by these modified surfaces were mainly but not solely due to changes in their physical properties. Differently, in the present study, both titanium substrates (treated and non treated) performed well either in presence or in absence of FBS, suggesting that the cell attachment and initial growth is not necessarily related to the specific topographical characteristics of titanium surfaces. However, both metal surfaces showed better performances as compared to glass, suggesting that the early cellular events are related to physical as well as chemical properties of the substrate.

Cells number was not affected by the absence of proteins during the pre-adsorption or during 3h of culture, regardless the substrate.

Nanotextured titanium surfaces has been claimed to allow increased layers of adsorbed proteins, as compared to smooth titanium surfaces, which in turn mediate en enhanced cells affinity for the implant surface (Deligianni et al. 2001, Sela et al. 2007). Although the amount of adsorbed proteins was not addressed in the present study, most experiments did not show significant differences in cell number between Poli-Ti and Nano-Ti and cells outcomes were optimal irrespective of the presence or absence of FBS on every substrate. This suggests that the presence of serum derived proteins and any resulting protein adsorption is not essential for the early cell attachment and growth on the tested substrates. Cell attachment seems not to be mediated by protein adsorption, although it can’t be excluded that higher volumes are adsorbed onto nanopatterned than smooth surfaces.

The challenge to achieve effective improvements of titanium biocompatibility and osteoconductivity should be rather avoiding the adsorption of molecules that may negatively impact the cueing that cells receive from the surface.

Initial cellular events are related to the physical-chemical characteristics of the substrate, as both titanium surfaces performed significantly better than glass, regardless the conditions.

In accordance with the quantitative analysis of cell number, the absence of serum-derived proteins did not affect the mitotic and osteogenic activities of the cells on metal substrates, but it did affect them on glass, as evaluated through quantification of Ki-67 expression (day 1) and mineral production of the cells (day 30).

OPN is one of the abundant noncollagenous proteins in bone matrix; it is able to bind to integrins, thus mediating bone cells adhesion to the mineralized matrix (Denhardt and Noda 1998). Recently, nanotexured surfaces have been found to enhance the adsorption and/or retention of specific extracellular matrix proteins, such as Osteopontin (OPN) and Bone Sialoprotein, both important osteoconductive proteins (de Oliveira and Nanci 2004, Vetrone, Variola et al. 2009). The authors hypothesized that the physical characteristics of the substrate, mainly due to its increased roughness and nanopitted topography, could selectively allow higher amounts of adsorbed proteins and that chemical properties of the surface may play a less important role.

Differently, the immunolabeling results of the present study did not reveal any considerable variance in OPN expression between Poli-Ti and Nano-Ti surfaces. Moreover, SEM analysis did not highlighted major differences in cell spreading and shape between metal surfaces, regardless the conditions. At day 1, notwithstanding the continuous absence of FBS for 24h, cell dimensions were increased as compared to those at 3h of culturing. SEM analysis also revealed that in absence of FBS during 3h and 24h the cell density was lower on glass than on metal surfaces. Moreover, cells were able to grow in dimensions and to better spread on metal surfaces than on glass after 3 days.

Thus, the optical and scanning electron microscopes qualitative analysis results are in accordance with the quantitative analysis results and confirm that the protein adsorption

on titanium surfaces seems not to be essential for cell adhesion and growth. Physical-chemical properties of the surfaces, rather than the presence or absence of specific serum proteins must be taken into account to achieve improvements in the osteogenesis events.

The presence of thickened cellular edges on Poli-Ti surfaces as revealed at optical microscope (Fig.4A) suggests that cells may need higher volumes of actin filaments to adequately adhere onto smooth than nanopatterned titanium surfaces. On the other hand, as highlighted by SEM, the cytoplasmatic extensions may hide inside the nano-holes and mould following the surface nano-cavities of the treated titanium surfaces (Fig.23). An increased cell surface area comes in contact with the nanotextured substrates, thus requiring less actin focal adhesions to attach on the nano-pitted than smooth titanium surfaces. The nanopitted topography with its enhanced roughness and higher surface area, can provide for more available sites for cell attachment. As described, nanotopograghy is ideal to match cell structures used to interact with the external environment and biomaterials. It may produce an enhanced affinity between the substrate and the cells, thus guiding desired and controlled biological reactions (de Oliveira and Nanci 2004, Curtis and Wilkinson 1999). For example, this may lead to mechanical modification of the cells shape and cytoskeleton, which may interfere on the cell growth and differentiation. Actually, it is well known that not only biological/chemical but also mechanical cuing may influence the cellular behaviors. Micromovements and primary stability are important factors in determining the clinical implant success. Thus, a further objective of nanomodifications for titanium surfaces may aim to influence mechanical stimulation of the osteogenic cells in order to guide their differentiation and maturation process. Host's quality and quantity bone defects could be then overcome as contraindication to the implant therapy. Proper implant osseointegration might be merely related to the type of titanium implants used, while clinical success would become independent from host-related factors.

It should be pointed out that the presented results pertain to MC3T3 cell line tested, and the universality of these results should be validated with other cell lines and in vivo. Moreover, assays readings must be cautiously interpreted, since different assays (MTT and Alamar Blue assays) used to test the same variable can lead to divergent results. Finally, a great attention must be paid when translating these results to *in vivo* conditions, since the composition of tissues fluids is complex and dynamic and in vitro studies are not able to fully demonstrate the complex tissue response to biomaterials.

Chapter 6

Comparison with in vivo studies

6.1 Advantages/Disadvantages of in vitro studies

Differently from the general documented belief that protein adsorption play a key role in the osteogenesis events (Lord et al 2008, Kasemo and Gold 1999, Liu and Webster 2006), the presented in vitro results showed that the presence or absence of serum proteins and any resulting protein adsorption was not critical for the initial cell interactions with the tested substrates. The physical-chemical properties of the biomaterials appear to be mainly responsible for the osteoblast cell adhesion without protein promoted mechanism.

Nevertheless, these results are limited to *in vitro* experiments outcomes. Although cell culture testing methods provide relevant information, they must be considered within the limits of acute toxicity testing (Granchi et al. 1995). *In vitro* studies are popular for the characterization of bone-contacting materials. They generate information about the immediate tissue response to avoid the unnecessary use of animals in the testing of cytologically inappropriate materials. However, as *in vitro* cell culture is based on growth of cells which are no longer organized into tissues, it is not helpful in studying the complex

interactions between material and host tissues (Hauman and Love 2003), thus being often inappropriate to be extrapolated to the *in vivo* situation.

In vitro assays are not able to demonstrate the complex tissue response to biomaterials in the body and are limited to acute studies of cytotoxic effects due to the relatively short lifespan of cultured cells (Pizzoferrato et al. 1994). Nevertheless*, in vitro* experiments have the advantage of ease of control of experimental factors, which is one of the most significant problems when performing experiments *in vivo*. They are also rapid, cheap and reproducible. Moreover, *in vitro* methods are frequently more sensitive and easily quantifiable than *in vivo* assays. For these reasons they are essential for evaluating biocompatibility and tissue response of biomaterials, prior of any clinical application on animals first, and finally on humans.

6.2 Brief analysis of in vivo studies on different implant surfaces

The success of the dental implants is related to the osseointegration that is defined as a direct bone-to-implant contact without interposition of any other tissue (Brånemark et al. 1969). The implant surface is the first component of dental implant that directly contacts with the surrounding tissues, thus a great interest has been grown towards improving its properties to enhance the tissues response. However, the quest for an optimal implant surface is still underway, especially for acceleration of the healing period and for compromised conditions.

For example, recent studies reported that the addition of bioactive substances might increase the osteoconductive property of the implant (Yoo et al. 2014, Jimbo, Sotres et al. 2012). Recent in vivo studies have shown that nanostructured implant surfaces enhance bone responses and promote interfacial strength (Wazen et al. 2013, Bjursten et al. 2010). Puckett et al. (2010) reported reduced bacterial attachment on nano-scale rough surfaces

than on other surfaces; furthermore, the same nano-scale surface showed higher affinity to fibronectin, which is essential for the initial osseointegration process (Jimbo et al. 2007). Meirelles et al. (2008) conducted an animal study with nanostructured and polished implants, and found that the former had higher bone-to-implant contact than the latter implant surfaces. This suggests that cells, particularly osteoblasts, respond to topographical alterations at the nanometer scale. A selective adsorption of beneficial molecules may be achieved as a result of implant surface modifications (Dee and Bizios 1996). Also, it was reported that nanoscale structures on Ti surfaces could selectively increase the adhesion and proliferation behaviors of pre-oesteoblasts, but not fibroblasts (Variola et al. 2008). Hence, nanoscale surface roughness may represent a pivotal factor in determining the bone and soft tissues healing around implants.

As said, the role of protein adsorption in the cell-substrate interactions is not yet clarified to a full extent. A recent study evaluated the *in vivo* effect of local application of the osteogenesis-promoting laminin-1 protein on bone healing response of nanotextured implants (Schwartz-Filho et al. 2012). The authors failed in demonstrating any significant difference in the osseointegration process around coated and noncoated implants. Moreover, in most studies concerning protein doping of implant surfaces, the beneficial enhancements were primarily restricted to the initial stages of healing and have been shown to have smaller effects when longer periods of experimental time were observed. (Schwartz-Filho et al. 2012, Yoo et al. 2014). A previous study showed that nanostructured surfaces allowed for less protein adsorption (Kam et al. 2014) resulting in decreased fibroblast proliferation, and induced lower gene expression of epidermal growth factor, key factors associated with an adverse fibrotic response. Thus, as also suggested by the present results, selected modified titanium surfaces (*i.e.* nanostructured) could be used to modulate the fibrotic behavior in cells and have the potential to be used as anti-fibrotic architecture for dental implants.

6.3 Potential impact in the field of implantology for medically compromised patients

The new goal of implant surface alterations should be controlling the early cellular events so that nonspecific adsorption of proteins would be minimized. In particular, the adsorption of molecules that may negatively affect the implant outcome would be avoided. This may play an important role in medically compromised patients (MCP), in which the composition of tissue fluids is altered. In contrast with the documented attempts in the literature to control cellular events and improve implant outcomes using the protein coating of titanium surfaces (Jimbo, Sotres et al. 2012, Rammelt et al. 2006, Hilbig et al. 2007), our results suggest that protein adsorption is not essential for the cellular events at the substrate-cell interface. Moreover, intentionally doping implant surfaces show some practical limits, such as the necessity of intermediary passages to obtain the surface coating prior to chirurgical implantation, whereas clinicians would preferably deal with ready-to-use implants. Several extra-cellular matrix proteins, such as vitronectin, fibronectin and collagen type-I, have been proven to promote adherence of potential pathogenic bacteria to titanium surfaces *in vitro*. Adhesion of bacteria, such as Prevotella denticola and Porfiromonas gingivalis, should be limited in order to reduce risk for peri-impantitis, which is particularly high in MCP, (Mahmoud et al. 2012). Some results that have shown opposing behavior of human osteoblasts and Staphylococcus epidermidis (Colon et al. 2006) or E. coli (Ploux et al. 2009) on nanostructured materials are particularly interesting, since they demonstrate that materials could be specifically designed to promote osteoblast function while reducing bacterial colonization. Such considerations together with the presented results indicating that protein adsorption does not have an effect on the osteogenic cells outcomes may have a relevant impact on the clinical application of dental implant therapy, especially for MCP. The therapeutic use of dental implants is often contraindicated for MCP (Smith et al. 1992).

MCP are at higher risk of implant loss, particularly when the general medical situation is associated with increased susceptibility to periodontal disease. (Morris et al. 2000; Olson et al. 2000). Because most inflammatory diseases are linked to a higher risk for peri-implant infections, which represents one of the main reasons for long-term implant loss, it becomes clear that these patients are at a higher risk of secondary implant failure, too (Renvert and Persson 2009). Patients suffering from diabetes mellitus, for example, are at a higher risk for post-surgical infection and typically present lower cicatrization rates (Goodson and Hunt 1979). Most studies suggest that implants may be used in diabetic patients, but only when their illness is well under control, (Balshi and Wolfinger 1999). Diabetes mellitus is one of the most common chronic metabolic disorders, and third common causes of disability and morbidity in the western world (Wild et al. 2004). The majority of experimental studies show that the effect of Diabetes mellitus on the implant osseointegration has been associated with impaired osseous wound and bone healing, decreased bone density and increased susceptibility to periodontal disease (Taylor et al. 2004).

Similarly, implant therapy is often contraindicated in aged individuals and patients suffering of osteoporosis or other diseases associated to poor bone quality, (Shapiro 1992). A lack of implant healing may be encountered. Osteoporosis is often observed among patients of advanced age, and in a population that lives longer, the possibility to offer them an implant therapy is becoming urgent.

The results of the presented *in vitro* study suggest that protein adsorption does not influence the implant outcome and physical-chemical cueing of nanomodified and conventional titanium surfaces may be sufficient to sustain the osteogenesis events. A previous *in vivo* study demonstrated that modified sandblasted and etched titanium surfaces that show increased hydrophilicity compensated for the negative influence of diabetes mellitus on osseointegration (Schlegel et al. 2013). Accordingly to the preliminary

in vitro results presented above, the improved osseointegration reported for these MCP has been solely related to enhanced surface properties of the implant surfaces, even if the protein adsorption effect was not investigated by the authors. If the presented in vitro results will be confirmed by in vivo studies and particularly in medically compromised situations, it might be expected that specifically phisico-chemically modified implant surfaces, capable of avoiding adsorption of undesirable molecules, may be helpful for a safe use of implant-supported prosthesis even in MCP. Such surfaces would be simultaneously capable of promoting bone healing and improving implant integration in not ideal medical conditions.

The opportunity to enlarge dental prosthetic options for individuals with compromised health conditions has important long-term effects, including a reduced incidence of complications related to the main disease that reflects a considerable improvement of the quality of life of these patients.

Chapter 7

Conclusions

- The fact that cells fared well under all conditions, and in particular in the total absence of FBS during the first 3h, suggests that physico-chemical interactions without mediation by proteins are sufficient to sustain this initial phase of culturing (day 3 and day 7) and guide osteogenic cells toward differentiation (day 30).
- Our results also suggest that topographical modifications of biomaterials, such as an increased nano-sized roughness of titanium surfaces, should not aim to enhance a wide spectrum protein adsorption, as this event may not be necessary to achieve a better implant outcome in normal serum conditions. The challenge is actually avoiding adsorption of molecules that may negatively impact the cueing that cells receive from the surface. This may not be a problem in healthy patients, but may have an important effect in medically compromised individuals in whom the composition of tissue fluids is altered.
- Finally, further studies analysing micro- and nano- surface modifications of biomaterials for implantations and their interactions with specific proteins are needed to better grasp the impact such modifications might have on the initial

healing phases, particularly for medically compromised patients. Thus, it is strongly recommended that these topics be further investigated through *in vivo studies* on animals and eventually on humans.

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Figures and graphics

Fig.1 Schematic drawing of experiments protocol

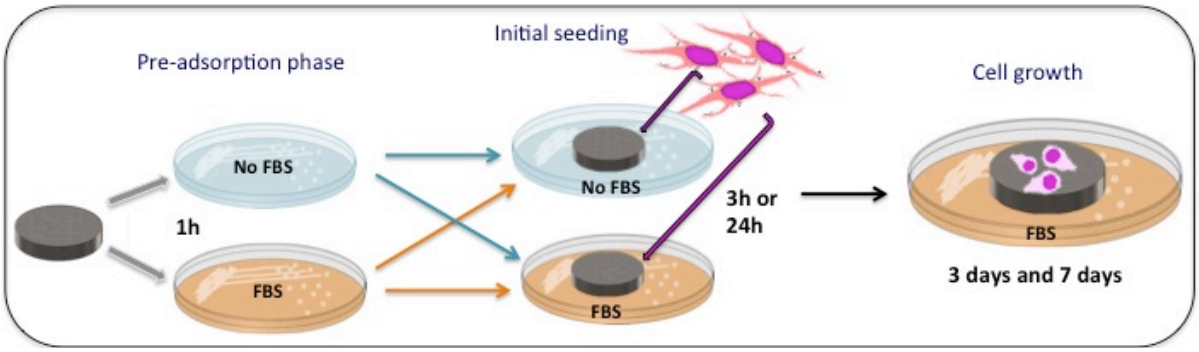


Fig.2 SEM images of Polished ***A)*** and Nanotextured ***B)*** substrates.

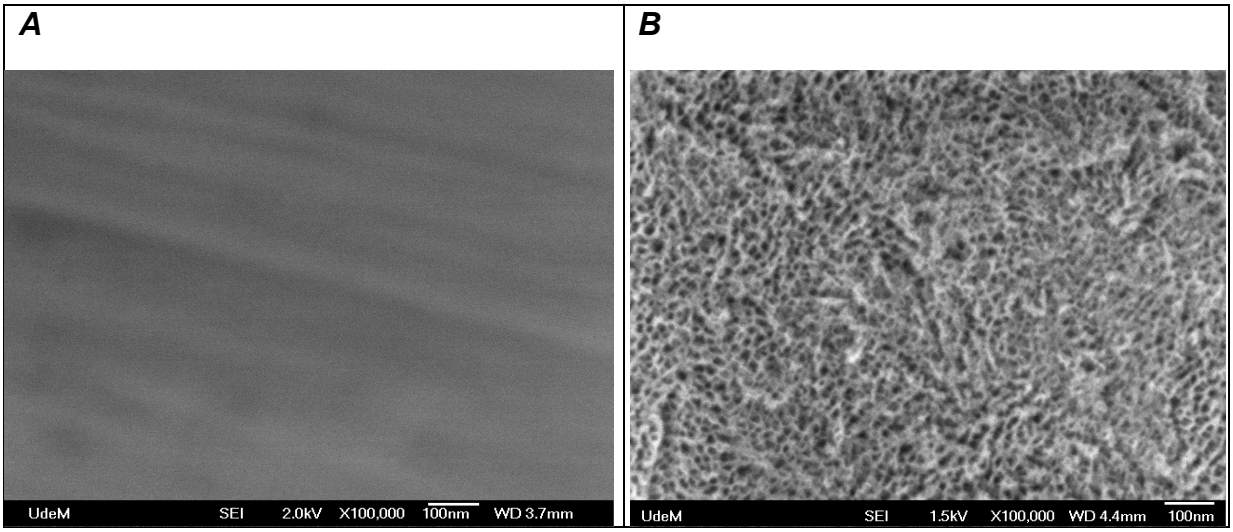


Fig.3 Fluorescence optical microscope imaging: Mosaic at 10X magnification of osteogenic cells grown on Poli-Ti (A) and Glass (B) in NoFBS-culture condition at day 1 of 1h/24 experiment.

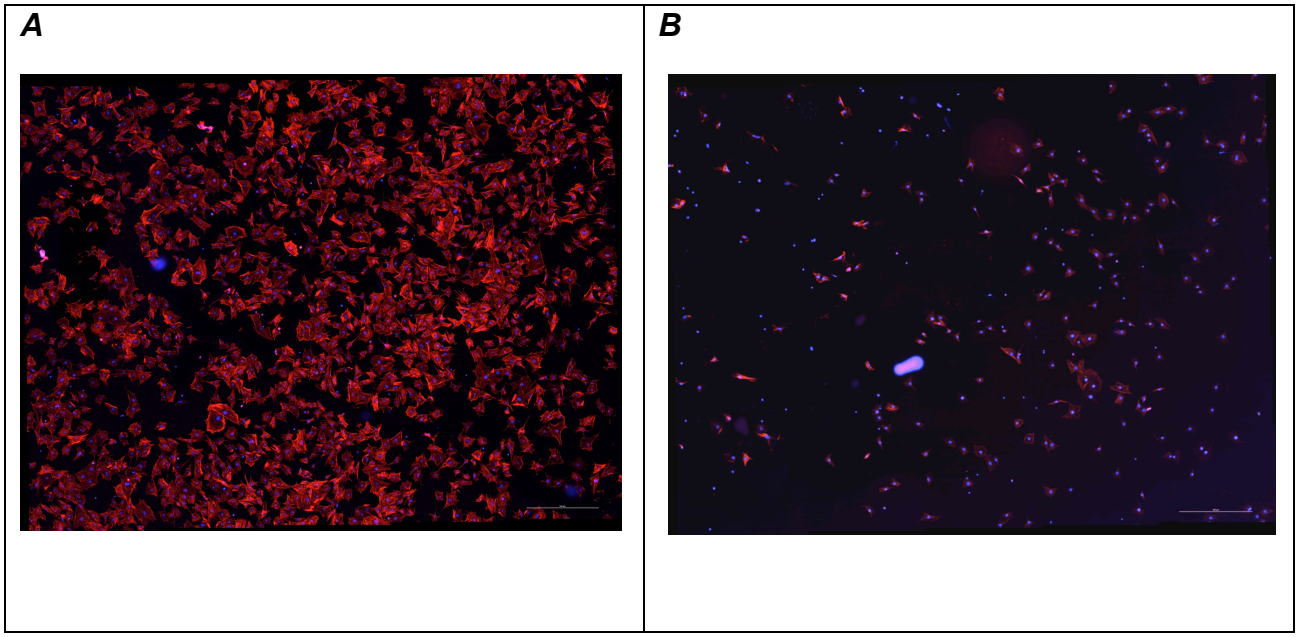


Fig.4 Immunolabeled preparation of osteogenic cells grown on Poli-Ti (A) and Nano-Ti (B) in FBS-culture condition at day 1 of 1h/24 experiment (20X). Actin cell outlines (red) and cell nuclei (blue) are well evidenced. On both surfaces cell shape was predominantly polygonal. A) Focal adhesions at cell edges were often observed on polished surfaces (arrows).

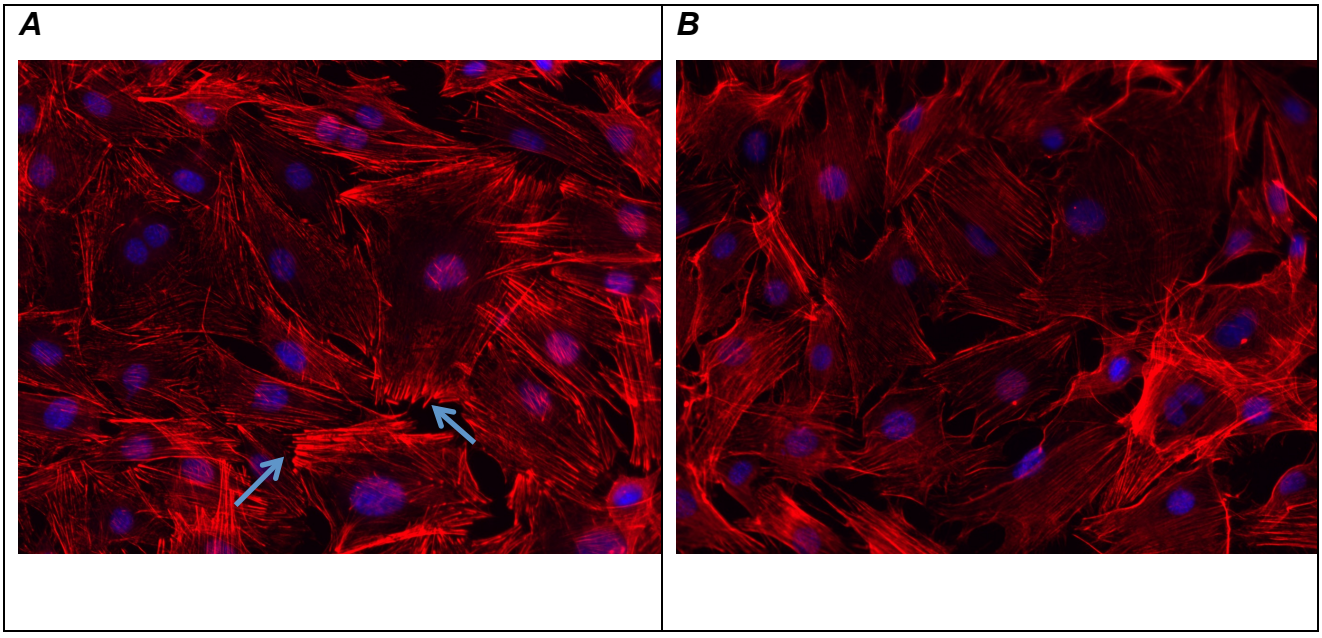


Fig.5 Immunolabeled preparation of osteogenic cells grown on Poli-Ti (A) and Nano-Ti (B) in NoFBS-culture condition at day 1 of 1h/24 experiment (20X): On both surfaces cell dimensions were smaller as compared to FBS-culture condition (Fig.4A and B).

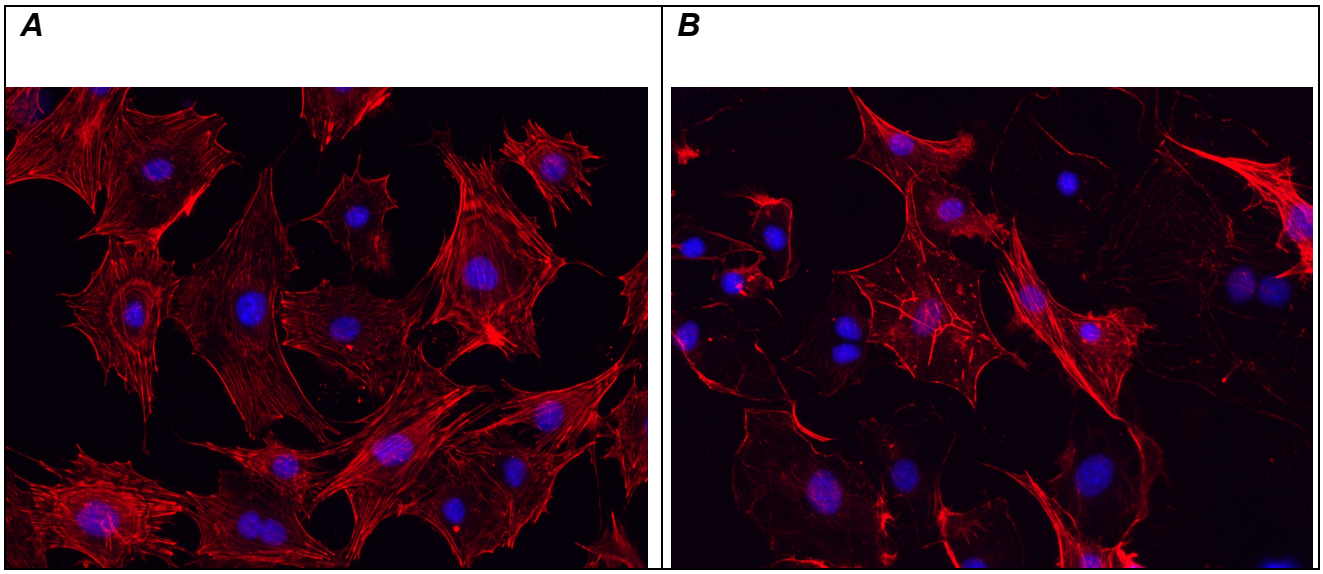


Fig.6 Immunolabeled preparation of osteogenic cells grown on Poli-Ti (A) and Nano-Ti (B) in NoFBS-culture condition at day 1 of 1h/24 experiment (10X). A) Short actin filaments, similar to dots, have been observed onto polished substrates (arrows); B) fusiform cells observed in absence of FBS during 24h of culture (arrow).

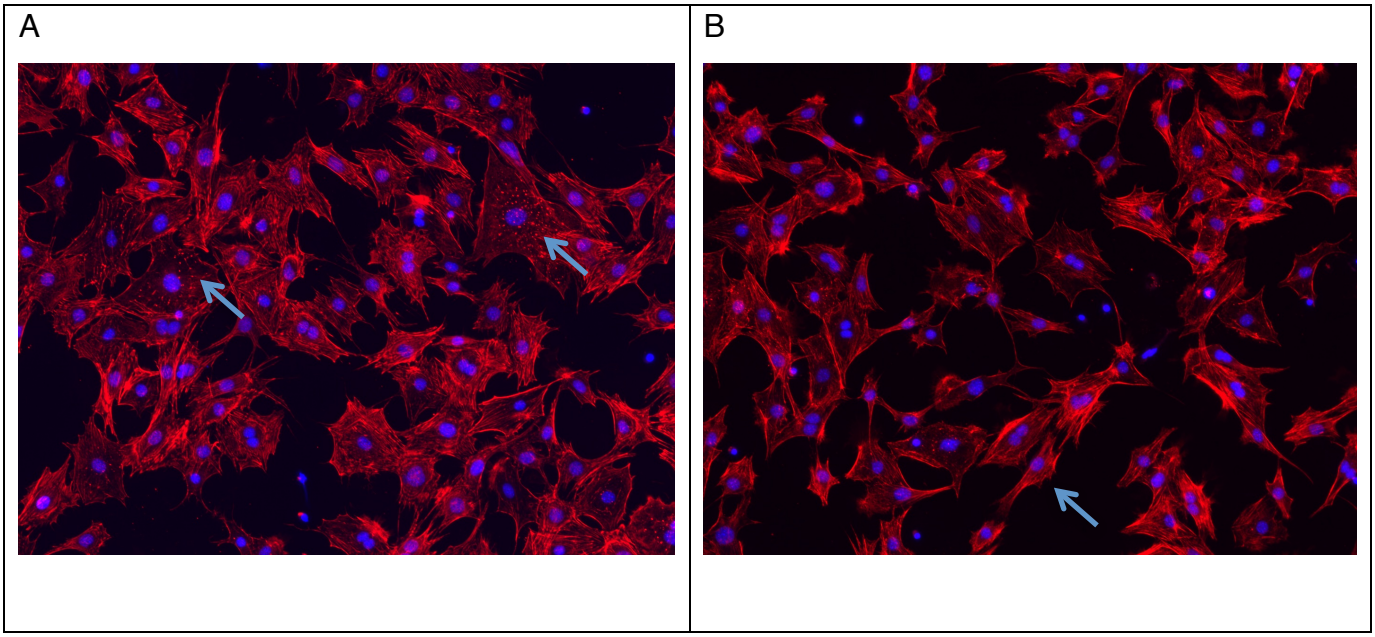


Fig7 Immunolabeled preparation of osteogenic cells grown on Poli-Ti (A) and Nano-Ti (B) surfaces in FBS-culture condition at day 3 of 1h/3h experiments (20X): Actin cell oulines (red), cell nuclei (blue), Ki-67 nuclear protein (green). Dividing cells (arrows) are present in both metal surfaces in all conditions (compare with Fig.8).

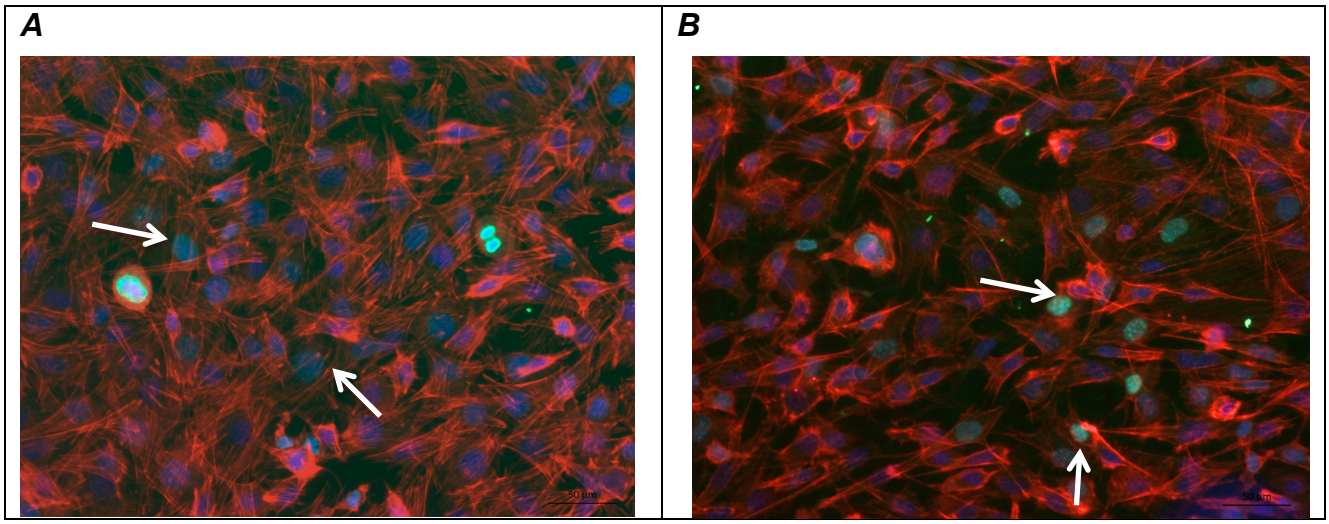


Fig.8 Immunolabeled preparation of osteogenic cells grown on Poli-Ti (A) and Nano-Ti (B) in NoFBS-culture condition at day 3 of 1h/3h experiments (20X): Actin cell oulines (red), cell nuclei (blue), Ki-67 nuclear protein (green).

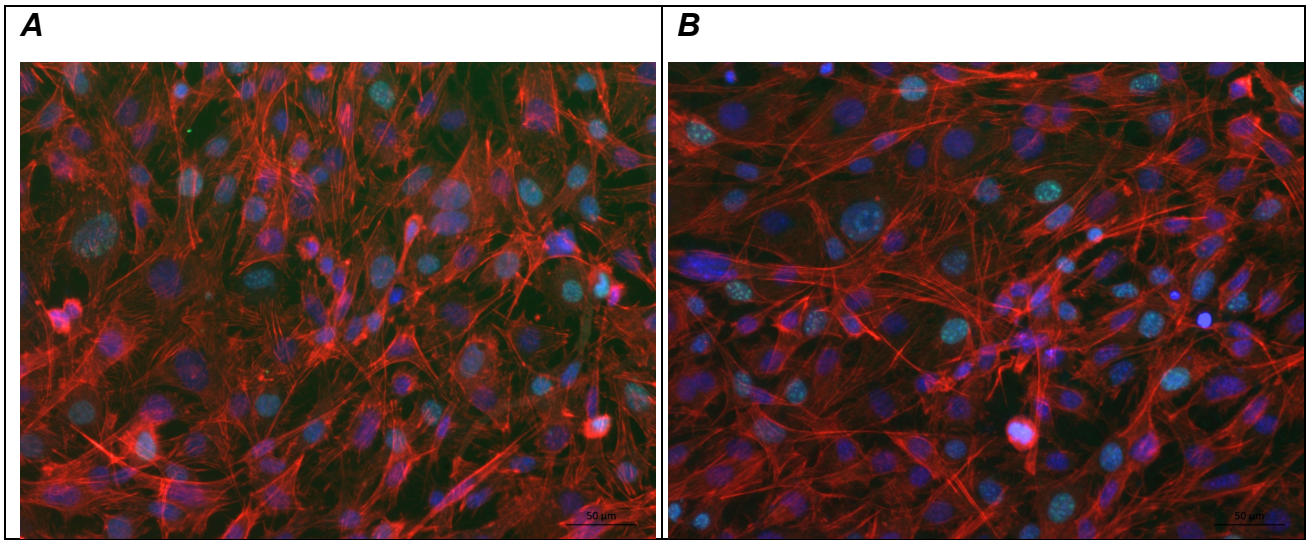


Fig.9 Immunolabeled preparation of osteogenic cells grown on Glass in NoFBS-culture condition at day 3 of 1h/3h experiments (20X): Actin cell outlines (red), cell nuclei (blue), Ki-67 nuclear protein (green). Dividing cells (arrows) are evident; cells on glass are less spread than on metal substrates (compare with Fig.8).

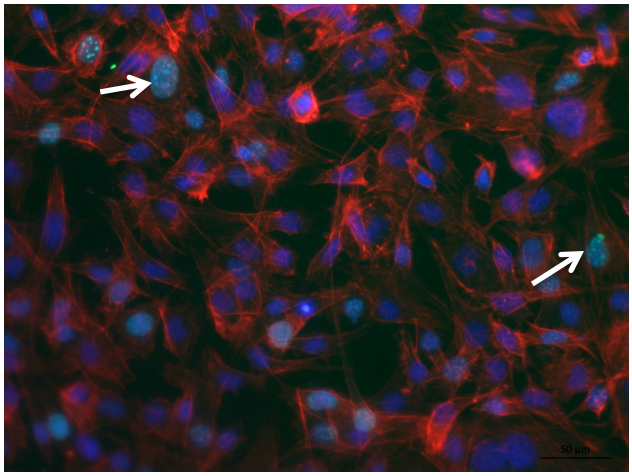


Fig.10 Immunolabeled preparation of osteogenic cells grown on Poli-Ti (A) and Nano-Ti (B) in FBS-culture condition at day 3 of 1h/24h experiments (20X): Actin cell outlines (red), cell nuclei (blue), Ki-67 nuclear protein (green).

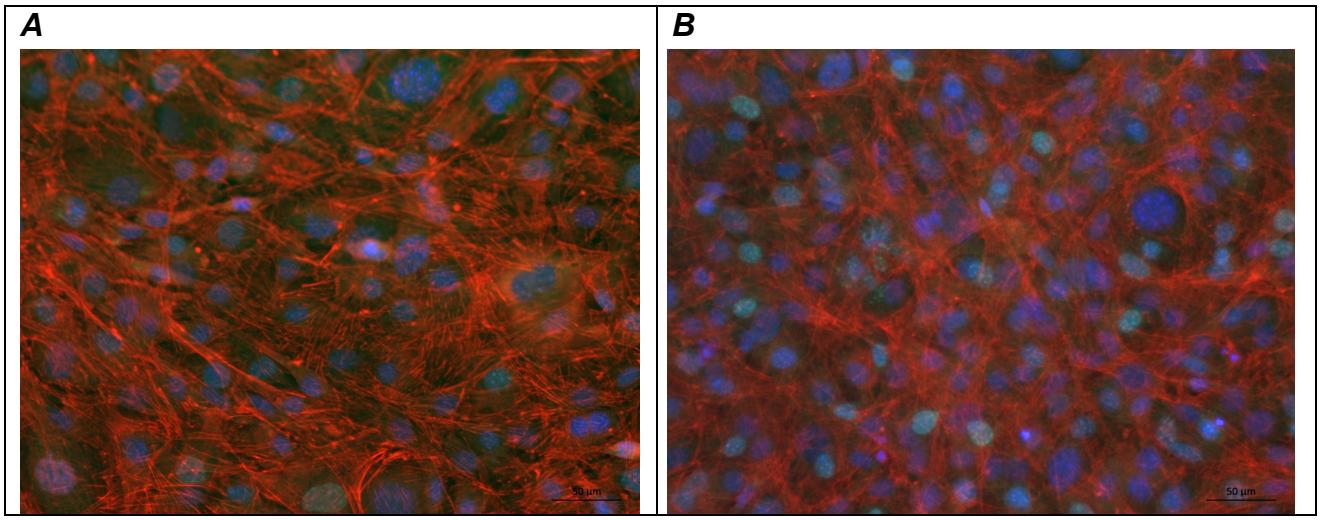


Fig.11 Immunolabeled preparation of osteogenic cells grown on Poli-Ti (A) and Nano-Ti (B) in NoFBS-culture condition at day 3 of 1h/24h experiments (20X): Actin cell outlines (red), cell nuclei (blue), Ki-67 nuclear protein (green). Some wrinkles in the cell cytoskeleton were observed, regardless the substrate (arrows).

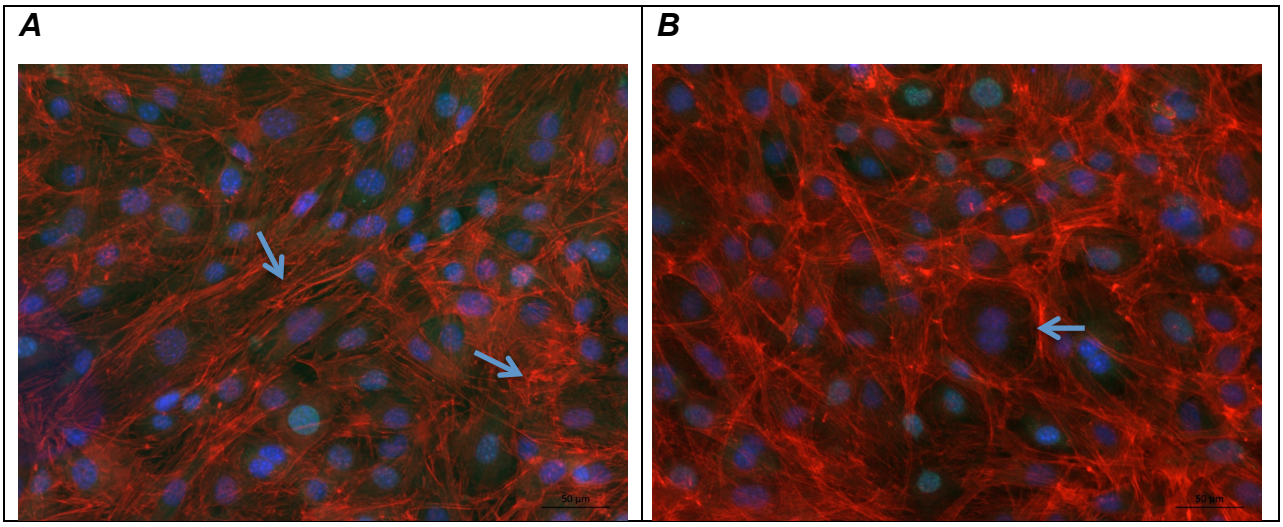


Fig.12 Immunolabeled preparation of osteogenic cells grown on Nano-Ti (A) and Glass (B) in NoFBS-preadsorp condition at day 1 of 1h/24h experiment (20X): Actin cell outlines (red), cell nuclei (blue), Ki-67 nuclear protein (green). Mitotic activity (green nuclei) was evident on all substrates in NoFBS-preadsorp condition.

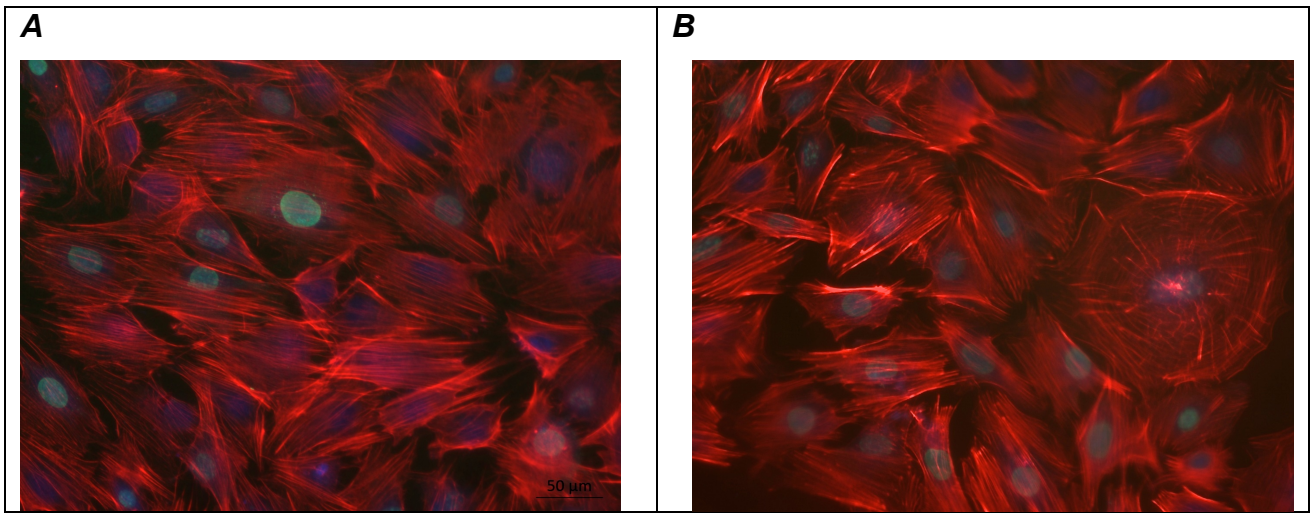


Fig.13 Immunolabeled preparation of osteogenic cells grown on Poli-Ti in FBS-culture (A) and NoFBS-culture (B) at day 1 of 1h/24h experiment (20X): Actin cell outlines (red), cell nuclei (blue), Ki-67 nuclear protein (green). Mitotic activity (green nuclei) was evident on all substrates in NoFBS-culture condition except for Glass.

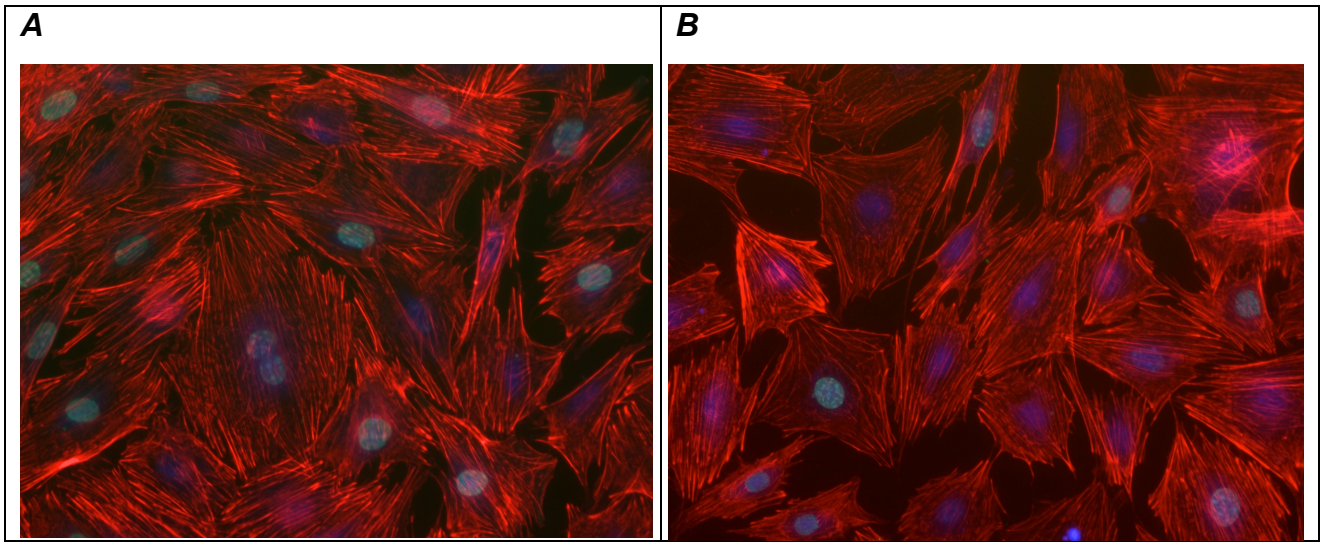


Fig.14 Osteogenic cells at day 3 of 1h/3h experiment (20X). A) Immunolabeled preparation on Poli-Ti in FBS-culture: Actin cell outlines (red), cell nuclei (blue), Osteopontin (OPN) bone related protein (green).
B) Staining for alkaline phosphatase (ALP) bone-marker protein (blue) and actin (pink) on Nano-Ti in FBS-culture. OPN (arrows in A) and ALP (arrows in B) positive cells indicate initial osteogenic activity on metal surfaces.

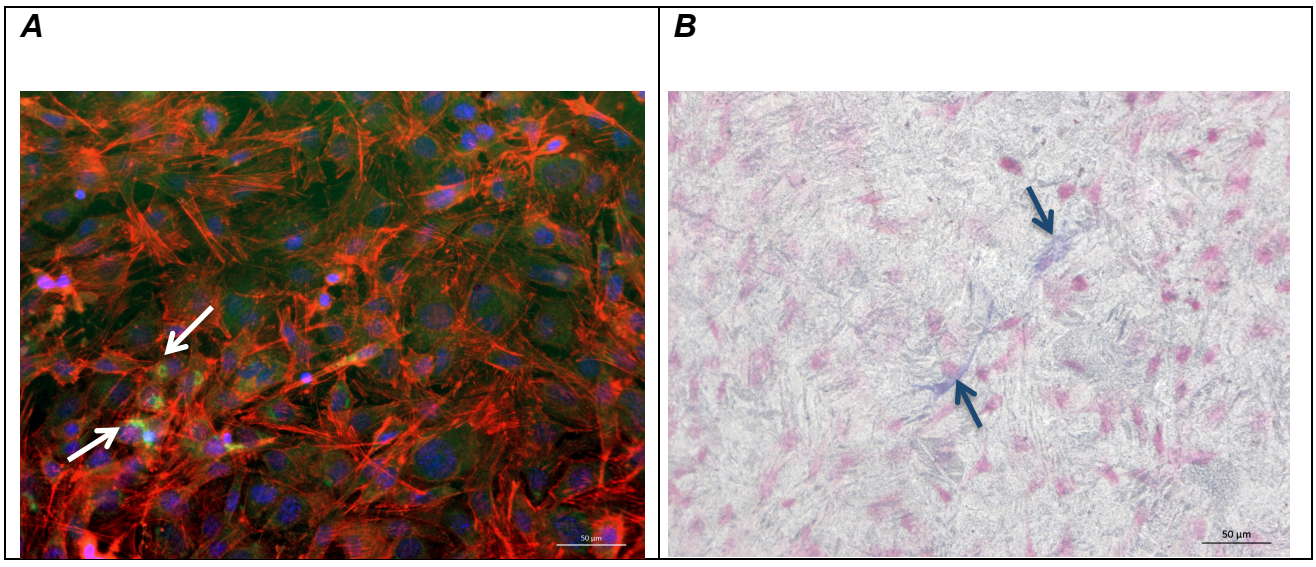


Fig.15 Single channel (green) image of immunolabeled preparation for OPN of osteogenic cells on Poli-Ti in FBS-culture (A) and in NoFBS-culture (B) at day 3 of 1h/3h experiment (20X): bright green dots (arrows) highlight the OPN positive cells.

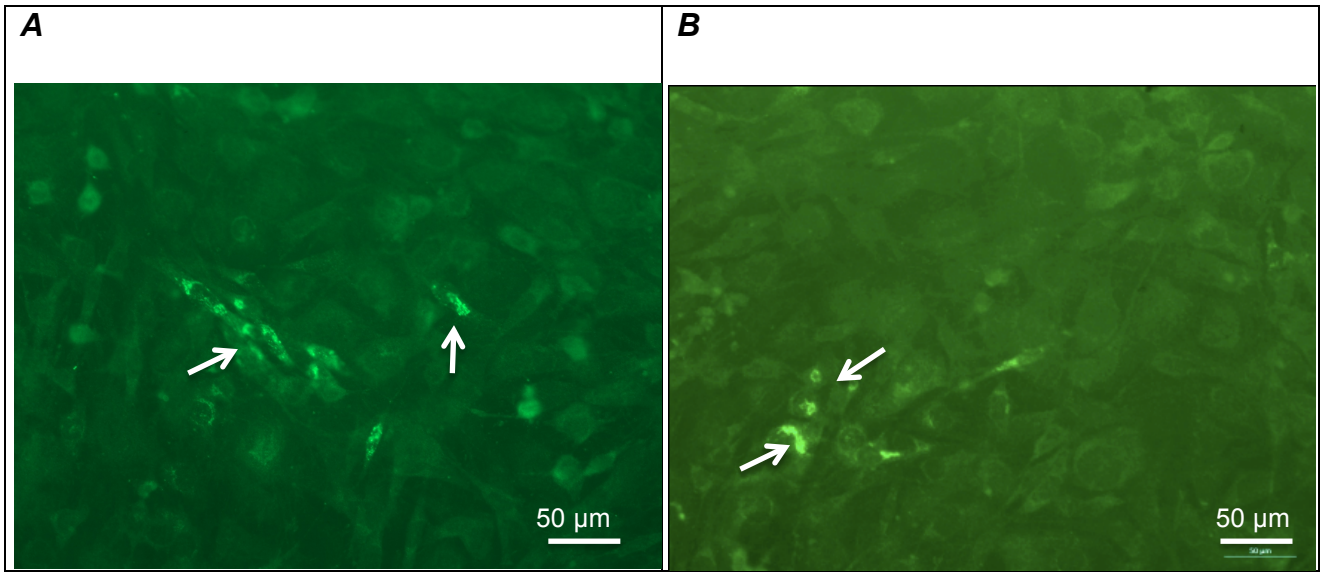


Fig.16 Scanning electron micrographs of osteogenic cells grown on Titanium surfaces at 3h of 1h/3h experiment, 500X: A) Poli-Ti in NoFBS-preadsorp; B) Nano-Ti in FBS-culture; C) Nano-Ti in NoFBS-culture. Round and irregular cell shape.

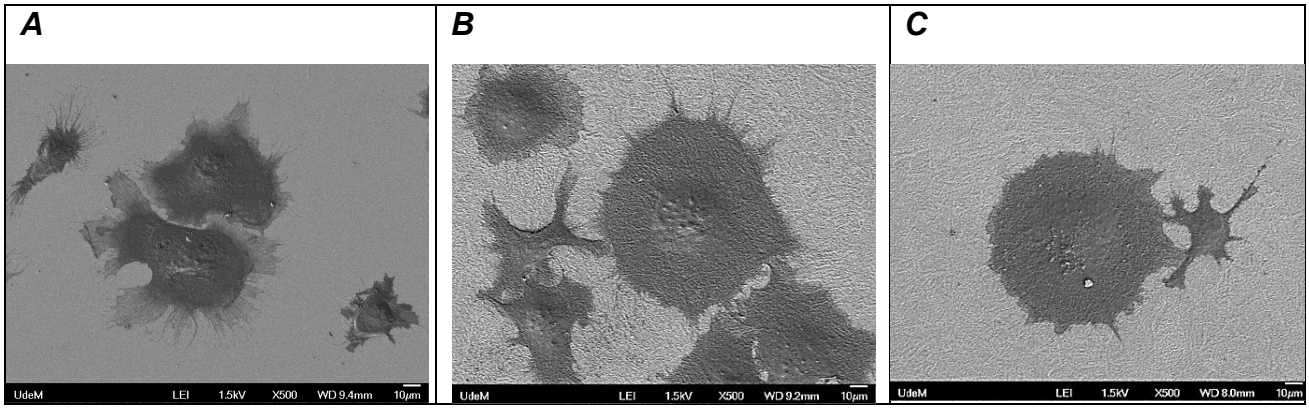


Fig.17 Scanning electron micrographs of osteogenic cells grown on Titanium surfaces at 3h of 1h/3h experiment: A) Poli-Ti in NoFBS-preadsorp; B) Nano-Ti in FBS-culture; A) Numerous, veil-like actin filaments at the periphery of the cell. B) Abundant cytoplasmatic interconnections among adjacent cells.

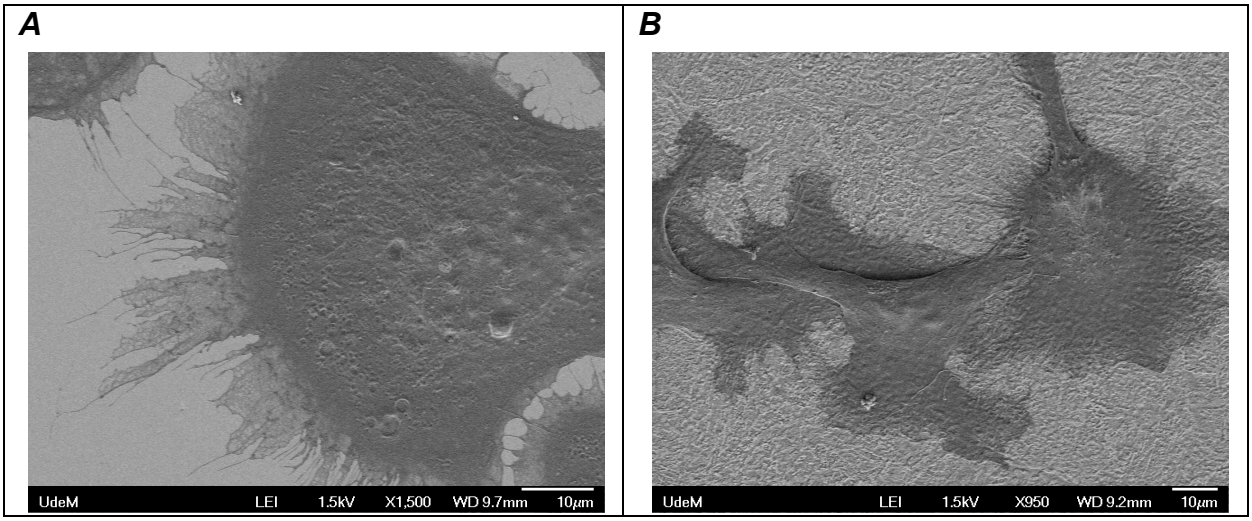


Fig.18 Scanning electron micrographs of osteogenic cells grown on Nano-Ti in NoFBS-culture at 3h of 1h/3h experiment: A) 200X: Irregular cells of small dimension; B) 1000X: Short actin filaments, most cytoskeleton concentrated around the cell nuclei.

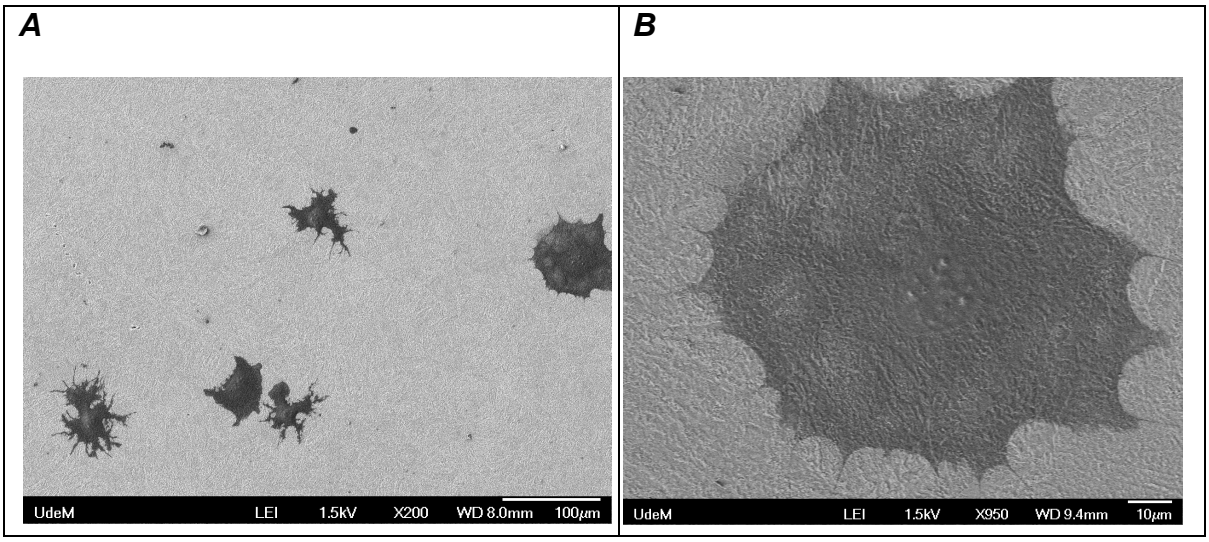
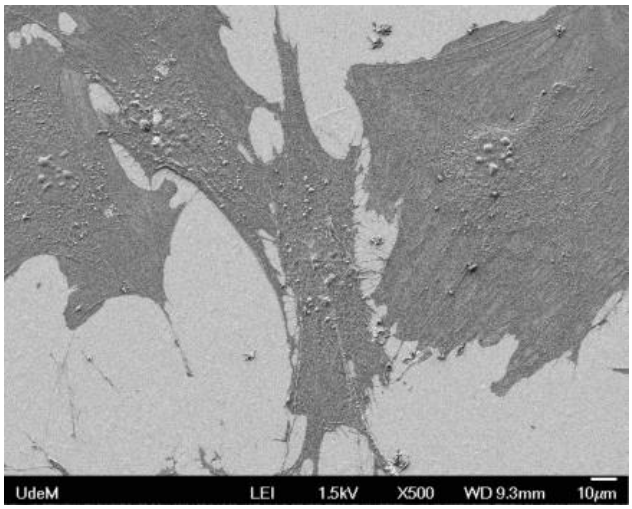


Fig.19 Scanning electron micrographs of osteogenic cells grown on Poli-Ti at day 1 of 1h/24h experiment, 500X: A) FBS-culture; B) NoFBS-culture. Predominant polygonal cell shape.

A



B

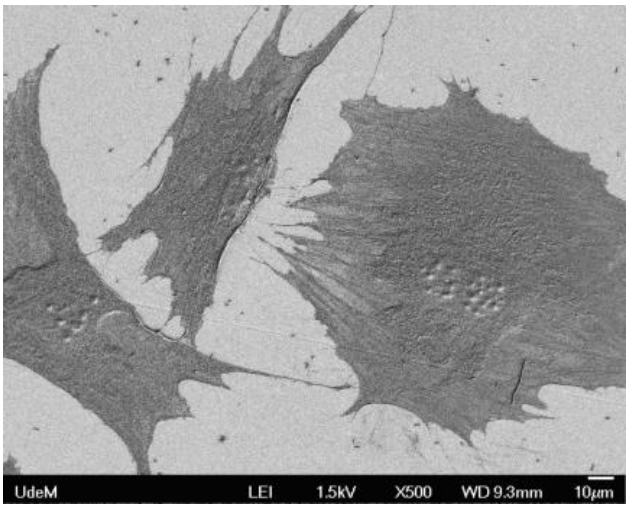


Fig.20 Scanning electron micrographs of osteogenic cells grown on Poli-Ti (A and C) and Nano-Ti (B and D) in FBS-culture (A and B, 1000X) and NoFBS-preadsorp (C and D, 5000X) at day 1 of 1h/24h experiment.

A, B) Cells on metal surfaces extended numerous projections. C, D) Higher magnification of large cell projections (star in C) and multiple thinner interconnections among cells (arrows in D).

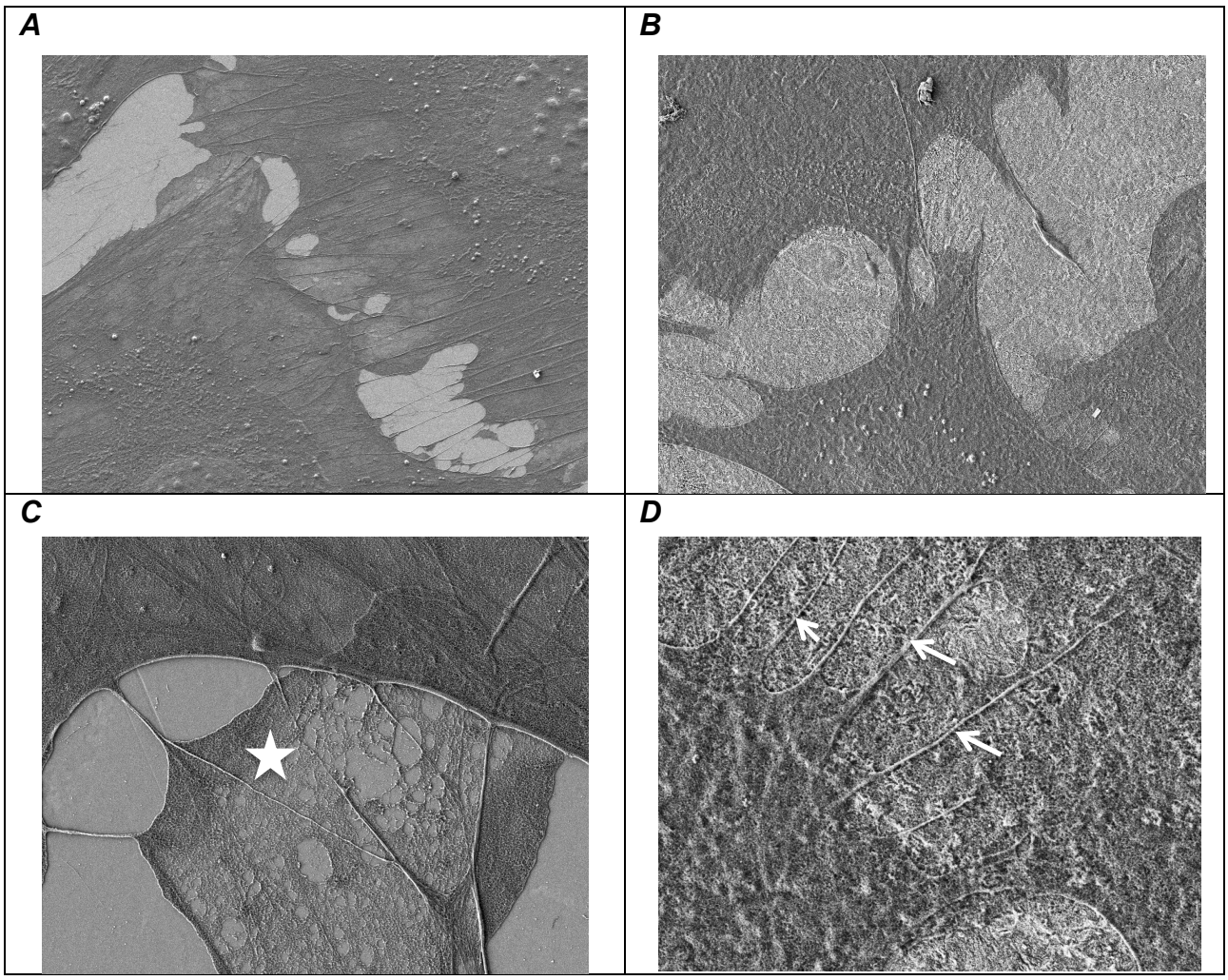


Fig.21 Scanning electron micrographs of osteogenic cells grown on Nano-Ti (A) in NoFBS-culture and in NoFBS-preadsorp (B) at day 1 of 1h/24h experiment, 1000X. A) Stellate cell shape with numerous thin lateral extensions. B) Uniform perinuclear actin spreading.

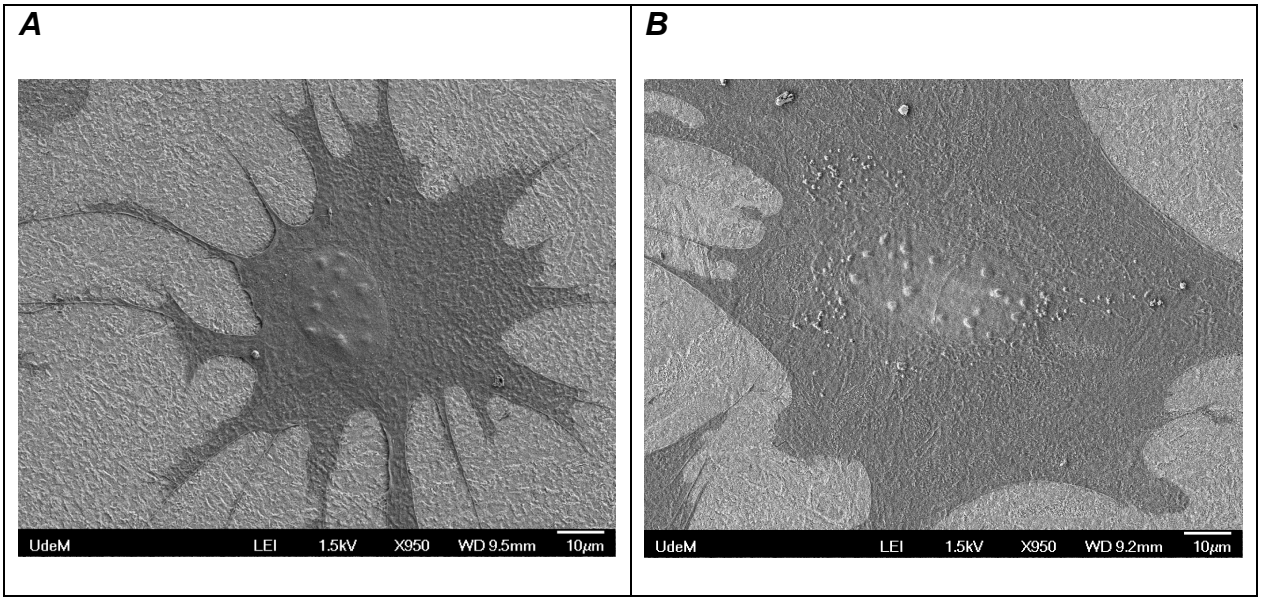


Fig.22 Scanning electron micrographs of osteogenic cells grown on Poli-Ti in NoFBS-preadsorp (A) and FBS-culture (B) at day 1 of 1h/24h experiment. A) 1000X. Large cytoplasmatic projection. B) 5000X. Higher magnification of long and straight filipodia emitted along the periphery of the cells,

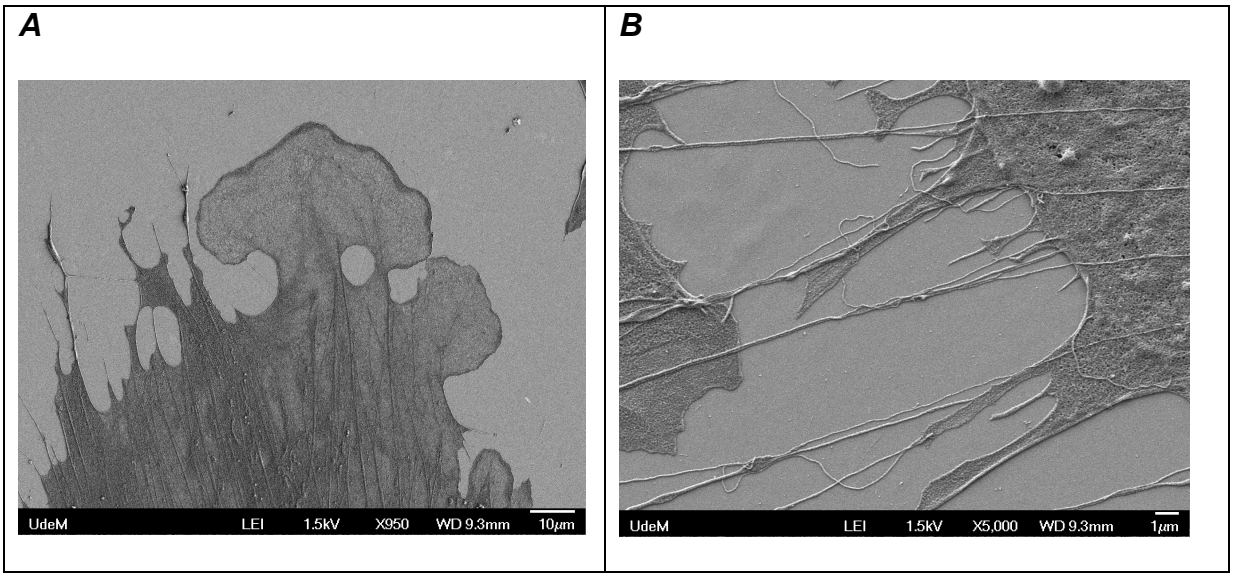


Fig.23 Scanning electron micrographs of osteogenic cells grown on Nano-Ti in NoFBS-preadsorp at day 1 of 1h/24h experiment. A) 1500X, co-presence of large, almost transparent projections and long thin filaments. B,C,D) Higher magnifications of the rectangular area in A. B) 10.000X. C) 30.000X. D) 60.000X. Little lateral filipodia (arrows in D) extending from the main filaments, attached and followed the underlying nanocavities of the surface.

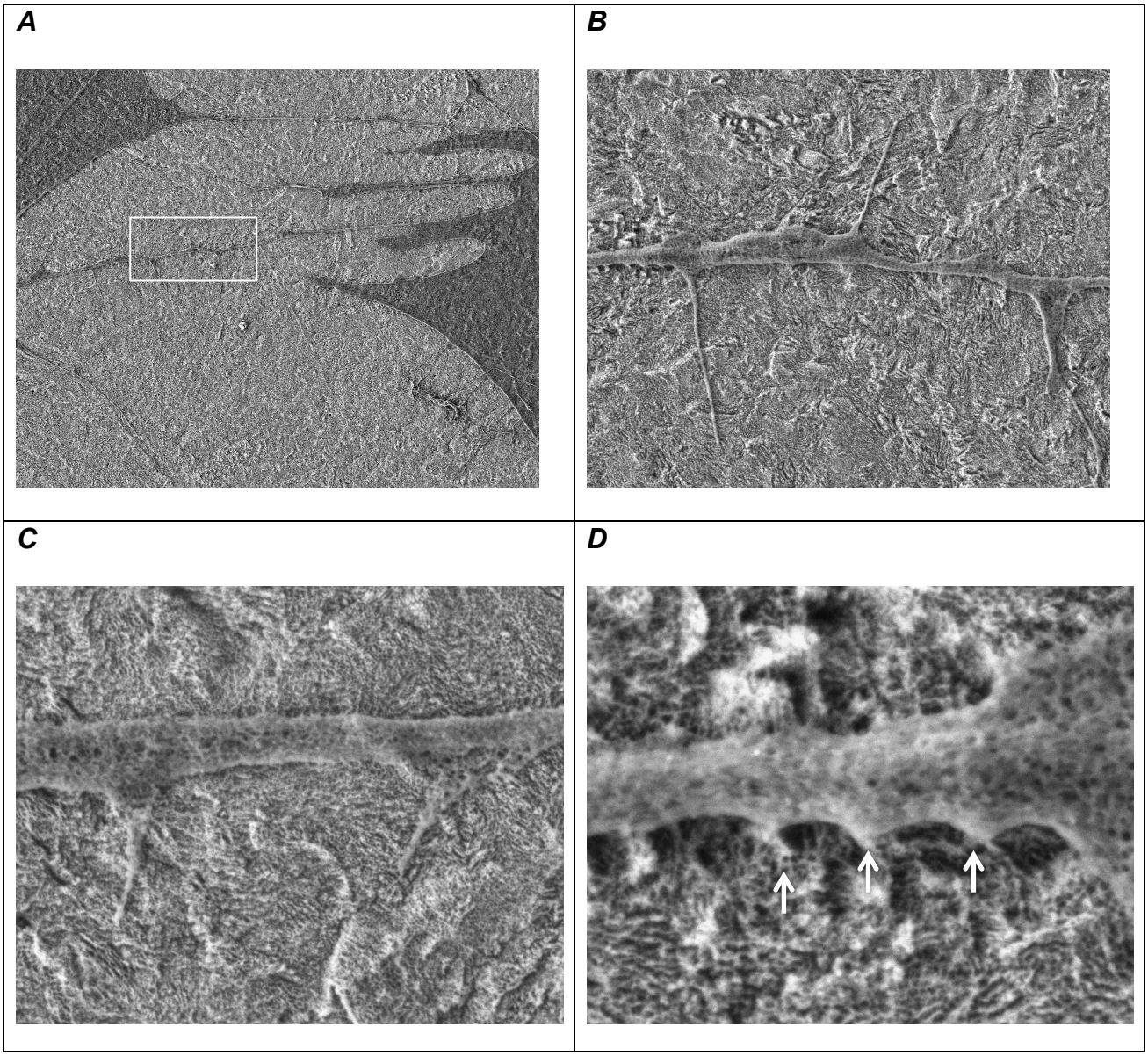


Fig.24 Scanning electron micrographs of osteogenic cells grown on Glass in FBS-culture at day 1 of 1h/24h experiment. A) 1000X. B) 3000X. Less abundant cytoplasmatic projections observed on Glass than on metal surfaces.

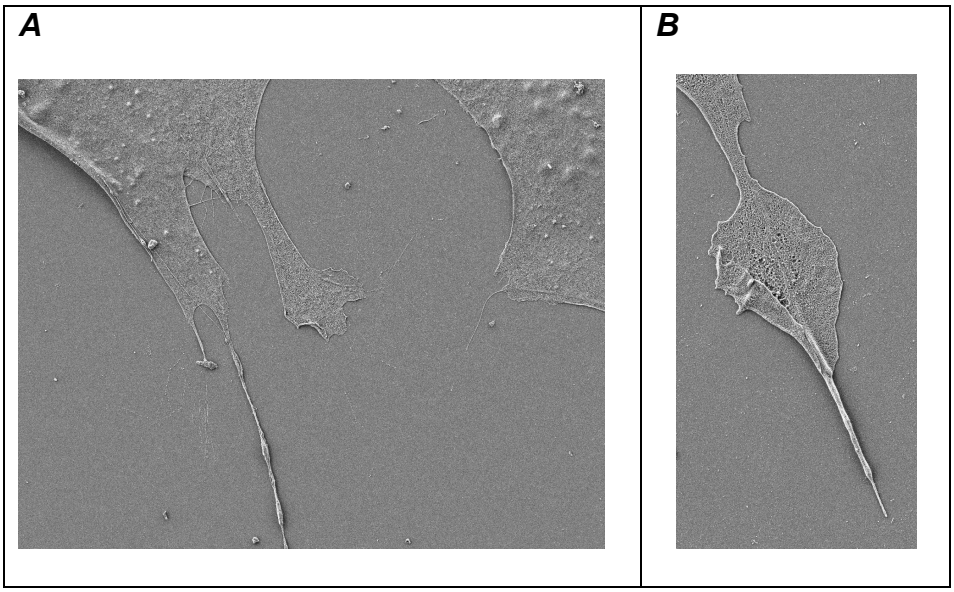


Fig.25 Scanning electron micrographs of osteogenic cells grown on titanium surfaces in NoFBS-culture at day 3 of 1h/3h experiment, 1000X. A) Poli-Ti. B) Nano-Ti. Cell nuclei (N). Cells superimposed each other and were more spread on the available surface than at day 1.

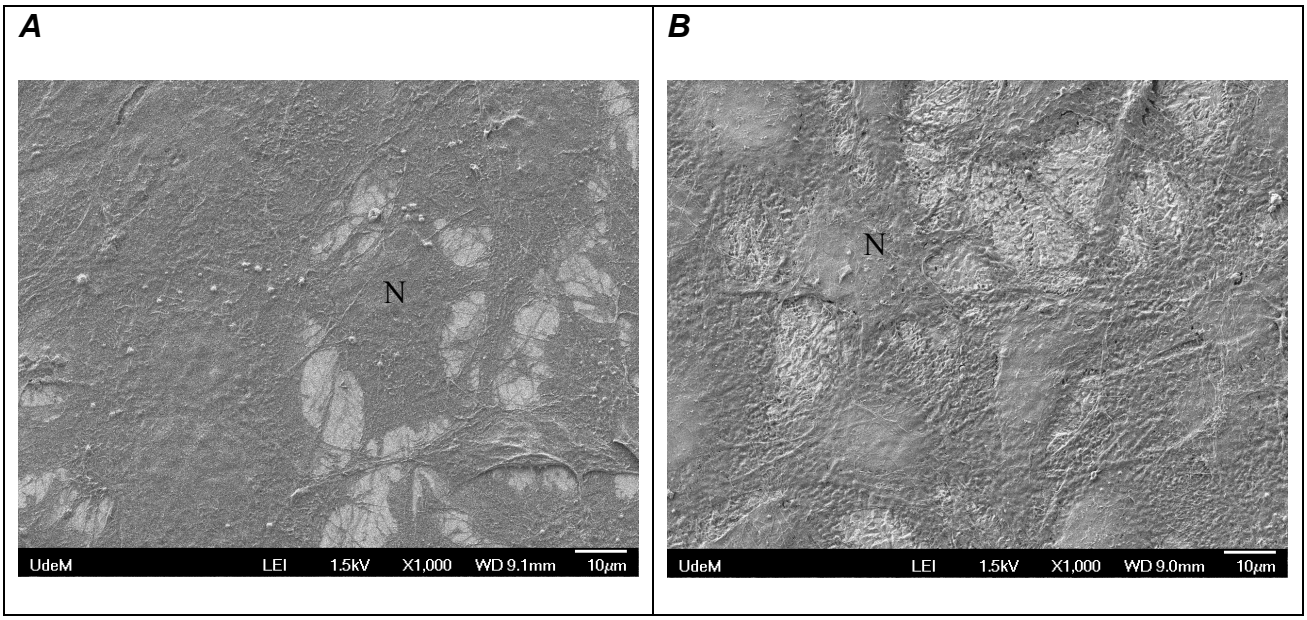
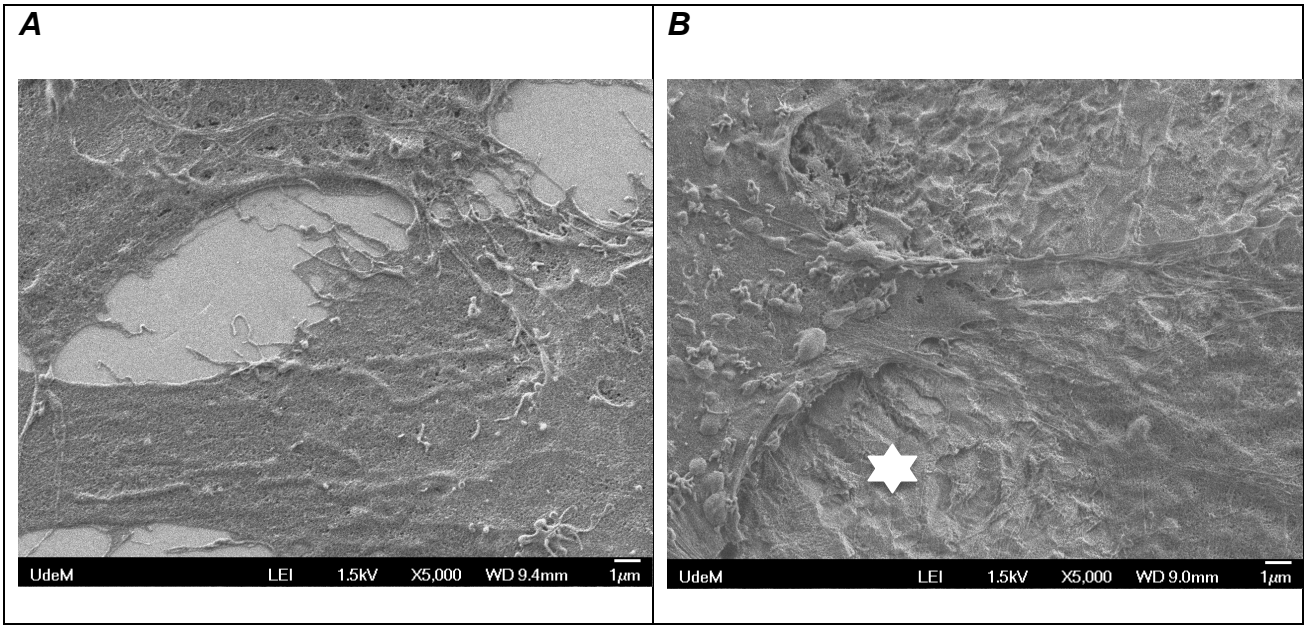
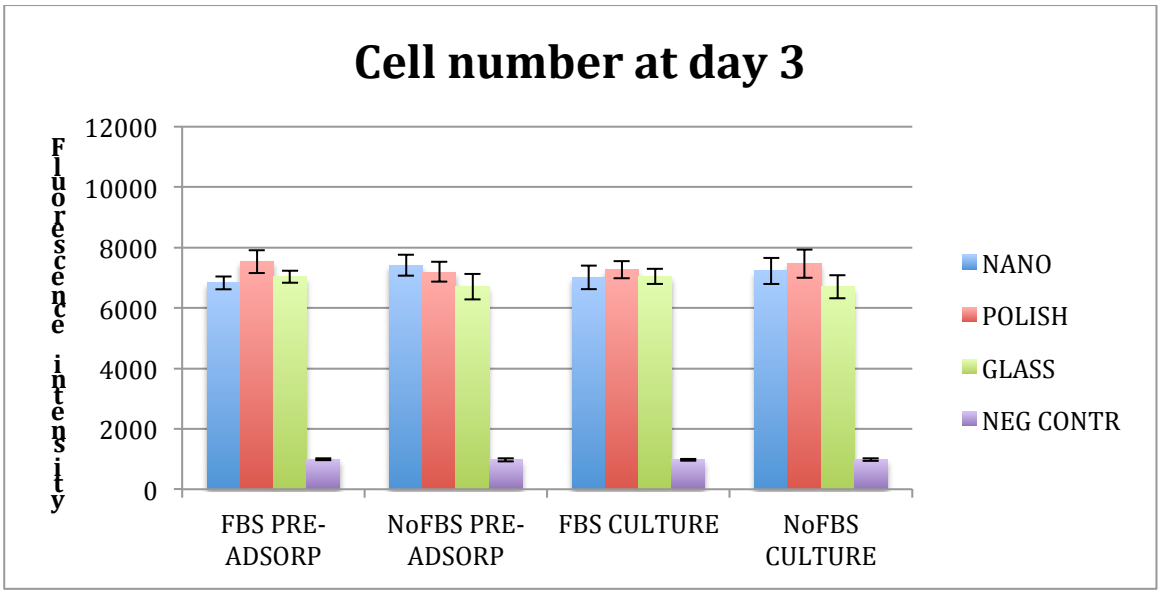


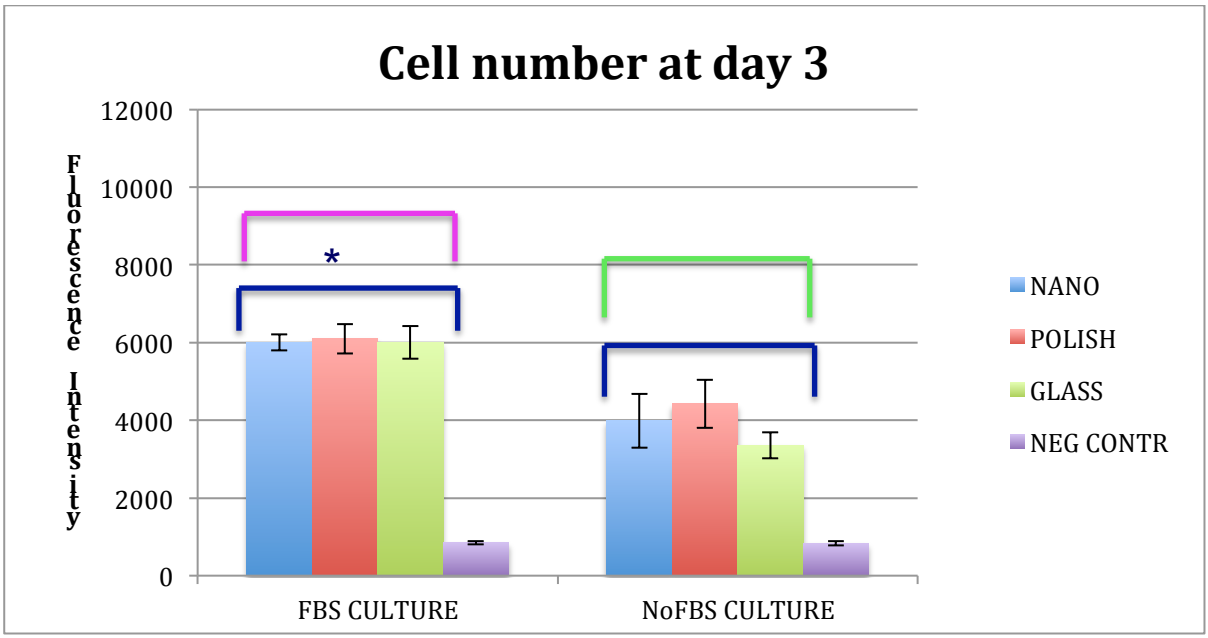
Fig.26 Scanning electron micrographs of osteogenic cells grown on titanium surfaces in NoFBS-culture at day 3 of 1h/3h experiment 5000X. A) Poli-Ti. Numerous cytoplasmatic interconnections. B) Nano-Ti. Extracellular accumulation of matrix (star).



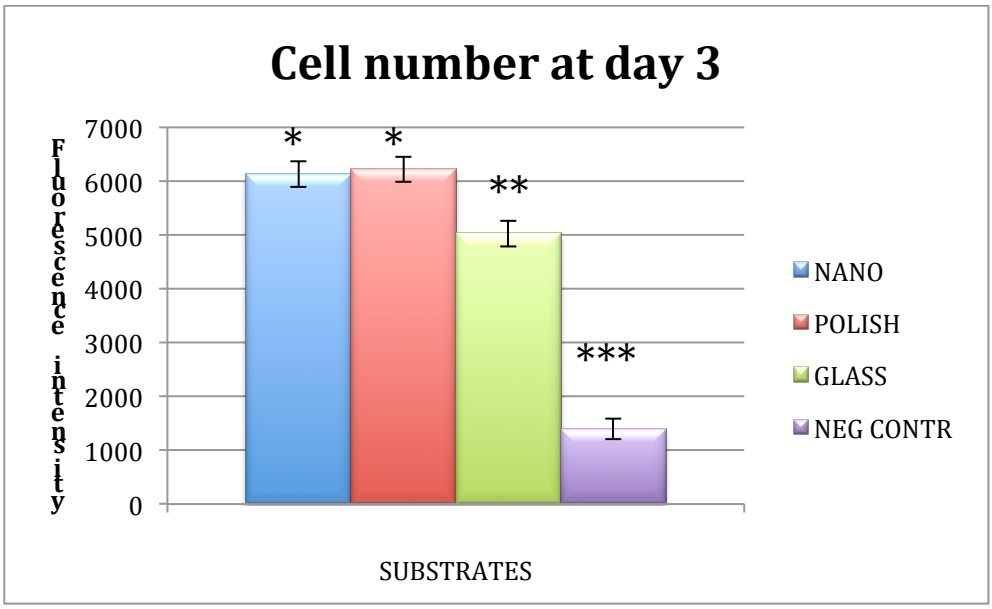
Graphic 1 Summary statistics of cell number (mean value ± standard deviation) as a function of FBS/NoFBS condition and substrate. Alamar Blue assay at day 3 of 1h/3h experiment. No significant differences were observed among conditions on each substrate.



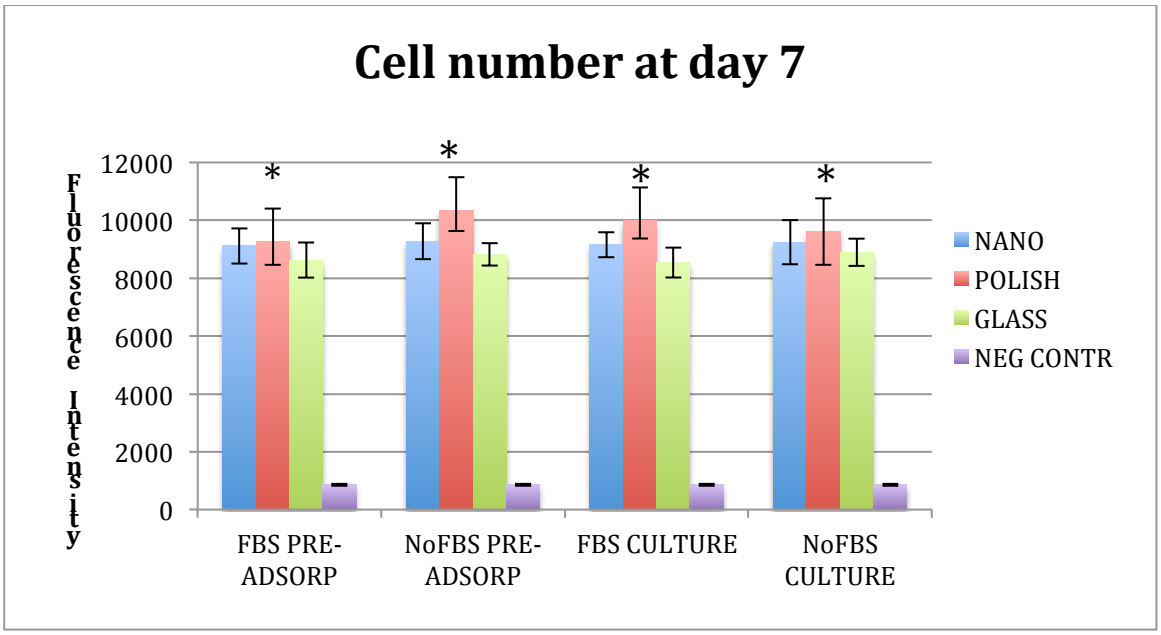
Graphic 2 Summary statistics of cell number (mean value ± standard deviation) as a function of culture condition and substrate. Alamar Blue assay at day 3 of 1h/24h experiment. A significantly different cell number was observed between FBS-culture and NoFBS-culture condition on each substrate (blue bars, *p<0.0001). Pink and green bars refer to comparison with Graphic 4.



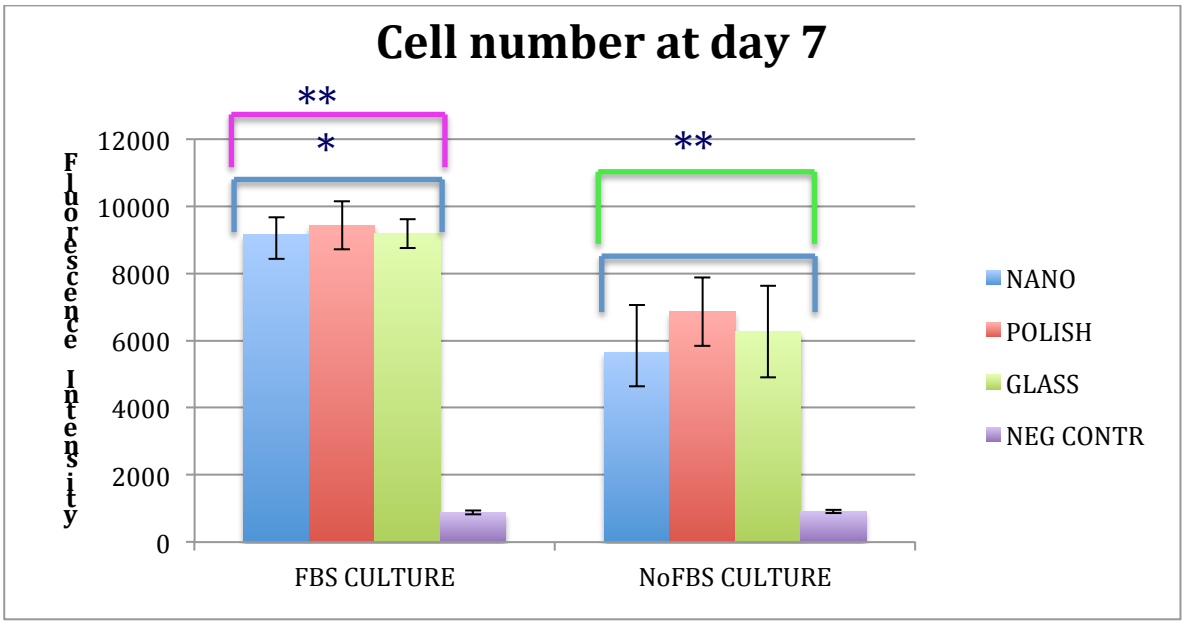
Graphic 3 Summary statistics of cell number (mean value ± standard deviation) as a function of substrate. Alamar Blue assay at day 3 of 1h/3h and 1h/24h experiments. Metal surfaces performed significantly better than glass surfaces. A significantly higher cell number was observed for Nano-Ti and Poli-Ti relative to Glass substrate; the number of asterisks depicts statistically homogeneous groups, (Ti vs Glass: $p<0.01$).



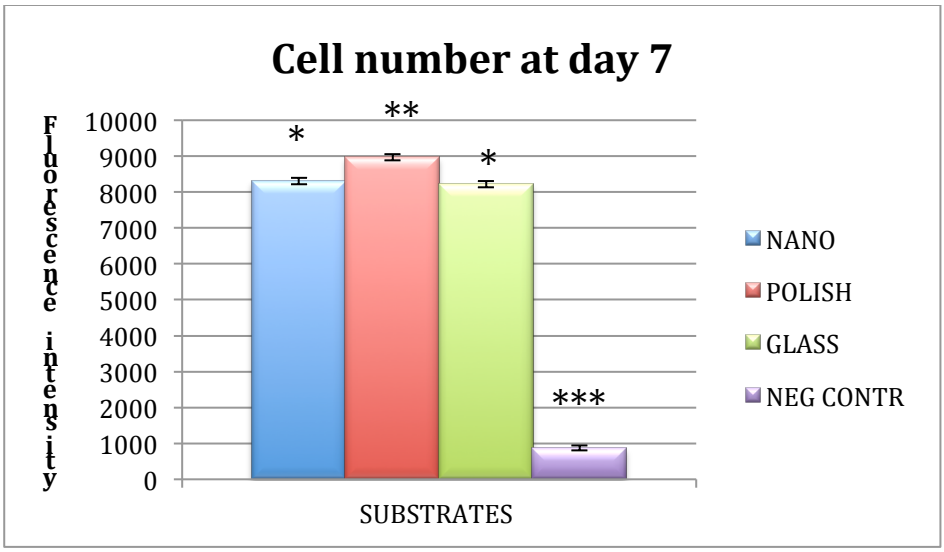
Graphic 4 Summary statistics of cell number (mean value ± standard deviation) as a function of FBS/NoFBS condition and substrate. Alamar Blue assay at day 7 of 1h/3h experiment. No significant differences were observed between conditions on each substrate. A significant increase in cell number was observed for each condition as compared to day 3 in Graphic 1, (*$p<0.0001$).



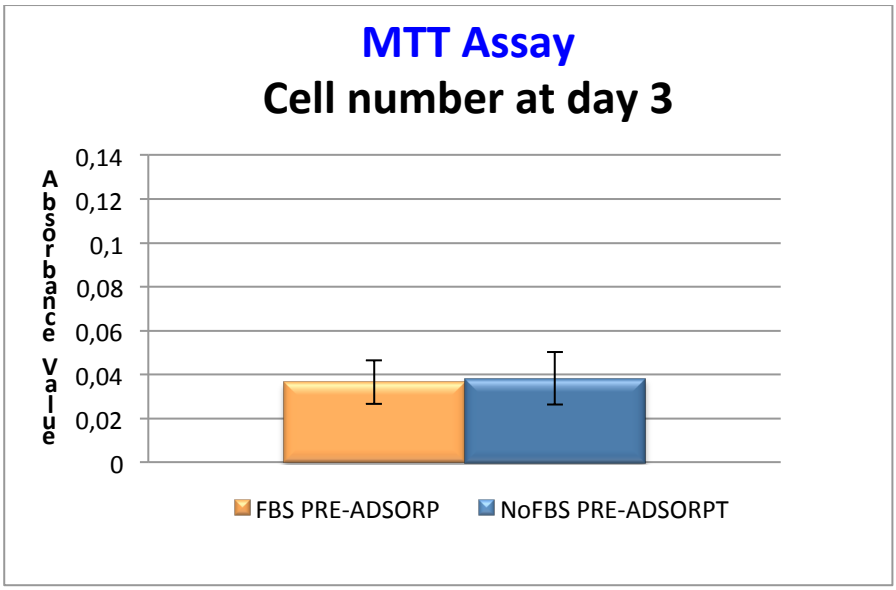
Graphic 5 Summary statistics of cell number (mean value ± standard deviation) as a function of culture condition and substrate. Alamar Blue assay at day 7 of 1h/24h experiment. A significantly higher cell number was observed for the FBS-culture condition relative to the NoFBS-culture condition (blue bars, *p<0.0001) and for each culture condition as compared to day 3 in Graphic 2 (pink and green bars, **p<0.0001).



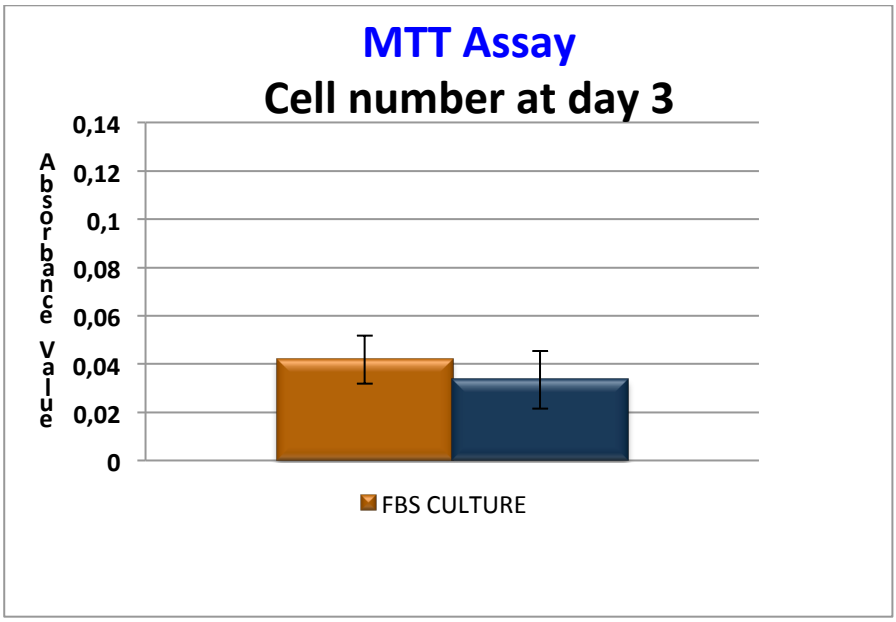
Graphic 6 Summary statistics of cell number (mean value ± standard deviation) as a function of substrate. Alamar Blue assay at day 7 of 1h/3h and 1h/24h experiments. A significantly higher cell number was observed for Poli-Ti relative to Nano-Ti and Glass substrates; the number of asterisks depicts statistically homogeneous groups, (p<0.0001).



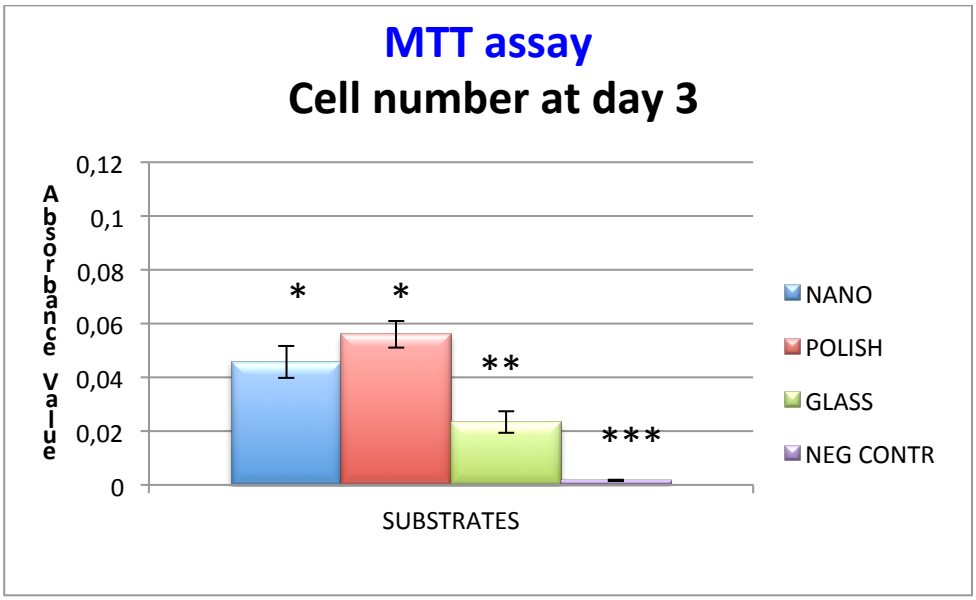
Graphic 7 Summary statistics of cell number (mean value ± standard deviation) as a function of pre-adsorption condition. MTT assay at day 3 of 1h/3h and 1h/24h experiments. No significant difference was observed between FBS-preadsorp and NoFBS-preadsorption condition, regardless the substrate.



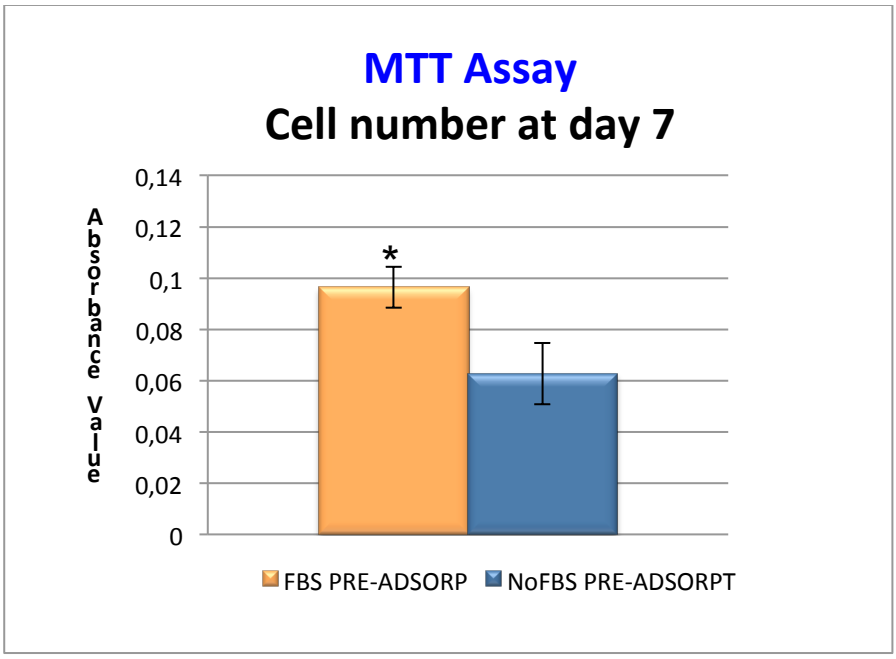
Graphic 8 Summary statistics of cell number (mean value ± standard deviation) as a function of culture condition. MTT assay at day 3 of 1h/3h and 1h/24h experiments. No significant difference was observed between FBS-culture and NoFBS-culture condition, regardless the substrate.



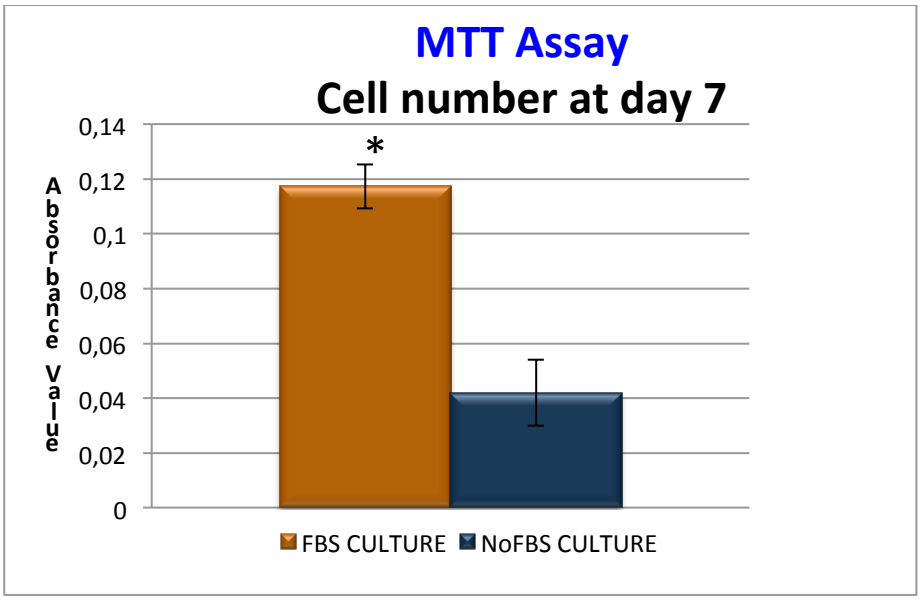
Graphic 9 Summary statistics of cell number (mean value ± standard deviation) as a function of substrate. MTT assay at day 3 of 1h/3h experiment. A significantly higher cell number was observed for Nano-Ti and Poli-Ti relative to Glass substrate; the number of asterisks depicts statistically homogeneous groups, (Nano-Ti and Poli-Ti vs Glass: $p<0.0001$).



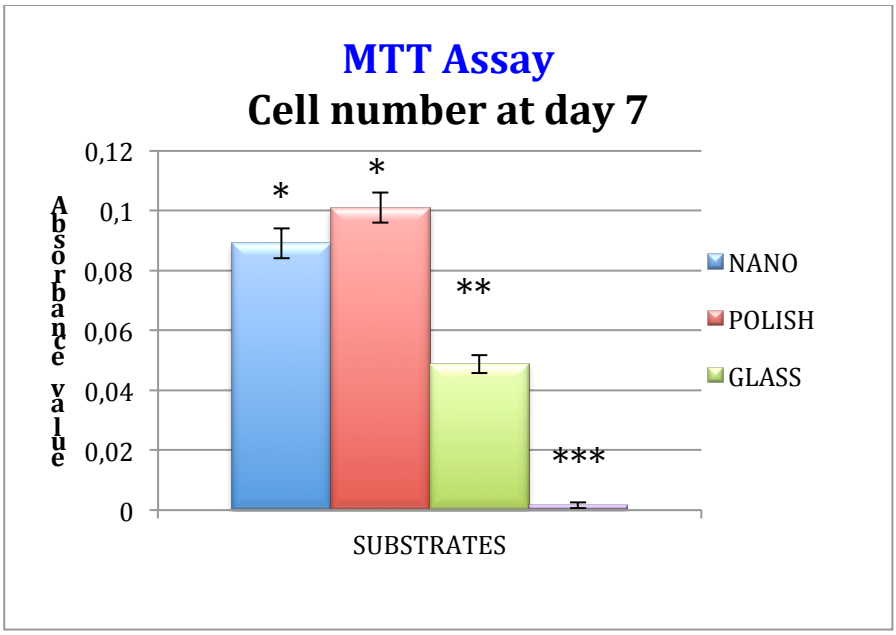
Graphic 10 Summary statistics of cell number (mean value ± standard deviation) as a function of pre-adsorption condition. MTT assay at day 7 of 1h/24h experiments. A significantly higher cell number was observed for the FBS-preadsorp relative to the NoFBS-preadsorp condition, on each substrate, (*$p<0.006$).



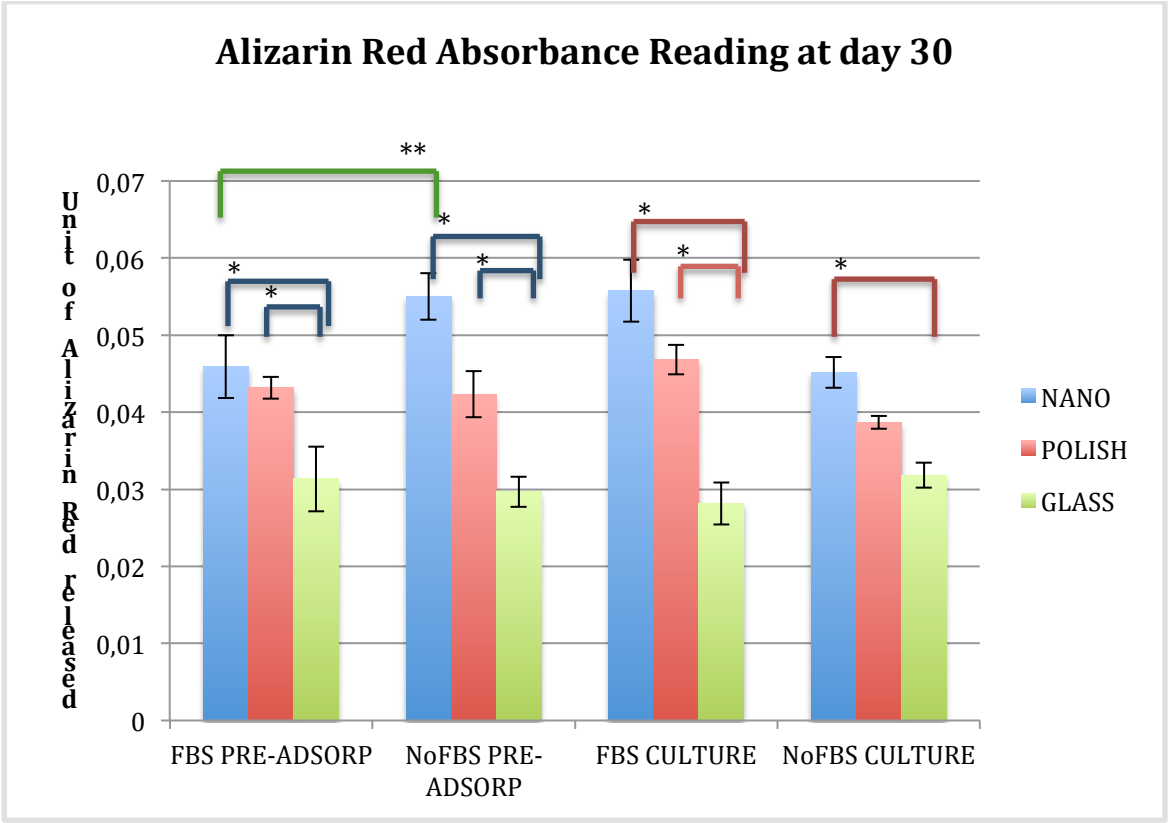
Graphic 11 Summary statistics of cell number (mean value ± standard deviation) as a function of culture condition. MTT assay at day 7 of 1h/24h experiments. A significantly higher cell number was observed for the FBS-culture as compared to NoFBS-culture condition, on each substrate, (*p<0.001).



Graphic 12 Summary statistics of cell number (mean value ± standard deviation) as a function of substrate. MTT assay at day 7 of 1h/24h experiment. A significantly higher cell number was observed for Nano-Ti and Poli-Ti relative to Glass substrate; the number of asterisks depicts statistically homogeneous groups, (p<0.0001).



Graphic 13 Summary statistics of cells mineral production (mean value ± standard deviation) as a function of condition and substrate. Alizarin red reading at day 30 of 1h/3h experiment. Cells on titanium substrates produced significantly more minerals than on glass under all conditions tested. Bars highlight the statistically different substrates, within each condition (blue and red bars, *p<0.0001), and among conditions (green bar, **p=0.004).



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