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# In vitro MC3T3 osteoblast adhesion with respect to surface roughness of Ti6Al4V substrates

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## Abstract

This work investigates the role of the surface roughness of Ti6Al4V on the cell morphology, proliferation and adhesion, and in particular on the variation of the expression of cell adhesion proteins. Standardised test samples with five different surface preparations are used: sandblasted, 80, 1200, and 4000 grade polished, mirror polished. Surface roughness is analysed by Scanning Electron Microscopy and LASER Confocal Microscopy. Cell culture experiments are performed with MC3T3-E1 mouse osteoblasts after 3 days culture: proliferation rate, morphology and adhesion are assessed. The variations of expression of cell adhesion proteins are evidenced by indirect immune fluorescence method: actin from the cytoskeleton, vinculin from the focal adhesion complex, fibronectin and collagen I from the extracellular matrix. The results reveal a clear influence of surface roughness of Ti6Al4V on cell proliferation, morphology and adhesion. A significant correlation is established between surface roughness and cell growth. More the surface is smooth more the osteoblasts proliferate and appear spread out on the test samples. In addition, the expression of adhesion proteins varies with respect to the surface roughness. These results indicate a direct relationship between the decrease of cell adhesion and the increase of cell proliferation on mirror polished materials. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Fractal dimension; Morphology; Proliferation; Actin; Vinculin; Collagen type-1; Fibronectin

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## 1. Introduction

Titanium-base biomaterials for endosseous implants have found widespread applications in the orthopaedic, maxillo-facial and dental domains. Indeed, titanium-base alloys bring up interesting mechanical proprieties. The passivation layer on the alloy surface generates an excellent resistance to corrosion and a quasi-perfect biocompatibility [1–3].

The long term stability of an endosseous implant in its receptor site is ensured by an intimate contact between the bone structure elements and the biomaterial, which is called osteointegration [4,5]. Optimal bone regeneration requires (i) the prevention of inflammatory reactions and bacterial infections; (ii) the isolation of the healing area from epithelial and connective tissue cells, the invasive behaviour of which may inhibit bone formation, and (iii) an optimised surface of the biomaterial itself.

Indeed, the surface characteristics such as the chemical specification, the energetic behaviour and the roughness are factors influencing considerably the cellular response and subsequently, the quantity and the quality of new-formed bone around an implant [6–8]. Cell adhesion, proliferation, differentiation, and metabolism as well as the synthesis of extracellular matrix proteins is very sensitive to the surface proprieties [9–12]. The cellular response to or interaction with a biomaterial is modulated by the cell adhesion phenomena. Cells adhere on the substrate by focal contacts which represent an entity structural proteins and enzymes [13,14]. Extracellular matrix proteins, e.g. fibronectin bind transmembranous receptors, i.e. integrins which are linked to the cytoskeleton via intracellular anchoring proteins such as talin, tensin, paxillin, vinculin,  $\alpha$ -actinin, etc. [15]. The interaction of extracellular matrix molecules with the cell generate signals which are transmitted via the integrins to the cytoplasm, to the cytoskeleton and finally to the nucleus. In this way, cell adhesion can be considered as the perception by the cell of its environmental milieu [16].

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The aim of the present investigation is to show the role of the surface roughness of a biomaterial on the cell proliferation, morphology and the expression of the different adhesion proteins. For this study, an osteoblast-like cell line MC3T3-E1 has been chosen [17] as target cell for any medical device for bone ingrowth. The biomaterial used is Ti6Al4V generally recognised as highly biocompatible, so that any effect observed can be considered as not cytotoxic and such owing to the surface structure.

## 2. Material and methods

### 2.1. Test samples

Test samples are Ti6Al4V discs of 15 mm in diameter and 1 mm thick from the same alloy charge, certified following the specifications ASTM F132-92 and ISO 5832-3-90. Five groups of 30 discs with different surface structure are prepared: (i) sandblasting with 500  $\mu\text{m}$  aluminium ( $\text{Al}_2\text{O}_3$ ) beads, (ii–iv) manual parallel polishing with silicium carbide papers of grade 80, 1200 and 4000, respectively, and (v) automatic mirror polish with 0.25  $\mu\text{m}$  diamond particles.

Prior to any analysis and in vitro experiment, test samples are cleaned each time by 10-min ultrasound treatment in pure acetone, pure alcohol and distilled water. For cell culture experiments, test samples are sterilised in an autoclave.

### 2.2. Surface structure analyses

Two samples of each group are examined by Scanning Electron Microscopy (SEM) at an accelerating voltage of 25 kV and a current of 100  $\mu\text{A}$ .

Roughness has been measured using a confocal microscope LASER (Lasertek) on 10 samples of each group randomly chosen. On each samples, five profiles have been recorded (evaluation length: 60  $\mu\text{m}$ , number of registered points: 1024). An analysis of variance (ANOVA) is performed to test the homogeneity of sandblasted or polishing process of each sample and the reproducibility of this process between samples of each group. The variance test ANOVA is then applied to usual roughness parameters ( $R_a$ ,  $R_t$ ,  $Z_{\text{max}}$ ,  $Z_{\text{min}}$ , ...) The homogeneity of these parameters could not be rejected at 95% confidence level for each sample and also between samples of a same group.

In general, the purpose of the ANOVA is to test for significant differences between mean values. This name is derived from the fact that in order to assess statistical significance between means; we are here comparing, i.e. analysing variances. The principle of ANOVA is the fact that variances can be divided or partitioned.  $\sigma_{\text{inter-group}}^2$  represents the variance of the proliferation rate between

the different classes of roughness and  $\sigma_{\text{intra-group}}^2$  the variance of the proliferation rate for a given class of roughness. The greater the variance factor  $F$  ( $\sigma_{\text{inter-group/intra-group}}$ ) is, the more discriminate is the fractal dimension. The value  $P$  is the critical probability and represents the probability to conclude to the significance of  $F$ , when  $F > 1$ .

### 2.3. Cell culture

MC3T3-E1 cells derived from mouse calvarium tissue and are an immortalised cell-line [17]. These cells described as osteoblast-like cells characterised by alkaline phosphatase activity and the synthesis of an extracellular matrix containing osteocalcin, osteopontin, osteonectin and type-1 collagen [18,19]. The original stem cells and the immortalised cells have an identical morphology. The cells are cultured in T75 plastic bottles (Nunc) in alpha MEM medium with glutamax (Gibco BRL) supplemented with 10% foetal calf serum (FCS) (Eurobio). The medium also contained penicillin (100 IU  $\text{ml}^{-1}$ ), streptomycin (0.1  $\text{g l}^{-1}$ ) and mycostatin (100 IU  $\text{ml}^{-1}$ ).

Cell proliferation is performed in 24-well plates (Nunc) following the international and European standards (ISO 10993-5/EN 30993-5). In order to ensure that all cells will adhere on the alloy surface,  $5 \times 10^3 \text{ cm}^{-2}$  growing cells contained in 0.1 ml culture medium are seeded in each well on the test samples and incubated at 37 °C and 5%  $\text{CO}_2$  atmosphere. After 3 h, time allowing complete adhesion of the cells on the test samples, the wells are carefully completed to 1 ml. After 3 days, cells are detached in a trypsin-EDTA solution and the number of cells in each well is determined with a cell counter Z1 (Coulter Electronics). Three samples of each group are used for the test. Minimum of five separate experiments is carried out.

Cell morphology was assessed after a 3-days culture by SEM. Cells grown on the different test samples and on borosilicate glass for the control are fixed with sodium phosphate buffered 2.5% glutaraldehyde, pH 7. After two washes in the same buffer, the cells are post-fixed with 1%  $\text{OsO}_4$  in saturated  $\text{HgCl}_2$ . After dehydration in graded ethanol, the cells are critical-point dried (EMSCOPE CPD 750), coated with gold to a thickness of approximately 100 nm (EMSCOPE SC 500) and examined in a scanning electron microscope at an accelerating voltage of 25 kV and a current of 100  $\mu\text{A}$ .

### 2.4. Fluorescence microscopy

Four different proteins have been visualised by fluorescence microscopy: cytoskeletal actin, vinculin containing focal adhesions, fibronectin and type-1 collagen. Osteoblasts are cultured upon the test substrates. After 3 days, the cells are fixed for 20 min, in 2%

paraformaldehyde at room temperature, permeabilized in a PBS–Triton X-100 buffer (Sigma) (10 mM PBS, 0.2% Triton), and then blocked with 1% bovine serum albumin (BSA) (Sigma) in PBS (Sigma).

**Cytoskeletal organisation:** actin filaments were labelled with  $1.2 \mu\text{g ml}^{-1}$  FITC-phalloidin (Sigma). **Focal contact formation:** cells are immune stained with 300  $\mu\text{l}$  of a 1:50 diluted mouse anti-chicken monoclonal antibody against vinculin (Sigma) followed by FITC-rabbit-anti-mouse antibody (Sigma). **Extra cellular matrix:** cells are immune stained with 300  $\mu\text{l}$  of a 1:200 diluted mouse anti-chicken monoclonal antibody against fibronectin (Sigma) followed by FITC-rabbit-anti-mouse antibody (Sigma). **Collagen synthesis:** osteoblasts are labelled with 300  $\mu\text{l}$  of a 1:50 diluted rabbit anti-human monoclonal antibody against type-1 collagen (Tebu), followed by rhodamine-goat-anti-rabbit antibody (Sigma).

After washes in buffer, the specimens are embedded in PBS–Glycerol-DABCO (1:1) (Sigma) mounting medium and examined in a ZEISS epifluorescence microscopy or in a LEICA TCS NT LASER scanning confocal microscope. For these observations, cell nuclei are previously labelled with propidium iodide (Sigma).

### 3. Results

#### 3.1. Surface analyses

Scanning electron microscopy shows a clear difference between each of the five surface structures (Fig. 1a–e). Sandblasted samples display a highly rugged and irregular surface. The surface roughness, i.e. the profile depth of polished test substrates decreases with respect to the grade of the silicium carbide papers. SEM also confirms the regular parallel oriented edges. Mirror polished waters have a very smooth surface, slight ridges are exceptional.

In a first part, homogeneity of the surface roughness is analysed according to the sandblasted or polishing process. To quantify whether a class of samples gets uniform roughness, numerous roughness parameters are calculated from each sample with five roughness recordings. When all parameters are statistically equal for a group of samples, then homogeneity of the process (polishing, sandblasting) is validated. To test the hypothesis, ANOVA are made for each parameter. It was proved (results not shown) that all values of roughness parameters of all samples of a roughness class are equal to the usual confidence level of 95%. As a consequence it could be demonstrated that all samples of a same roughness class are homogenous and will not skew biological results.

LASER Confocal Microscopy allows to measure precisely the surface roughness without damage and to

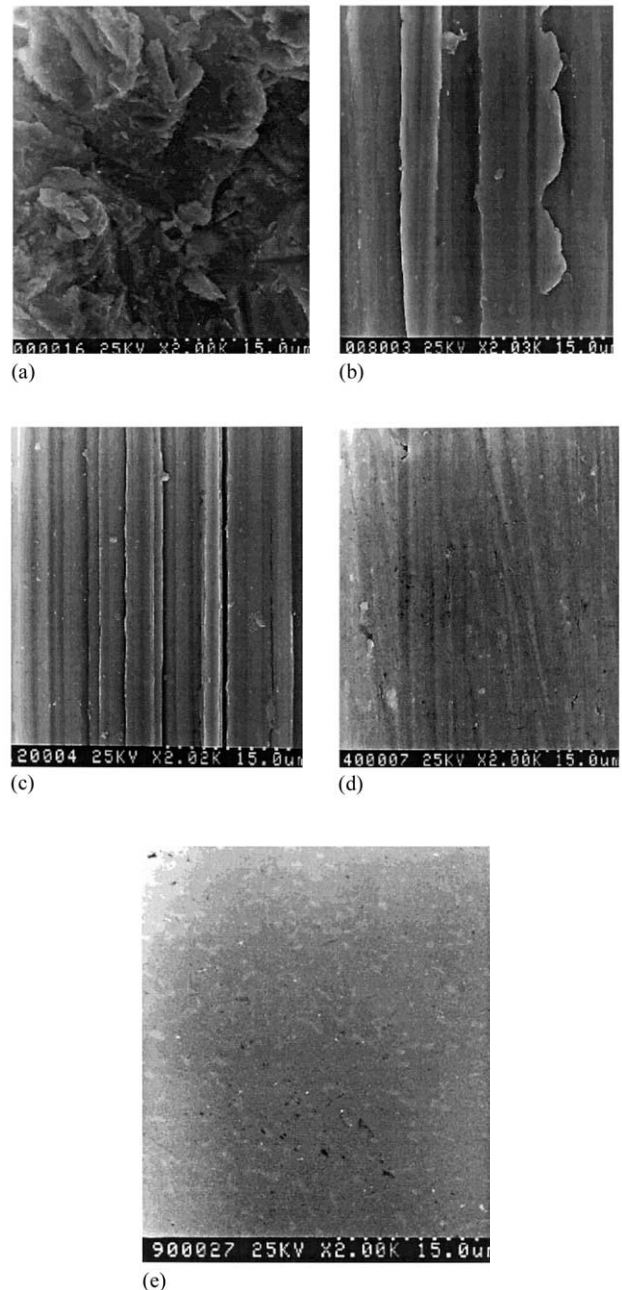


Fig. 1. SEM micrographs showing the surface roughness of the different test samples; (a) sandblasted, (b) grade 80, (c) grade 1200, (d) grade 4000, (e) mirror polished.

establish the principal parameters of surface roughness amplitude:  $R_a$ ,  $Z_{\text{max}}$ ,  $Z_{\text{min}}$ , and  $R_t$ . As expected, the amplitude of these parameters decreases from the sandblasted to the mirror polished test samples (Table 1, Fig. 2).

A tridimensional analysis of the successively recorded profiles makes evidence of the difference between sandblasted and polished surfaces. Indeed, sandblasting results in a very irregular surface, whereas polishing allows obtaining regular and homogenous surfaces (Fig. 3a–c).

Table 1  
Mean amplitude of the surface roughness of Ti6Al4V test samples

Roughness	Sandblasted	Grade 80	Grade 1200	Grade 4000	Mirror polished
$R_a$	0.62	0.61	0.43	0.3	0.16
$Z_{max}$	4.21	2.27	2.21	1.53	0.73
$Z_{min}$	-4.55	-3.02	-3.48	-3.16	-0.73
$R_t$	8.76	5.29	5.69	4.69	1.46

### 3.2. Cell proliferation

The cell proliferation rate is dependent on the surface roughness. It decreases with respect to the increase of roughness and it could be shown by ANOVA that the proliferation rate on mirror polished samples is higher than in control cultures. It is significantly different ( $P < 0.01$ ) from the cell growth on sandblasted samples (Fig. 4).

The ANOVA of the experimental data considers the three main parameters, i.e. the experiments iteration, the number of analysed test samples and the surface roughness classes. By this mean of analysis, evidence is shown that the roughness is a factor influencing significantly cell proliferation ( $P < 0.03$ ) and that the influence force is significant itself ( $F = 18.34$ ).

To quantify the roughness influence described above; the correlation was established between the arithmetic means of roughness ( $R_a$ ) and the mean values of cell proliferation. This negative correlation is shown to be highly significant:  $R^2 = 0.92$  (Fig. 5). This means that the lower the roughness amplitude is, the more cell proliferation increases.

### 3.3. Cell morphology

Cell morphology has been assessed by SEM and results are shown in Fig. 6a–e. On sandblasted samples, cells have a quite round form and exhibit sphere-like surface evaginations but very few other cytoplasmic prolongations (Fig. 6a). Cells grown on grade 80

polished samples have a spread out aspect and show multiple microvilli on the surface and long fine cytoplasmic extensions in multiple directions. In addition, these cells are oriented with respect to the polishing edges (Fig. 6b). Cells cultured on 1200, 4000 and mirror polished samples demonstrate apparently greater spreading with large lamellipodes indicating an active cell migration. Intercellular connections can frequently be observed (Fig. 6c). Cell orientation is still obvious on 1200 grade polished samples, becomes occasional on 4000 grade polished samples and completely disappears on mirror polished surfaces (Fig. 6d and e). In addition, the number of microvilli and in particular cytoplasmic extensions tend to diminish on cells grown on mirror polished samples.

### 3.4. Qualitative assessment of cell adhesion

The cytoskeletal organisation is determined by actin labelling with FITC-phalloidin. This staining demonstrates the presence of stress fibres in all cells grown on any sample. On sandblasted surfaces, actin filaments appear with lower organisation than on polished surfaces where the fibres are generally oriented in a parallel direction following the main cellular axis. Cortical filament bundles at the cell periphery are frequently observed (Fig. 7).

The focal adhesion plaques are evidenced by the use of anti-vinculin antibodies. They appear in form of patches essentially disposed on the cell periphery and at the extremities of cellular extensions. In cells grown on

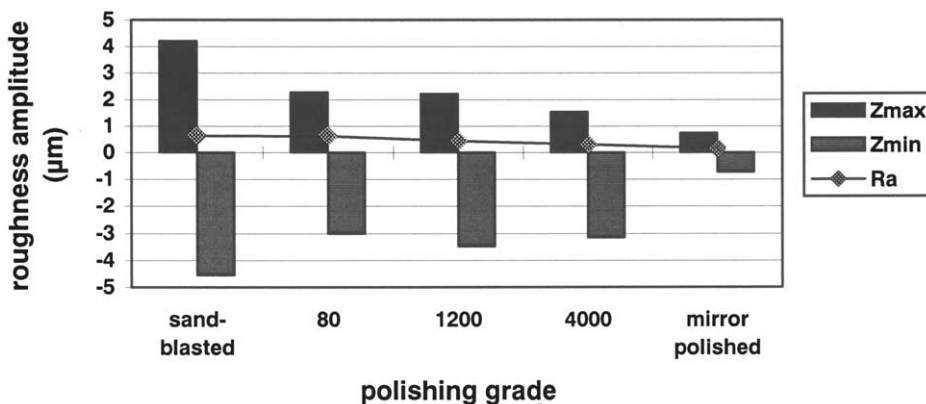
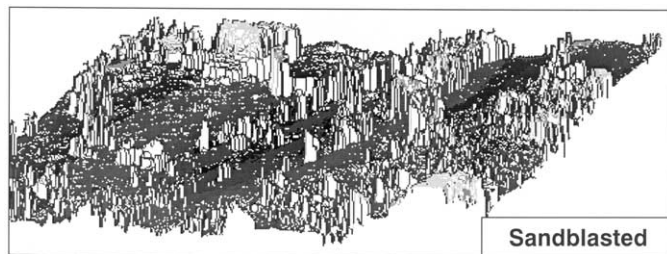
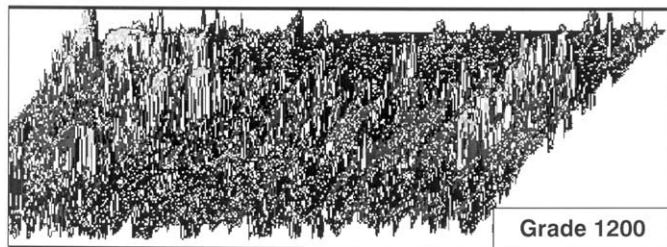


Fig. 2. Medium principal roughness parameters of each grade.

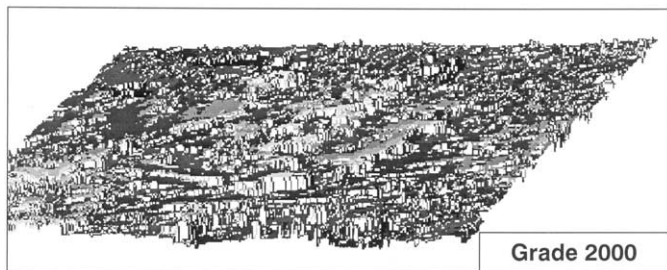




(a)



(b)



(c)

Fig. 3. Tridimensional representation of the surface roughness of samples obtained by LASER confocal multiprofile recording: (a) sandblasted, (b) grade 1200, (c) grade 4000.

polished surfaces, the focal adhesion plaques are distributed all over the area occupied by the adherent cell. The intensity of these plaques is less marked in cells on mirror polished samples (Fig. 8).

After a 3 days culture, type-1 collagen is still intracellular with a specific perinuclear localisation. It is present in cells grown on any sample, with a lower labelling in cells on the mirror polished surface (Fig. 9).

Fibronectin is demonstrated on all samples with a specific perinuclear localisation comparable to that of collagen (Fig. 10). No difference could be established between the cells cultured on the different test samples.

#### 4. Discussion

SEM analyses of cell morphology show that cells grown on a very irregular, i.e. sandblasted surface exhibit a quite round form with long evaginations predicting a good adhesion. The multiple surface microvilli indicate an important cell activity. Polished surfaces with variable roughness generate a more wide spread form of cells characterised by the formation of lamellipodes and fine spicula-like pseudopodia. Cells exhibit multiple microvilli and inter-cellular links [20–22]. Microvilli and pseudopodia tend; however, to diminish on cells grown on mirror-polished substrates, indicating a lower adhesion performance of osteoblasts and a decreased cell activity [23].

A similar and more significant relationship between cell growth and the substrate roughness is revealed by the proliferation test: the more the surface is smooth, the more cells proliferate. This proliferation rate can qualitatively be related smooth surfaces and produce less adhering pseudopodia, thus promoting their own proliferation.

The surface of an endosseous implant and in particular of a dental implant must allow an optimal attachment to bone, but also to connective tissue and epithelium. Although the epithelium needs a very smooth surface for an optimised cell adhesion [24],

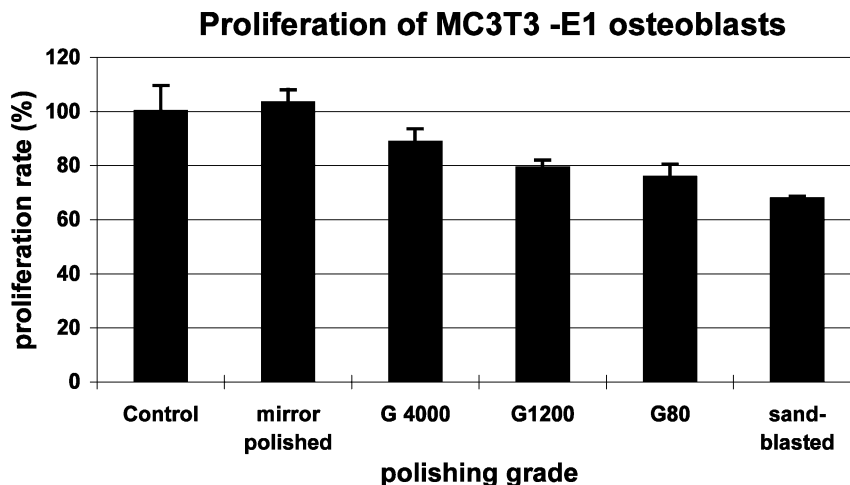


Fig. 4. Proliferation rate of MC3T3-E1 osteoblasts grown for 72 h in direct contact on Ti6Al4V with different surface roughness ( $n = 5$ ).

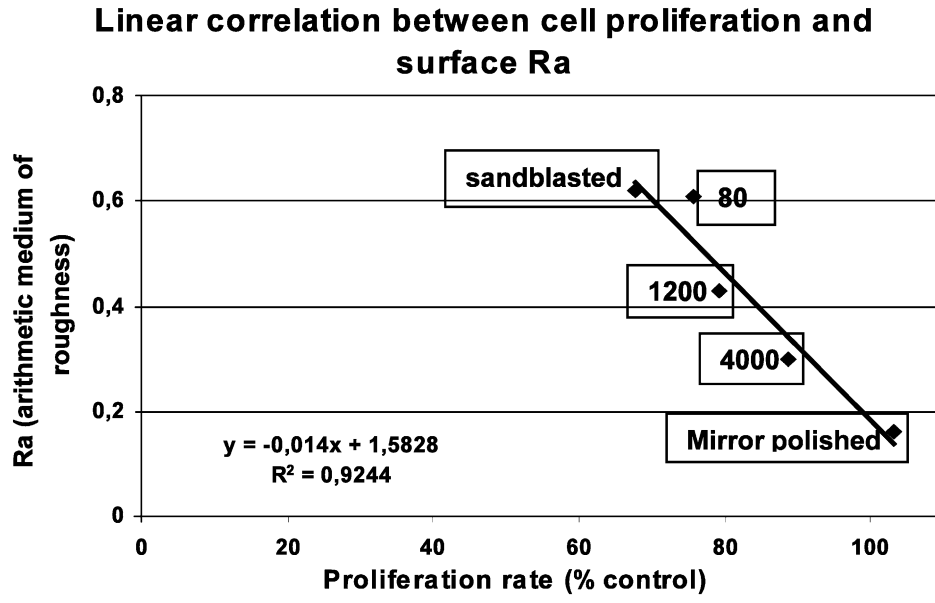


Fig. 5. Linear correlation between the arithmetic mean values of surface roughness and the proliferation rate of osteoblasts.

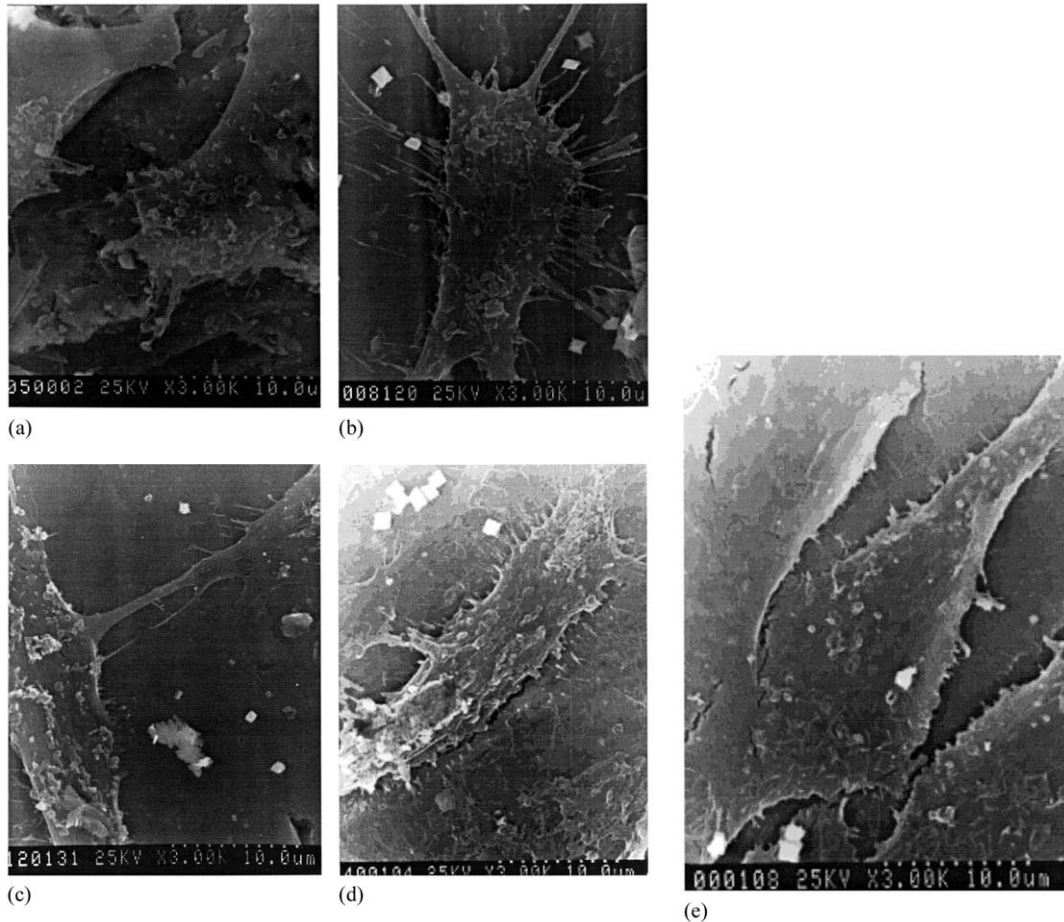


Fig. 6. SEM micrographs showing the osteoblast morphology after a 3-days culture on Ti6Al4V samples with different surface roughness. (a) Sandblasted, (b) grade 80, (c) grade 1200, (d) grade 4000, (e) mirror polished.

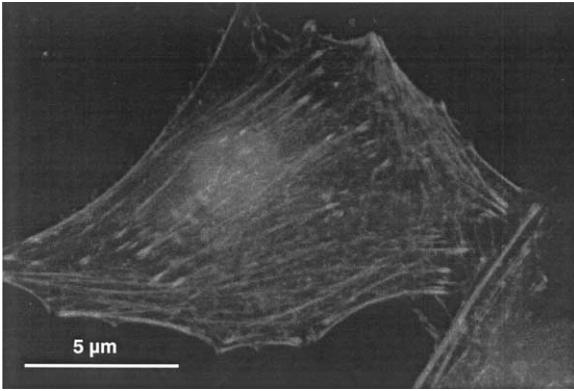


Fig. 7. Actin labelling with phalloidin of osteoblasts grown for 72 h on 1200-grade polished Ti6Al4V samples. Note the regular orientation of the actin stress fibres. The red colour of the nucleus appears after propidium iodide staining.

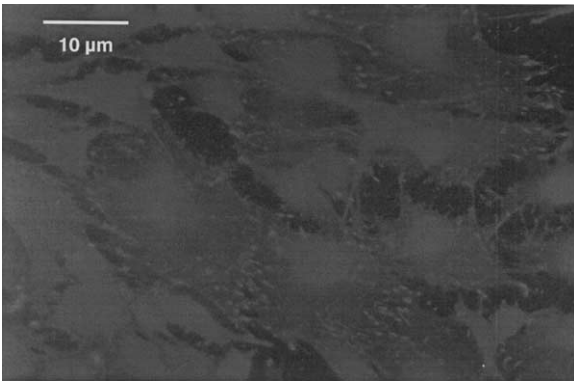


Fig. 8. Immune labelling of vinculin in osteoblasts grown for 72 h on 1200-grade polished Ti6Al4V samples. Numerous patches representing focal adhesion contacts are observed in spread cells.

hard tissue growth is activated more rapidly by a rough surface [25,26], with subsequent accelerated cell differentiation [11], simulated synthesis of extra-cellular matrix and improved mineralisation [27]. These phenomena may explain the differences observed within the proliferation assays.

In the present investigation, cell adhesion has been qualitatively analysed by the immune labelling of proteins participating in the attachment of cells to the substrate. In addition to previous studies considering in particular the cytoplasmic proteins, actin and vinculin, our specific interest was also directed to fibronectin and collagen 1, two extra-cellular matrix proteins playing an important role in the conditioning of cell adhesion.

At least, on the qualitative level, the expression of all these proteins varies with respect to the substrate roughness. The cytoskeleton seems less organised on the sandblasted samples than on the others. In all experiments, vinculin and collagen-1 labelling diminished with respect to the surface smoothness and in

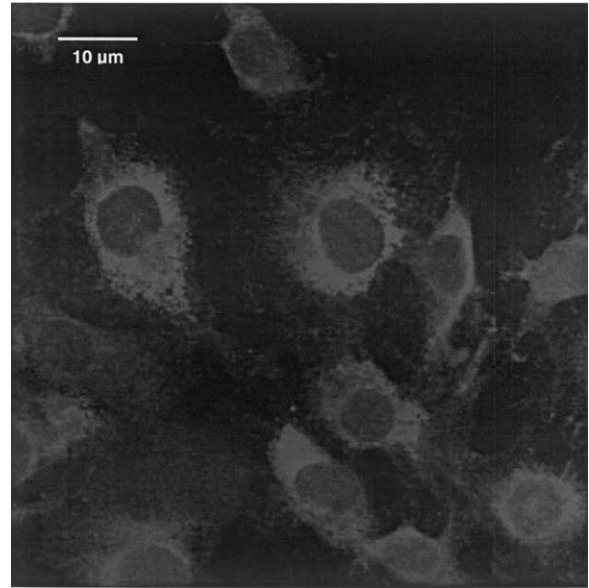


Fig. 9. Immune labelling of Type-1 collagen in osteoblasts grown for 72 h on 1200-grade polished Ti6Al4V samples. Note the perinuclear localisation of intracellular collagen.

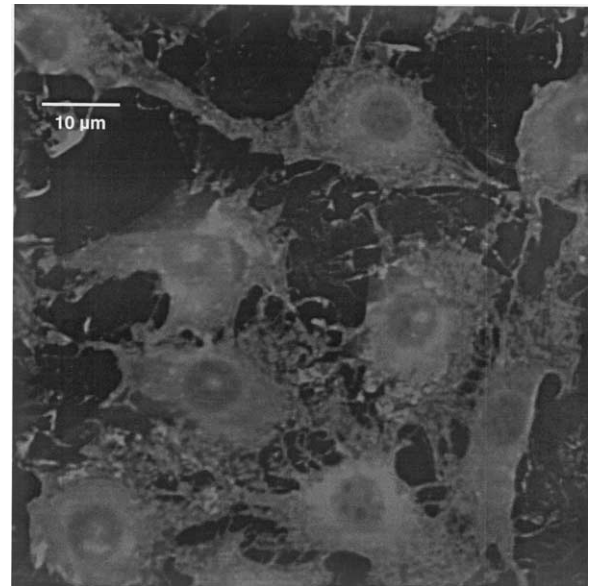


Fig. 10. Immune labelling of fibronectin in osteoblasts grown for 72 h on 1200-grade polished Ti6Al4V samples. Note the perinuclear localisation of intracellular fibronectin.

particular on mirror-polished test samples. Only fibronectin expression remains unchanged.

In contrast to collagen 1, the synthesis of which depends from the cell type and the nature of biomaterial [28], the synthesis of fibronectin by the osteoblasts does not appear to be specifically related to the substrate. It is possible, that other extra-cellular matrix proteins, e.g. vitronectin, lamellin, are more specific for osteoblasts grown on titanium alloys.



A relationship can be established between the immune labelling, the morphological modifications and the cell growth: on mirror-polished samples, the lower frequency of adhering pseudopodia and of focal adhesions as well is correlated to an increase of cell proliferation. Indeed, cell growth better occurs when cell adhesion is decreased. This has been demonstrated for cell/substrate (biomaterials) and cell/cell interactions [29].

Following our experimental results, sandblasted or mirror polished surfaces seem not to be convenient to obtain an optimal osteointegration of the implants. Although sandblasted surfaces induce a good cell adhesion, the rapid cell differentiation and the lower proliferation rate may generate a bone healing with lower cell density and subsequently result in reduced implant stability. In contrast, decreased adherence forces on mirror polished samples and slowed cell differentiation may lead to a retarded hard tissue formation and subsequently to a delayed implant load. This aspect is also unfavourable for optimal tissue stimulation.

From the present experimental in vitro conditions, the 1200 grade polished surface seems to be a convenient compromise on the cell adhesion level and on the cell proliferation level as well. In addition, the orientation of osteoblasts along the polishing ridges is an interesting factor susceptible to increase the resistance of new formed bone tissue with regard to mechanical stress [22,30].

From biochemical point of view, cell adhesion is conditioned by an entirety of cytoplasmic proteins and extracellular matrix. The assemblage of focal adhesion contacts is realised via transmembranous integrins [28]. In this way, focal contacts play a role in the signal transduction from the environmental milieu up to the cell nucleus resulting in an appropriate cell response such as the modification of proliferation rates, the differentiation behaviour and the morphological appearance of cells. This internal signalisation seems to be a consequence of the activation of different molecules forming with integrins the adherence complexes. Some of these molecules, e.g. enzymes of the Src family, contribute to the activated signalisation pathway by various growth factors [16]. Other molecules of the extracellular matrix and the growth factors control reciprocally their messages by emitting convergent signals or by inhibiting certain transmission pathways. The activity of other molecules of the focal adhesion complex which are activated by integrins such as focal adhesion kinase (FAK), paxillin, tensin... are scarcely modified by the growth factors, but probably respond to more extracellular matrix constituents. As mentioned above, these molecules contain binding sites for signal transducing molecules, they certainly play a role for the forwarding of messages from integrins to genes or to other cellular elements [16,20].

## 5. Conclusion

In conclusion, this present study on osteoblast adhesion on Ti-alloys with variable surface roughness reveal, on the one hand, a significant correlation between the material surface roughness and the cell proliferation rate, and the other hand, a qualitative relationship between roughness and cell adhesion. Indeed, on very smooth surfaces, the increase of cell growth is directly related to a decrease of cell adhesion. It is important to confirm these results by more quantitative analyses of the adhesion process.

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