Cell interaction with nanopatterned surface of implants

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Cell interaction with nanopatterned implants

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Abstract

Metals such as titanium and alloys are commonly used for manufacturing orthopedic and dental implants because their surface properties provide a biocompatible interface with peri-implant tissues. Strategies for modifying the nature of this interface frequently involve changes to the surface at the nanometer level, thereby affecting protein adsorption, cell–substrate interactions, and tissue development. Recent methods to control these biological interactions at the nanometer-scale on the surface of implants are reviewed. Future strategies to control peri-implant tissue healing are also discussed.

Keywords

Nanostructured surface, Titanium implants, Protein adsorption, Cell adhesion, Cell differentiation, Osseointegration

Introduction

As populations age in industrial countries, the market for orthopedic prostheses and dental implants is growing steadily at a rate of 15 % per year. Total hip and knee joint replacements are routinely performed worldwide with high clinical success rates. In response to millions of edentulous patients, artificial dental roots are implanted in the maxillary and mandible bone to restore dentition (Figure 1). Many studies have shown that there is a strong correlation between clinical success rates over long periods and the early events of osseointegration of implants. In order to promote the biological anchoring of metal implants to the skeleton, different surface treatments have been developed and tested. The aim of this research is to favor protein adsorption, cell adhesion and bone tissue apposition. These biological properties are related to the chemical composition, wettability and roughness of metal surfaces. Grid-blasting, acid etching, chemical grafting, ionic implantation and calcium phosphate coatings are the most commonly used surface treatments on metal implants. Despite being the subject of considerable interest, the precise role of the surface properties of implants in the biological response remains poorly understood. As all the interactions between proteins and focal adhesion points of cells occur at the nanometer scale, it may be possible to activate intracellular molecular signaling pathways and, thus, to control cell fate using nanostructured surface implants.

Recent literature reports several methods for preparing surfaces structured at the nanometer scale and studying the behavior of cells on these surfaces. Most of these nanostructured surfaces have been obtained on polymers [1] but seldom on metals [2, 3]. On metal surfaces, enhanced cell metabolic activity has been observed, such as the up regulation of bone sialoprotein and osteopontin [1], as well as a threefold increase in osteoblastic cell adhesion as compared to the surfaces without nanostructure. Nevertheless, none of these studies have investigated reproducible networks of nanometer sized features using a systematic and high throughput methodology. For the purpose of studying proteins, cells and tissues interactions, surfaces should exhibit repetitive ordered features such as pores, holes or lanes in the nanometer range. However, standard physical and chemical treatments usually produce surfaces with random, highly variable topologies making the assessment of biological phenomena rather unpredictable. It is thus difficult to establish a precise correlation between biological activities and implant surfaces due to the absence of standardized surfaces with repetitive topography at the nanometer size level (e.g. pits with fixed diameters and depth, lanes with controlled profiles). Furthermore, implant surfaces are often developed using a ‘trial and error’ approach sacrificing many animals during *in vivo* testing.

Nanotechnology derived from the microelectronics industry may facilitate the development of nanoscale topographical manipulation, and the fabrication of highly controlled experimental substrates for biological assays. Processing methods such as lithography, ionic implantation, and radio frequency plasma treatments may produce surfaces with controlled properties at the nanometer scale. These advances in nanotechnology can help us understand biological interactions and develop new implant surfaces with predictable tissue integrative properties. Topographical modification at the nanoscale level may be a proficient way of guiding tissue integration on an implant surface. The "ideal surface" directing a specific cell lineage, and thus the formation of a specific tissue, remains a challenge in the implant industry.

This paper reviews the most recent literature related to the different methods for obtaining nanostructured surfaces and current knowledge of interactions with proteins, and cells at the stage of adhesion, proliferation and differentiation. As the differentiation of cells conditions the formation of a specific tissue around implants, predictable tissue integrative surfaces are envisioned as future trends.

Preparation methods for nanopatterned titanium implant surfaces

Several methods are generally used to prepare nanopatterned surfaces but most of these methods are difficult to apply to metal implants. Photolithography using direct contact masks has been widely used for patterning surfaces and studying interactions with cells [4]. However, the resolution of photolithography is usually limited to several hundred nanometers (≈ 600 nm). Chemical etching methods using strong acids such as HCl, H_2SO_4, HNO_3 and HF may be used to refine the size of the textures after lithography. Nanoimprint lithography can be performed on spin-coated titanium films to obtain nanometer-scale features with a range of 100 to 200 nm [5]. Another approach for studying biological interactions is to use nanostructured substrates made by other methods and then deposit a metal layer using electron beam evaporation. Puckett *et al*. [6] showed that aligned nanophase metal features improved early osteoblast functions (morphology and adhesion). After electron beam evaporation, different chemical moieties such as $–CH=CH_2$, -OH, $-NH_2$, -COOH have also been used in order to produce surfaces with a variety of electric charge properties [7]. Radio frequency plasma or glow discharge plasma may also be used to chemically modify the surface of titanium (Ti) implants and then enhance the surface energy [2, 8]. Plasma treatment in different gas atmospheres has been used for cleaning, activation and sterilization of implants. It has been shown that plasma treatment at low atmospheric pressure can effectively increase the wettability of titanium surfaces [8]. By changing the gas atmosphere, different chemical groups may be grafted to the titanium surface

giving a variety of charge properties. This technique could improve the adhesion and differentiation of osteoblasts to titanium plates [8, 9]. Nevertheless, the method is limited by the non-specific treatment of the surfaces. It is indeed difficult to selectively attach chemical groups at precise locations to nanopattern the surfaces. As in photolithography, masking may be used, but again, it is limited to a resolution of approximately 1 μm [10].

The most promising methodology giving features of nanometer size dimensions on titanium implants is anodization. This consists of developing a titanium oxide layer using a platinum counter electrode in acidic solutions at a potential of 5-25 V. By changing experimental conditions (i.e. potential, temperature, electrolyte), several authors have produced an oxide layer a few microns in thickness and composed of a regular array of nanometer sized pores with diameters in the 30-100 nm range, growing perpendicular to the titanium surface [3]. The pore sizes are compatible with those of proteins such as fibronectin (FN) and vitronectin which play an important role in cell adhesion. Recent reports have shown that not only can cell adhesion be controlled by nanometer sized features, but also cell differentiation [2]. Furthermore, certain chemical elements from the electrolyte may be present in the porous oxide layer modifying surface properties [11-14]. Kim *et al*. [15] showed that water contact angle and elastic modulus were significantly reduced on the nanotubular surface compared with those prior to anodizing.

The challenges for the coming years are to optimize the preparation of nanopatterned surfaces on titanium implants for biological assays.

Analysis of implant surfaces

As shown in Figure 2, surface properties such as roughness, chemical composition, wettability and charges have a profound influence on cell behavior [16].

Atomic Force Microscopy (AFM) [17] and profilometry [18] are widely used to measure surface roughness at the micro- and nanolevel. Le Guehennec *et al*. [19] characterized polished and nanostructured metal surfaces by using AFM and measured roughness of 1.4 and 6.2 nm, respectively. Topography is known to modify cell adhesion and, indirectly, cell phenotype. It is generally accepted that the adhesion and proliferation of fibroblastic or epithelial cells is promoted on smooth surfaces [20] while osteoblastic cells adhere and proliferate better on rough surfaces than on smooth ones. Moreover, osteogenic differentiation is more rapid on grit blasted, acid etched, rough titanium [21].

Energy dispersive X-ray spectrometry (EDX) and X-ray photoelectron spectroscopy (XPS) are commonly used to investigate the chemical composition of surfaces. For instance, Lee *et al*. [22] examined the cell attachment and proliferation of osteoblastic cells on a Ti6Al4V alloy after

different surface treatments. They found elemental modifications in the titanium oxide layer by EDX and XPS with the presence of amphoteric OH groups. The wettability of surfaces seems to play a major role in the interactions of implants with biological fluids [23]. Surfaces with a low water contact angle or high surface energy, have been shown to enhance protein adsorption, cell adhesion, proliferation and osteoblastic differentiation [24, 25]. Zeta potential, measuring electric charges on surfaces, is also of a great importance in proteins and cell interactions. For instance, positively charged surfaces will interact specifically with negatively charged proteins at physiological pH and *vice versa* [26].

Protein adsorption on implant surfaces

Immediately after implantation, implants are in contact with a diversity of biomolecules present in blood that will interact differently depending on their surface properties. The nature, conformation and orientation of the proteins on the surface of implants will have a direct consequence on the recruitment, attachment, proliferation and differentiation of cells through integrin binding sites. As these cells will then ensure the development of a tissue in the peri-implant area, surface properties that can control protein adsorption are of key importance in the cascade of biological responses. Healing of tissues around implants is a complex phenomenon in which various populations of cells, cytokines, growth factors and extracellular matrix are involved. For instance, cell recruitment is determined by the presence of appropriate chemotactic factors that attract the circulating mesenchymal stem cells (MSCs) involved in tissue healing. These specific binding proteins should be present at the surface of implants for cell adhesion while appropriate growth factors and cytokines may be secreted for the proliferation and differentiation of cells. Thus, the material itself may induce a multiplicity of responses from the cells, which may achieve the desired tissue healing [27]. It has been shown that the adsorption of proteins is related to surface properties [28]. The wettability and Zeta potential of surfaces are major surface properties for interactions with proteins. These properties influence the nature, quantity, density and orientation of proteins by modifying attractive and repulsive changes, led by chemistry and topography [29, 30]. Titanium is a hydrophobic surface [26] with a contact angle of around 80°, a negative charge and a Zeta potential at -9 mV at physiological pH of 7.5. Most proteins present in blood are negatively charged at physiological pH.

Cellular interactions with implants are primarily led by proteins, through a complex series of adsorption and displacement steps known as the Vroman effect [31]. It has been shown that differences in surface wettability or hydrophilicity (low contact angle) result in modifications in protein absorption on implants [32]. Cai *et al*. [26] showed that Ti functionalized with -NH_2

groups had a contact angle similar to Ti (70° instead of 80°) but improved protein adsorption. In this example, the Zeta potential was 0 mV for Ti-NH_2 as compared to -8mV for Ti. On the other hand, Ti functionalized with -COOH has similar Zeta potential and protein adsorption but the contact angle was different (80° for Ti and 50° for Ti-COOH). In this study, they showed that protein adsorption depends more on Zeta potential than contact angle. Fibronectin (FN) has an isoelectric point (pI) between 4.9 and 6.3 at pH 7.4, thus resulting in a negative charge. Albumin is the most abundant protein in blood and is also negatively charged at physiological pH (pI: 4.9) and it may compete with FN for absorbing on titanium surfaces. Sousa *et al*. [33] showed that FN is less resistant to displacement by other serum or cell derived proteins during initial cell attachment and later during spreading and growth phases. Both the orientation and distribution of FN on to nanostructured surfaces have recently been proposed to control not only the adhesion but also the differentiation of mesenchymal stem cells into osteoblasts [12]. The authors have hypothesized that spatial localization of FN on surfaces may elongate cells through focal points, a stress cytoskeleton through actin fibers which in turn induces the expression of osteoblastic genes. This hypothesis is illustrated in Figure 3.

In this diagram, proteins such as FN are adsorbed on to smooth titanium with random distributions and orientations that are influenced by the chemistry and wettability of the surfaces. On the other hand, proteins could specifically interact with nanopores and exhibit the RGD (Arg-Gly-Asp) cell-adhesion sites on nanostructured titanium through different conformations. These differences in protein adsorption may direct the differentiation of cells. This hypothesis should, however, be supported by stem cell gene expression on different surfaces. For instance, non differentiated mesenchymal stem cells are characterized by the expression of α-smooth muscle actin (αSMA), CD90, CD49a and CD105 cell surface markers. Osteogenic differentiation of stem cells is related to the expression of osteogenic markers such as Runx2, alkaline phosphatase (ALP), osteocalcin (OCN) and osteopontin (OPN). In order to validate the hypothesis of directing cell differentiation by surface properties, comparative studies should be performed using gene expression assays as described by Dalby [55]. Schneider *et al*. [34] also investigated the behavior of osteoblasts on titanium surfaces and found that the adhesion and proliferation of osteoblasts was better in the presence of serum due to the rapid formation of focal adhesions. Webster *et al*. [32] corroborated these results using osteoblasts, fibroblasts and endothelial cells seeded on nanophase ceramics (alumina, titanium, hydroxyapatite) and showed the ability of nanophase ceramics to selectively adsorb vitronectin, a mediator of cell adhesion. In addition to the nature and amount of proteins adsorbed on implants, the orientation of these biomolecules is of a great importance for cell adhesion on surfaces. For instance, differences in fibronectin (FN)

protein conformation on surfaces can affect the availability of RGD binding domains for cell adhesion [35]. Understanding of the effect of surface properties and nanostructures on protein adsorption is still a challenge for research and should be an approach to develop implant surfaces which allow better cellular interaction and thus better implant integration [36].

Effects of nanostructured implants on cell adhesion, proliferation and differentiation

As illustrated in Figure 4, the initial contact of cells on surfaces will determine their behavior. It is well known that cells interact with surfaces at the micro or sub-micrometer scale. Cells initially interact with surfaces through Van der Wall's forces. The presence of components of extracellular matrix such as FN and vitronectin will condition their anchoring to the surface through cell-membrane integrins. Integrin fixation is triggered by a complex cascade of biochemical and biomechanical pathways that culminate with the formation of focal points between cells and microenvironment [37]. After initial contact, cells produce filopodia that transiently sense the surface enabling optimum anchorage and spreading [38]. These cell-surface interactions have a direct effect on the spatial organization of the cytoskeleton. Several studies have illustrated that surface properties, particularly surface roughness, play a major role in cell adhesion: for instance, cells aligned along lanes on microstructured surfaces [3]. This phenomenon is called contact guidance and has also been observed on nanogrooved surfaces [39]. Anselme *et al*. [40] recently showed that surface chemistry at the nanometer scale rather than microtopography (Ra>1 µm) may explain why cell attachment is not systematically increased by rough surfaces [41-46]. This work corroborated that cells interact with surfaces at the micrometer and the nanometer range. It seems that cells respond to surface topography at both the micro and nanometer level but are sensitive to surface chemistry at the nanometer scale.

The difficulty in studying cell behaviors on implants relates to the use of standardized surfaces and quantitative biological assay methods. However, most physical and chemical surface treatments produce random, highly variable topologies making the assessment of biological phenomena rather unpredictable. In order to decipher the cell-surface interactions, normalized, well-characterized and reproducible surfaces must be used. For this purpose, nanotechnology should make it possible to obtain surfaces which are reproducible at the nanometer scale.

After standardization of surfaces, the numbers of adhering and proliferating cells on surfaces could be directly counted or determined by using metabolic assays [47]. Contact guidance, which is the ability of the cell to use substrate morphology for orientation and migration, could be visualized by the organization of actin fibers and cell morphology. Cell morphology and differentiation can be approached by immunostaining techniques. The cytoskeleton is usually

visualized by using actin antibody while focal adhesion points are stained with Vinculin. The osteogenic differentiation of cells on different substrates could be evaluated by the expression of markers such as OCN and OPN [1, 48], the mineralization of extracellular matrix using Alizarin red [49] or Von Kossa staining [50], or EDX or Fourier transform infrared spectroscopy (FTIR) mapping [50]. Bone morphogenetic protein-2 (BMP-2) and ALP production are both considered to be early bone formation markers. ALP activity could be histochemically stained [51, 52] or measured using p-nitrophenyl phosphate assay [52]. Polymerase chain reaction (PCR) arrays have recently been used to elucidate the molecular mechanisms implicated in cell response to nanostructured surfaces [1]. This technique is useful for screening limited gene sets associated with extracellular matrix, cell adhesion molecules and proliferating genes [5, 53]. The genes associated with adhesion and proliferation were up-regulated on nanostructured surfaces compared with the other genes. PCR array, DNA chips and real time PCR determination of osteogenic markers such as BMPs, bone sialoprotein (BSP), OCN, OPN, etc. could be carried out for studying the differentiation of cells on nanostructured surfaces [3, 54].

The influence of surface properties on cell behavior has been studied using many surfaces and cell types such as mesenchymal stem cells (MSCs), osteoblasts and fibroblasts. MSCs are stem cells derived from somatic tissue which can be differentiated into mesenchymal lineages such bone, cartilage, fat and skin. Dalby *et al*. [55] showed that MSCs differentiate better on random nanostructured surfaces without stimulation by osteogenic factors. Several papers have reported that increasing surface roughness enhanced osteoblastic differentiation while inhibiting cell proliferation [56-59]. However, others have shown that high surface roughness improved proliferation [13, 60]. Lipski *et al*. [48] observed the effect of nanoroughness on osteoblasts by immunostaining of actin. They found that cells decreased their surface areas when roughness increased from 50 to 300 nm. Differential protein adsorption on nanometer sized topographies influenced the density of FN adsorption and the focal adhesion points of osteoblasts [61]. Santiago *et al*. [62] showed that osteoblastic cell differentiation/viability/proliferation was greater for Ti samples treated with HCl / H_2SO_4 in comparison to surfaces immersed in sodium fluoride solution. Wang *et al*. [63] confirmed the role of chemistry on cell behavior. They found that positively charged functional groups promoted fibroblast attachment and spreading. OCN and ALP activity were highest on smooth polished surfaces and lowest on rough plasma-cleaned Ti, indicating that bone cell phenotypic expression can be altered by surface preparation techniques [27, 43]. Fibroblast adhesion and proliferation have been shown to be lower on nanoscale surfaces compared to smooth surfaces [64, 65].

Numerous reports have therefore demonstrated that nanometer sized features may control cell fate through protein adsorption and cell signaling pathways. It is possible to imaging developing new implant surfaces with predictable tissue integrative properties.

Future trends: controlling implant tissue integration with nanostructured surfaces

The nanostructure of implants seems to play a key role in controlling the differentiation of cells. In turn, it may correlate to the tissue integrative properties of implants. However, there is still a lack of correspondence between *in vitro* and *in vivo* experiments in relation to nanostructured surfaces. Ogawa T *et al*. [66] prepared Ti nanostructures by physical vapor deposition and tested their osseointegration in rat femurs. They found a surface area increased by up to 40 % and a greater strength of osseointegration for the nanostructured compared to an acid-etched surface. Sul *et al*. [67] prepared anodized surfaces with an oxide thickness varying between 200 and 1000 nm and implanted them into rabbit tibias for 6 weeks. They observed that implants with thick oxide layers were better osseointegrated than implants with an oxide layer more than 600 nm in thickness. Furthermore, Son *et al*. [12] showed that the removal torque strength was significantly higher for anodized implants (48.02 ± 5.92 N/cm) than the untreated implants (controls) (27.83 ± 1.78 N/cm) at 6 weeks after implantation in a rabbit model. It was concluded that the surface anodized implants resulted in a high interfacial strength at an early implantation period as compared to the non treated titanium implants. Eriksson *et al*. [68] examined both cell behavior and the healing response between hydrophilic and hydrophobic titanium implants after 3 weeks in rat tibias. They observed no significant difference in cell numbers between the two types of surface, but an increase in the expression of certain osteogenic markers on hydrophilic surfaces. Several studies have demonstrated that hydrophilic surfaces play a crucial role in the initial stage of wound healing due to higher thrombogenic properties than hydrophobic surfaces [69, 70]. Sawase *et al*. [71] showed that photo-induced hydrophilic implants in rabbit tibias enhanced bone formation with bone-to-metal contact of 28.2 % after 2 weeks of healing (control at 17.97 %). Certain authors have correlated the initial events in bone formation adjacent to the surface with the long term tissue response to these materials in humans [72, 73]. Schwartz *et al*. [72, 74] investigated the initial events in bone formation adjacent to glass ceramic, Ti, CaP and stainless-steel implants in rat tibias following bone marrow ablation.

All these studies tend to demonstrate that surface properties at the nanometer scale could determine the nature of the peri-implant tissue. Although the hypothesis is not fully validated to date, it may be possible to promote early osteogenic differentiation of cells and bone apposition rather than a fibrous encapsulation by using nanostructured implant surfaces [1, 27]. The

capacity of surfaces to direct MSCs into a specific lineage remains a challenge as few correlations between *in vitro* and *in vivo* experiments have been made. Furthermore, a systematic approach using standardized surfaces is usually not carried out.

Conclusion

Nanostructuring the surfaces of implants is a subject receiving considerable interest but the mechanisms leading to protein adsorption, cell attachment proliferation and differentiation, as well as tissue integration, remain poorly understood. Careful preparation of standardized nanostructured surfaces with repetitive topography may elicit protein adsorption, cell response and cell differentiation. Further research aiming at correlating cell behavior and tissue integration will be required in the future to decipher the role of surface nanostructures in these biological responses. It is possible to imagine controlling peri-implant tissue healing by changing the surface properties at the nanometer scale. Despite active research in implants, the ideal surface for predictive tissue integration remains a challenge.

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Executive summary

Introduction

- The precise role of the surface properties of implants in biological response remains poorly understood.
- Nanotechnology may facilitate the development of a controlled nanoscale for biological assays.

Methods of preparation of nanopatterned titanium implant surfaces

- Several methods are generally used for the preparation of nanopatterned surfaces such as photolithography or anodization.

Analysis of implant surfaces

- Roughness, chemical composition, surface energy and Zeta potential should be measured to characterize the surface properties of implants.

Protein adsorption on implant surfaces

- Immediately after implantation, implants are in contact with a wide range of biomolecules present in blood that will interact differently depending on their surface properties.
- The nature, conformation and orientation of proteins on the surface of implants will have a direct consequence on cell behavior.

Effects of nanostructured implants on cell adhesion, proliferation and differentiation

- Nanometer sized features may control cell fate through protein adsorption and cell signaling pathways

Future trends: controlling implant tissue integration with nanostructured surfaces

- The nanostructure of implants seems to play a key role in controlling the differentiation of cells.

Conclusion

- Further research aiming at correlating cell behavior and tissue integration will be required in the future to decipher the role of surface nanostructure in these biological responses.

Figure caption

Figure 1: Tissue – dental implant interactions at both gingival and bone sites.

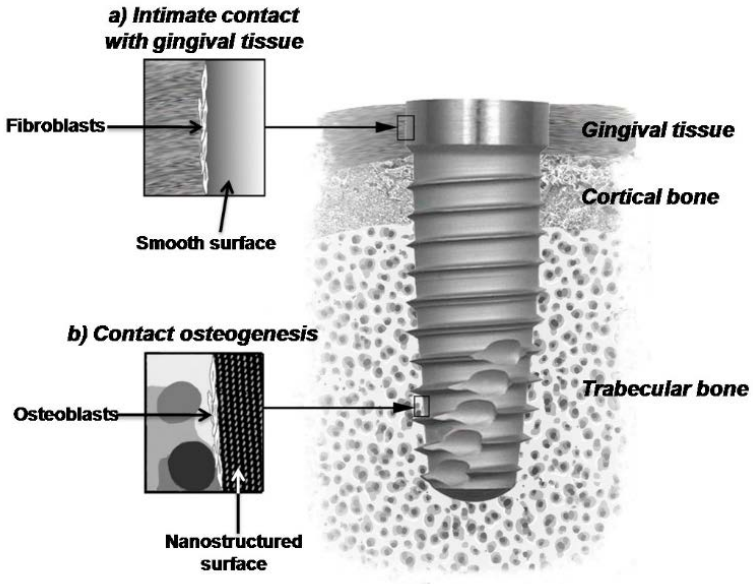


Figure 2: Physical and chemical analysis of surface properties. a) roughness by AFM, b) analysis of chemical composition by XPS or EDX, c) surface energy by contact angle and d) charges by using Zeta potential.

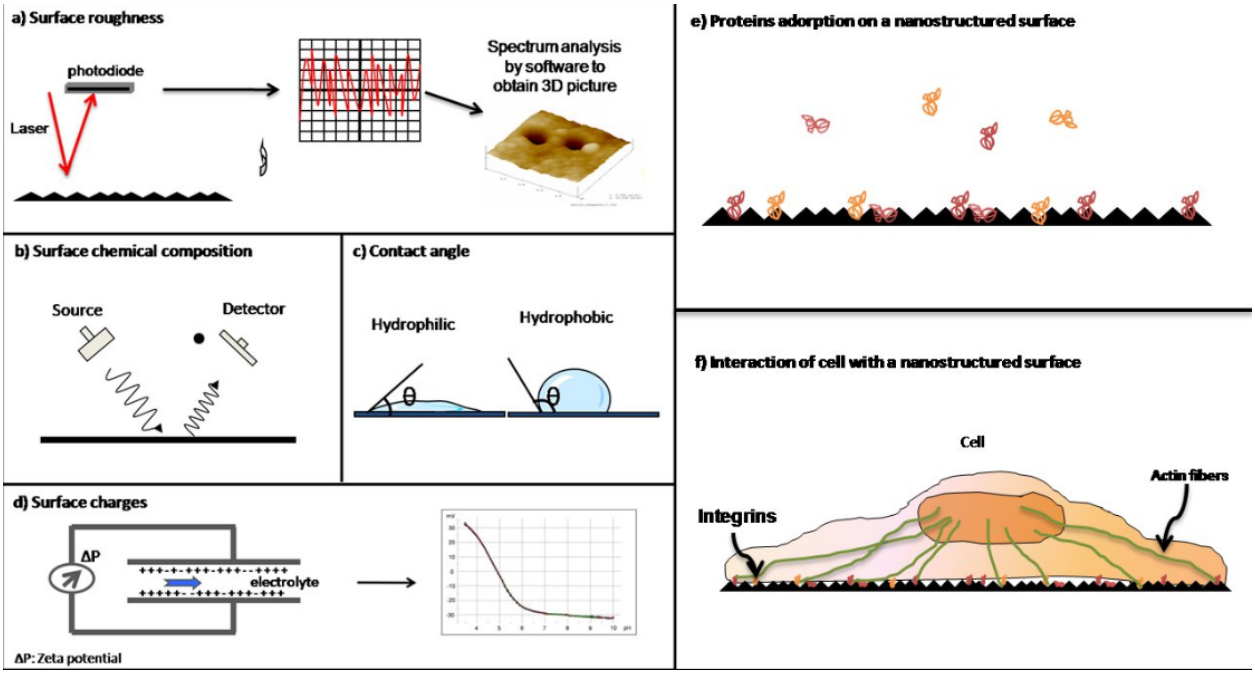


Figure 3: Influence of surface on cell morphology and differentiation a) adsorption of proteins and stem cell adhesion on smooth surfaces b) specific adsorption of proteins on nanostructured surfaces and osteoblastic differentiation.

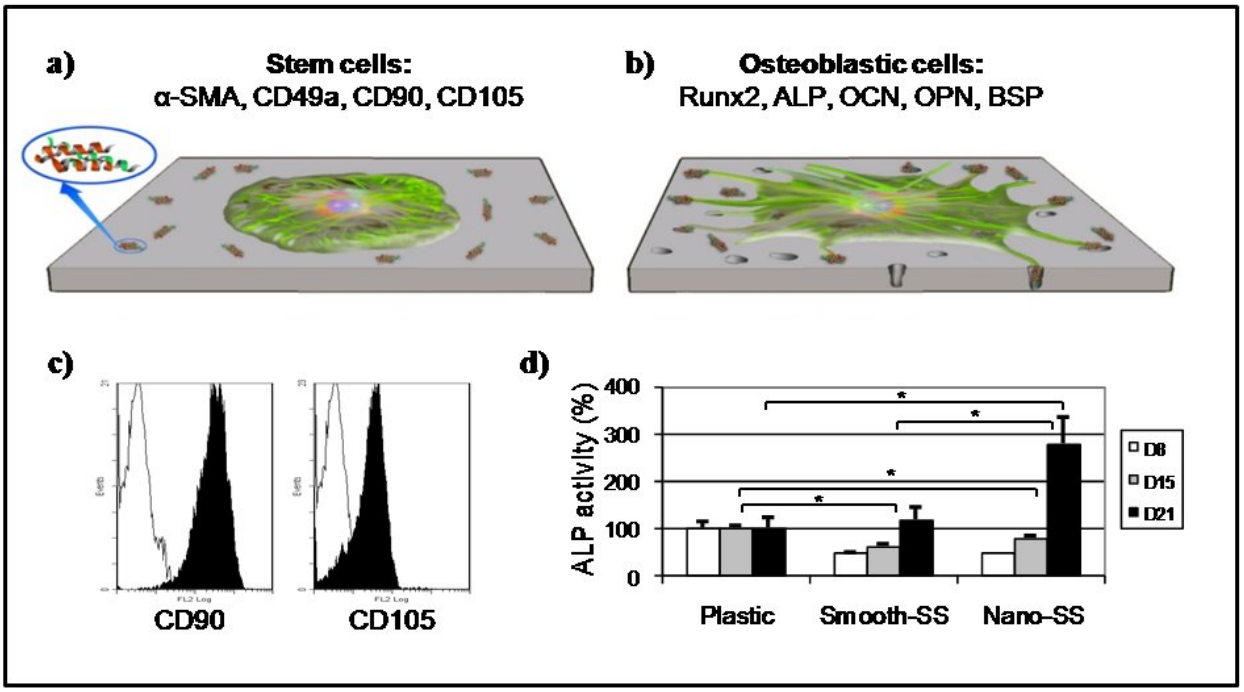
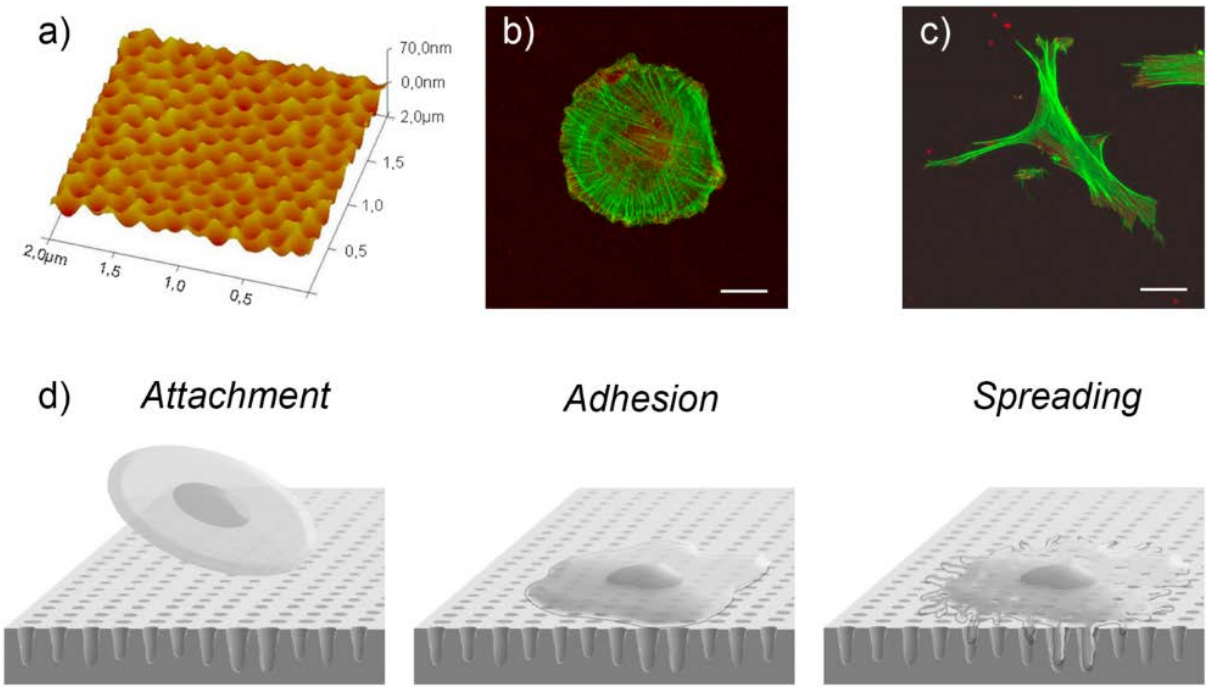


Figure 4: The successive steps of cell adhesion on nanostructured surfaces. a) AFM image of a nanostructured substrate, b and c) Confocal images of human mesenchymal stem cells after 2 and 4 h on surfaces. Immunostaining of Actin (green) and Vinculin focal points (red) are shown (bare scale: 20µm). Diagram showing initial attachment, adhesion and spreading of cells on nanostructured surfaces.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1,0
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2,0µm
1,5
1,0
0,5
b)
c)
d)
Attachment
Adhesion
Spreading

Bibliography

1. **Biggs Mj, Richards Rg, Gadegaard N, Wilkinson Cd, Dalby Mj: The effects of nanoscale pits on primary human osteoblast adhesion formation and cellular spreading. *Journal of materials science* 18(2), 399-404 (2007).**
2. **Le Guehennec L, Lopez-Heredia Ma, Enkel B, Weiss P, Amouriq Y, Layrolle P: Osteoblastic cell behaviour on different titanium implant surfaces. *Acta Biomater* 4(3), 535-543 (2008).**
3. **Oh S, Brammer Ks, Li Ys *et al.*: Stem cell fate dictated solely by altered nanotube dimension. *Proceedings of the National Academy of Sciences of the United States of America* 106(7), 2130-2135 (2009).**
4. **Bhatia Sn, Yarmush Ml, Toner M: Controlling cell interactions by micropatterning in co-cultures: Hepatocytes and 3t3 fibroblasts. *J Biomed Mater Res* 34(2), 189-199 (1997).**
5. **Covas Dt, Panepucci Ra, Fontes Am *et al.*: Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with cd146+ perivascular cells and fibroblasts. *Experimental hematology* 36(5), 642-654 (2008).**
6. **Puckett S, Pareta R, Webster Tj: Nano rough micron patterned titanium for directing osteoblast morphology and adhesion. *International journal of nanomedicine* 3(2), 229-241 (2008).**
7. **Cai K, Frant M, Bossert J, Hildebrand G, Liefeith K, Jandt Kd: Surface functionalized titanium thin films: Zeta-potential, protein adsorption and cell proliferation. *Colloids Surf B Biointerfaces* 50(1), 1-8 (2006).**
8. **Shibata Y, Hosaka M, Kawai H, Miyazaki T: Glow discharge plasma treatment of titanium plates enhances adhesion of osteoblast-like cells to the plates through the integrin-mediated mechanism. *The International journal of oral & maxillofacial implants* 17(6), 771-777 (2002).**
9. **Swart Km, Keller Jc, Wightman Jp, Draughn Ra, Stanford Cm, Michaels Cm: Short-term plasma-cleaning treatments enhance in vitro osteoblast attachment to titanium. *The Journal of oral implantology* 18(2), 130-137 (1992).**
10. **Curtis As, Casey B, Gallagher Jo, Pasqui D, Wood Ma, Wilkinson Cd: Substratum nanotopography and the adhesion of biological cells. Are symmetry or regularity of nanotopography important? *Biophysical chemistry* 94(3), 275-283 (2001).**
11. **Kurze P, Drysmann W, Knofler W: [anodic oxidation using spark discharge--a new surface treatment method for medical technology]. *Stomatologie der DDR* 36(10), 549-554 (1986).**
12. **Andersson As, Brink J, Lidberg U, Sutherland Ds: Influence of systematically varied nanoscale topography on the morphology of epithelial cells. *IEEE transactions on nanobioscience* 2(2), 49-57 (2003).**
13. **Suh Jy, Jang Bc, Zhu X, Ong Jl, Kim K: Effect of hydrothermally treated anodic oxide films on osteoblast attachment and proliferation. *Biomaterials* 24(2), 347-355 (2003).**
14. **Zhu B, Zhang Q, Lu Q *et al.*: Nanotopographical guidance of c6 glioma cell alignment and oriented growth. *Biomaterials* 25(18), 4215-4223 (2004).**
15. **Her Hj, Kim Jm, Kang Cj, Kim Ys: Fabrication of thin film titania with nanopores, nanopoles, and nanopipes by nanoporous alumina template. *Journal of nanoscience and nanotechnology* 8(9), 4808-4812 (2008).**
16. **Streicher R: Nanosurfaces and nanostructures for artificial orthopedic implants. *Nanomedicine* 2(6), 861-874 (2007).**

17. **Covani U, Giacomelli L, Krajewski A *et al.*: Biomaterials for orthopedics: A roughness analysis by atomic force microscopy. *Journal of biomedical materials research* 82(3), 723-730 (2007).**
18. **Anselme K, Bigerelle M: Modelling approach in cell/material interactions studies. *Biomaterials* 27(8), 1187-1199 (2006).**
19. **Le Guehennec L, Martin F, Lopez-Heredia Ma *et al.*: Osteoblastic cell behavior on nanostructured metal implants. *Nanomedicine (London, England)* 3(1), 61-71 (2008).**
20. **Eisenbarth: Influence of the surface structure of titanium materials on the adhesion of fibroblast. *Biomaterials*, (1996).**
21. **Citeau A, Guicheux J, Vinatier C *et al.*: In vitro biological effects of titanium rough surface obtained by calcium phosphate grid blasting. *Biomaterials* 26(2), 157-165 (2005).**
22. **Lee Tm, Chang E, Yang Cy: Attachment and proliferation of neonatal rat calvarial osteoblasts on ti6al4v: Effect of surface chemistries of the alloy. *Biomaterials* 25(1), 23-32 (2004).**
23. **Olivares-Navarrete R, Raz P, Zhao G *et al.*: Integrin alpha2beta1 plays a critical role in osteoblast response to micron-scale surface structure and surface energy of titanium substrates. *Proceedings of the National Academy of Sciences of the United States of America* 105(41), 15767-15772 (2008).**
24. **Faghihi S, Azari F, Zhilyaev Ap, Szpunar Ja, Vali H, Tabrizian M: Cellular and molecular interactions between mc3t3-e1 pre-osteoblasts and nanostructured titanium produced by high-pressure torsion. *Biomaterials* 28(27), 3887-3895 (2007).**
25. **Zhao G, Schwartz Z, Wieland M *et al.*: High surface energy enhances cell response to titanium substrate microstructure. *Journal of biomedical materials research* 74(1), 49-58 (2005).**
26. **Cai K: Does the nanometre scale topography of titanium influence protein adsorption and cell proliferation. *Colloids and surfaces*, (2006).**
27. **Boyan Bd, Hummert Tw, Dean Dd, Schwartz Z: Role of material surfaces in regulating bone and cartilage cell response. *Biomaterials* 17(2), 137-146 (1996).**
28. **Sigal Gb, Mrksich M, Whitesides Gm: Effect of surface wettability on the adsorption of proteins and detergens. *J. Am. Chem. Soc.* 120, 3464-3473 (1998).**
29. **Roach P, Eglin D, Rohde K, Perry Cc: Modern biomaterials: A review - bulk properties and implications of surface modifications. *J Mater Sci Mater Med* 18(7), 1263-1277 (2007).**
30. **Woo Km, Chen Vj, Ma Px: Nano-fibrous scaffolding architecture selectively enhances protein adsorption contributing to cell attachment. *J Biomed Mater Res A* 67(2), 531-537 (2003).**
31. **Miller R, Guo Z, Vogler Ea, Siedlecki Ca: Plasma coagulation responses to surfaces with nanoscale chemical heterogeneity. *Biomaterials* 27(2), 208-215 (Jan 2006).**
32. **Webster Tj, Ergun C, Doremus Rh, Siegel Rw, Bizios R: Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics. *J Biomed Mater Res* 51(3), 475-483 (2000).**
33. **Sousa Sr, Moradas-Ferreira P, Barbosa Ma: Tio2 type influences fibronectin adsorption. *Journal of materials science* 16(12), 1173-1178 (2005).**
34. **Schneider G, Burridge K: Formation of focal adhesions by osteoblasts adhering to different substrata. *Experimental cell research* 214(1), 264-269 (1994).**
35. **Filho Fces, Menezes Gc: Osteoblasts attachment and adhesion: How bone cells fit fibronectin-coated surfaces. *Materials science & Engineering C* 24, 637-641 (2004).**
36. **Lord M, Foss M, Besenbacher F: Influence of nanoscale surface topography on protein adsortpion and cellular response. *Nanotoday* 5, 66-78 (2010).**

37. Kim Mh, Kino-Oka M, Taya M: Designing culture surfaces based on cell anchoring mechanisms to regulate cell morphologies and functions. *Biotechnology advances* 28(1), 7-16 (2010).
38. Zhu X, Chen J, Scheideler L, Altebaeumer T, Geis-Gerstorfer J, Kern D: Cellular reactions of osteoblasts to micron- and submicron-scale porous structures of titanium surfaces. *Cells, tissues, organs* 178(1), 13-22 (2004).
39. Teixeira Ai, Abrams Ga, Bertics Pj, Murphy Cj, Nealey Pf: Epithelial contact guidance on well-defined micro- and nanostructured substrates. *Journal of cell science* 116(Pt 10), 1881-1892 (2003).
40. Anselme K, Bigerelle M: Statistical demonstration of the relative effect of surface chemistry and roughness on human osteoblast short-term adhesion. *Journal of materials science* 17, 471-479 (2006).
41. Mustafa K, Wennerberg A, Wroblewski J, Hultenby K, Lopez Bs, Arvidson K: Determining optimal surface roughness of tio(2) blasted titanium implant material for attachment, proliferation and differentiation of cells derived from human mandibular alveolar bone. *Clinical oral implants research* 12(5), 515-525 (2001).
42. Broberg M, Eriksson C, Nygren H: Gpiib/iiia is the main receptor for initial platelet adhesion to glass and titanium surfaces in contact with whole blood. *The Journal of laboratory and clinical medicine* 139(3), 163-172 (2002).
43. Keller Jc, Stanford Cm, Wightman Jp, Draughn Ra, Zaharias R: Characterizations of titanium implant surfaces. Iii. *J Biomed Mater Res* 28(8), 939-946 (1994).
44. Bowers Kt, Keller Jc, Randolph Ba, Wick Dg, Michaels Cm: Optimization of surface micromorphology for enhanced osteoblast responses in vitro. *The International journal of oral & maxillofacial implants* 7(3), 302-310 (1992).
45. Deligianni Dd, Katsala N, Ladas S, Sotiropoulou D, Amedee J, Missirlis Yf: Effect of surface roughness of the titanium alloy ti-6al-4v on human bone marrow cell response and on protein adsorption. *Biomaterials* 22(11), 1241-1251 (2001).
46. Xavier Sp, Carvalho Ps, Beloti Mm, Rosa Al: Response of rat bone marrow cells to commercially pure titanium submitted to different surface treatments. *Journal of dentistry* 31(3), 173-180 (2003).
47. Anselme K, Noel B, Flautre B *et al.*: Association of porous hydroxyapatite and bone marrow cells for bone regeneration. *Bone* 25(2 Suppl), 51S-54S (1999).
48. Lipski Am, Pino Cj, Haselton Fr, Chen Iw, Shastri Vp: The effect of silica nanoparticle-modified surfaces on cell morphology, cytoskeletal organization and function. *Biomaterials*, (2008).
49. Gregory Ca, Gunn Wg, Peister A, Prockop Dj: An alizarin red-based assay of mineralization by adherent cells in culture: Comparison with cetylpyridinium chloride extraction. *Analytical biochemistry* 329(1), 77-84 (2004).
50. Bonewald Lf, Harris Se, Rosser J *et al.*: Von kossa staining alone is not sufficient to confirm that mineralization in vitro represents bone formation. *Calcified tissue international* 72(5), 537-547 (2003).
51. Akiyama S, Katagiri T, Namiki M *et al.*: Constitutively active bmp type i receptors transduce bmp-2 signals without the ligand in c2c12 myoblasts. *Experimental cell research* 235(2), 362-369 (1997).
52. Aoki H, Fujii M, Imamura T *et al.*: Synergistic effects of different bone morphogenetic protein type i receptors on alkaline phosphatase induction. *J Cell Sci* 114(Pt 8), 1483-1489 (2001).
53. Vetrone F, Variola F, Tambasco De Oliveira P *et al.*: Nanoscale oxidative patterning of metallic surfaces to modulate cell activity and fate. *Nano letters* 9(2), 659-665 (2009).

54. Mendonca G, Mendonca Db, Simoes Lg *et al.*: The effects of implant surface nanoscale features on osteoblast-specific gene expression. *Biomaterials* 30(25), 4053-4062 (2009).
55. Dalby Mj, Gadegaard N, Tare R *et al.*: The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. *Nat Mater*, (2007).
56. Anselme K: Osteoblast adhesion on biomaterials. *Biomaterials* 21(7), 667-681 (2000).
57. Lange R, Luthen F, Beck U, Rychly J, Baumann A, Nebe B: Cell-extracellular matrix interaction and physico-chemical characteristics of titanium surfaces depend on the roughness of the material. *Biomolecular engineering* 19(2-6), 255-261 (2002).
58. Martin Jy, Schwartz Z, Hummert Tw *et al.*: Effect of titanium surface roughness on proliferation, differentiation, and protein synthesis of human osteoblast-like cells (mg63). *J Biomed Mater Res* 29(3), 389-401 (1995).
59. Perizzolo D, Lacefield Wr, Brunette Dm: Interaction between topography and coating in the formation of bone nodules in culture for hydroxyapatite- and titanium-coated micromachined surfaces. *J Biomed Mater Res* 56(4), 494-503 (2001).
60. Chehroudi B, Brunette Dm: Subcutaneous microfabricated surfaces inhibit epithelial recession and promote long-term survival of percutaneous implants. *Biomaterials* 23(1), 229-237 (2002).
61. Gonzalez-Garcia C, Sousa Sr, Moratal D, Rico P, Salmeron-Sanchez M: Effect of nanoscale topography on fibronectin adsorption, focal adhesion size and matrix organisation. *Colloids and surfaces*, (2010).
62. Santiago As, Santos Ea, Sader Ms, Santiago Mf, Soares Gde A: Response of osteoblastic cells to titanium submitted to three different surface treatments. *Brazilian oral research* 19(3), 203-208 (2005).
63. Wang Cc, Hsu Yc, Su Fc, Lu Sc, Lee Tm: Effects of passivation treatments on titanium alloy with nanometric scale roughness and induced changes in fibroblast initial adhesion evaluated by a cytodetacher. *Journal of biomedical materials research*, (2008).
64. Cohen A, Liu-Synder P, Webster T: Decreased fibroblast and increased osteoblast functions on ionic plasma deposited nanostructured ti coatings. *Nanoscale Res Lett.* 2, 385-390 (2007).
65. Miller D, Vance R, Thapa A, Webster T, Haberstroch K: Comparaison of fibroblast and vascular cell adhesion to nano-structured poly(lactic co glycolic acid)films. *Applied Bionics and Biochemics* 2, 1-7 (2005).
66. Ogawa T, Saruwatari L, Takeuchi K, Aita H, Ohno N: Ti nano-nodular structuring for bone integration and regeneration. *Journal of dental research* 87(8), 751-756 (2008).
67. Sul Yt, Johansson Cb, Roser K, Albrektsson T: Qualitative and quantitative observations of bone tissue reactions to anodised implants. *Biomaterials* 23(8), 1809-1817 (2002).
68. Eriksson C, Nygren H, Ohlson K: Implantation of hydrophilic and hydrophobic titanium discs in rat tibia: Cellular reactions on the surfaces during the first 3 weeks in bone. *Biomaterials* 25(19), 4759-4766 (2004).
69. Vogler Ea, Graper Jc, Harper Gr, Sugg Hw, Lander Lm, Brittain Wj: Contact activation of the plasma coagulation cascade. I. Procoagulant surface chemistry and energy. *J Biomed Mater Res* 29(8), 1005-1016 (1995).
70. Hunt Bj, Parratt R, Cable M, Finch D, Yacoub M: Activation of coagulation and platelets is affected by the hydrophobicity of artificial surfaces. *Blood Coagul Fibrinolysis* 8(4), 223-231 (1997).

71. Sawase T, Jimbo R, Baba K, Shibata Y, Ikeda T, Atsuta M: Photo-induced hydrophilicity enhances initial cell behavior and early bone apposition. *Clinical oral implants research* 19(5), 491-496 (2008).
72. Kohavi D, Schwartz Z, Amir D, Mai Cm, Gross U, Sela J: Effect of titanium implants on primary mineralization following 6 and 14 days of rat tibial healing. *Biomaterials* 13(4), 255-260 (1992).
73. Boyan Bd, Schwartz Z, Hambleton Jc: Response of bone and cartilage cells to biomaterials in vivo and in vitro. *The Journal of oral implantology* 19(2), 116-122; discussion 136-117 (1993).
74. Schwartz Z, Amir D, Boyan Bd *et al.*: Effect of glass ceramic and titanium implants on primary calcification during rat tibial bone healing. *Calcified tissue international* 49(5), 359-364 (1991).