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Topography effects of pure titanium substrates on human osteoblast long-term adhesion

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Abstract

Classically various treatments are applied to increase the roughness of titanium implants and improve their integration in the tissues. Many in vitro studies have been performed to better understand the mechanisms underlying the adhesion of cells on materials. Frequently, the adhesion is related to the attachment of cells during the first hours of contact with the substrate. For several years, our objective has been to develop experimental methods to evaluate the long-term adhesion of human osteoblasts from some hours to several weeks in order to model in vitro a tissue-like interface. This culture model allows for the formation over 21 days of a complex osteoblast/extracellular matrix/material interface. We recently developed a new parameter called adhesion power (AP) to evaluate this long-term adhesion. In this study, our objective is to check its efficiency in discriminating the long-term adhesion of human osteoblasts on pure titanium substrates with seven different surface morphologies obtained by electro-erosion, sandblasting, polishing, acid-etching and machine-tooling. By scanning electron microscopy, we observed that the human osteoblasts did spread more intimately on surface with low roughness amplitude than on rough ones. However, the AP was higher on rough isotropic surfaces obtained by electro-erosion, sandblasting or acid-etching and lower on smoother surfaces obtained by polishing and machine-tooling. We demonstrated that the AP was pertinent for evaluating human osteoblast's long-term adhesion on pure titanium surfaces with various roughness parameters. Its correlation with the order parameter, which describes the organization of the roughness, confirmed once more that human osteoblasts are more sensitive to the organization and morphology of the roughness than to its amplitude.

Keywords: Surface topography; Titanium; Cell adhesion; Osteoblast; Human

1. Introduction

Titanium (Ti) and titanium alloys are widely used as implant materials. Various treatments have been applied

to increase the roughness of implants and improve their integration in the tissues. In vivo, classically, the integration of implants in bone tissue is positively correlated with increasing roughness of the implant surface [1–6]. Many in vitro studies have been performed to better understand the mechanisms underlying cell–material interaction. It is now well understood that surface morphology influences the response of cells [7–10] and particularly their adhesion, which is one of the most critical initial events occurring during the interactions of

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cells with implants and has further influence on the proliferation and differentiation of bone cells before bone tissue formation [11].

Frequently, adhesion is related by authors to the short-term adhesion or attachment of cells during the first hours of contact with the substrate. The effects on cell attachment of materials composition [12–14], as well as the effects of surface chemistry [15–17] or surface topography [18–22] have been largely studied on bone-derived cells. The material composition always influences cell attachment [12–14] whereas variations of surface chemistry of titanium-based substrates following surface treatments like anodization have generally little influence on the attachment capacity of osteoblasts [16,17]. Likewise, Ahmad et al. described an insignificant difference of osteoblastic cell attachment between Grade 1 and Grade 4 pure titanium [15]. In contrast, the surface roughness of titanium substrates is known to have a considerable effect on osteoblastic cell attachment as well as on cell adhesion, proliferation and differentiation [12,18,21,23–28]. Attachment is generally increased on rough surfaces ($S_a > 1 \mu\text{m}$), produced for example by sandblasting, compared to smooth ones [10,18–20,29,30] but sometimes no effects are observed [22,31].

For several years, our objective has been to develop a human bone cell culture model allowing the *in vitro* formation of a cell/material interface that would be as close as possible to the implant/tissue interface existing *in vivo* during the first days of contact. Our *in vitro* model consists of the culture of primary human-derived osteoblasts on model substrates from some hours to several weeks, to take into account not only the short-term adhesion of cells in direct contact with the native surface or after adsorption of serum proteins, but also the adhesion of cells in the longer term. By this long-term adhesion, we understand the cell/material interface strength at times when the cells have elaborated an interfacial extracellular matrix (ECM) which is an intricate arrangement of glycoproteins, collagens, proteoglycans and growth factors that act not only as a physical scaffold for cell attachment and organization of cellular structures, but also as a mediator of intracellular signaling through cell surface receptors. In our *in vitro* model, a thick ECM formed by fibronectin, type I collagen and osteopontin is synthesized by the osteoblast cell layer on the material's surface [24]. Besides, it was demonstrated recently by Meyer et al. that cells and extracellular matrix proteins (fibronectin, osteonectin) were present from day one of the implant/bone contact after implantation of titanium dental implant in the mandible of mini-pigs. Over the first 14 days of contact mineralization occurred, the crystals growing along newly synthesized collagen fibers [32]. Moreover, the relatively rare studies interested in the early tissue response to titanium implants have also demonstrated new bone formation with

direct bone-to-metal contact after 1 week of implantation [2] and a thickening of trabeculae formed by immature woven bone with frequent osteocytes after 3 weeks [1,2]. The major differences in our culture model compared to these *in vivo* observations are the absence of mineralization, which is related to the absence in our culture medium of essential additives for mineralization like ascorbic acid and inorganic phosphate, and the absence of interactions with other cell types such as blood cells, macrophages, fibroblasts, and endothelial cells.

It is important to note that to date and to our knowledge, few studies have focused on the study of the long-term evolution of adhesion, although some authors interested in interactions between osteoblasts and bone replacing materials have recently underlined that the differences found in the initial responses of cells cannot predict the cell's behaviour after longer periods [33]. To quantify the long-term adhesion, we developed several years ago an original progressive enzymatic method to detach cells and to evaluate the cell/ECM/material interface strength. In this paper, we use a new adhesion parameter named adhesion power (AP) to evaluate the long-term adhesion of human osteoblasts on pure Ti substrates with different surface morphologies. The topography of these surfaces were extensively analysed and roughness parameters were correlated with adhesion power to determine the one having the highest influence on cell adhesion. This approach of correlating the physical surface parameters with biological parameters describing cell behaviour on surfaces is an original feature of this work, since few attempts have been made to this date to statistically correlate physical and biological parameters [26,34].

2. Materials and methods

2.1. Surface preparation

Pure Ti bars were polished, acid-etched, machine-tooled, electro-eroded or sandblasted to obtain various surface morphologies (Table 1). In brief, pure titanium Ti40 bars (12 mm in diameter) were machine-tooled using a numeric lathe Cazeneuve HB725 to obtain samples measuring 2 mm in thickness. The conditions for machine-tooling were established to obtain either parallel grooves (200 μm in width and 14.5 μm depth) (TU0) or cross-grooves forming sorts of “nests” (200 μm in width and 5.5 μm in depth) (TUX), by using, respectively, one passing or two perpendicular passings. A wire cutting machine was used for electro-erosion processing (AGIECUT, Premier Equipment, Altamonte Springs, FL, USA). Two process conditions were used to obtain two different roughness amplitudes ($S_a = 0.7 \mu\text{m}$ and $S_a = 2.4 \mu\text{m}$). The first samples were cut at 3A and then the tooled face was electro-eroded twice at 0.25A (TE1).

Table 1
Identities and characteristics of the seven different samples tested

Name	Method	Sa (μm)	Procedure
TU0	Machine-tooling (U)	0.7	One passing: parallel grooves
TUX			Two perpendicular passing: cross-grooves
TE0	Electro-erosion (E)	0.7	Cutting: 3A Face treatment: 1A, 0.25A
TE1		2.4	Cutting: 3A Face treatment: 0.25A, 0.25A
TP	Polishing (P)	0.7	Polishing with silicon carbide paper P40
TA	Etching (A)	0.7	Polishing with silicon carbide paper (grade P220, 320, 500, 100, 4000) + 300 s treatment with 10% fluorhydric acid at room temperature
TS	Sandblasting (S)	2.4	Polishing with silicon carbide paper (grade P220, 320, 500, 100, 4000) + sandblasting with silicon carbide particles (400 μm in diameter)

The second samples were cut at 3A and then the tooled face was electro-eroded twice more at decreasing powers (1A and 0.25A) (TE0). Samples were polished using grade 40 silicon carbide paper (TP) using a Pedemax 2 automatic polishing machine (Struers S.A.S, Champigny sur Marne, France). To eliminate any risk of contamination by machine-tooling residues, all the samples were extensively polished using grade 220, 320, 500, 1000 and 4000 before being treated for 300 s with 10% fluorhydric acid at room temperature (TA) or before being sandblasted using silicon carbide particles measuring 400 μm in diameter giving the samples (TS1). Finally, seven different substrates were compared using the same cellular model and the long-term adhesion of cells was quantified on these substrates. The cell culture treated plastic (Thermanox[®], Fisher Labosi, France) was systematically used as control.

2.2. Roughness measurement

As the objective of this work is to check the efficiency of our new AP parameter for discriminating the adhesion of human osteoblasts on pure Ti substrates with different surface morphologies, the topography of these surfaces will be extensively analysed using not only classical amplitude and frequency parameters but also using new original parameters describing surface organization. Roughness was measured using a tactile profilometer (Tencor P-10, KLA Tencor, USA) on a surface of 1 mm \times 1 mm with one measurement every two micrometres on horizontal and vertical scanning. Three-dimensional profiles were drawn and analysed on a computer using personal software. One hundred roughness parameters were computed. Classical roughness amplitude parameters were used like Ra, Sk and Rt. Frequency roughness parameters were also analysed like the autocorrelation length (lac) [26]. As the bibliography did not provide us with any parameter to quantify the order of a surface with scaling invariance both in amplitude and position, we created a new original scale invariant roughness parameter that quantifies the

organization of surfaces. Our main idea was to find a parameter without resorting to Fourier's analysis (since spectrum parameters have little robustness and sinus-cosinus basis is not always appropriate to characterise surface roughness), which gave an upper limit value (100%) if surfaces were periodic, a medium value if surfaces had a nonneglected first order autocorrelation and a lower limit zero value for uncorrelated random surfaces (white noise). This parameter called order is now used in our laboratory to quantify the impact of different processes on the topography of the surface [25,26,35]. The most important quality of this parameter is to be mathematically independent of the amplitude parameter and of the autocorrelation length of the surface. Thus, the effect of the order of surface and both the scaled amplitude parameter and autocorrelation length could be analysed without any correlation bias.

2.3. Cell culture

Human osteoblasts were obtained from trabecular bone taken from the iliac crest of young patients. Briefly, bone was minced in about 5 mm³ pieces, extensively rinsed under stirring in phosphate buffered saline (PBS), distributed in 100 mm dishes (about 30 explants/dish) and recovered by a Dulbecco's modified Eagle's medium (DMEM, Eurobio, Les Ulis, France) containing 10% fetal bovine serum (FBS) + 2 mM L-glutamin + 50 IU/ml penicillin + 50 $\mu\text{g}/\text{ml}$ streptomycin. Culture medium was changed twice a week and cultures were maintained until confluence (about 3 weeks). Then, cells were used immediately for in vitro characterization of phenotype and frozen in liquid nitrogen by keeping for 2 h in vapour before immersion.

For characterization of cell phenotype, cells were inoculated at 2×10^4 cells/well in 24-multiwell plates. After 28 days the wells were rinsed in PBS and recovered by DMEM + bovine serum albumin (BSA) 0.1% over 24 h. Half of the wells were then treated with calcitriol (1,25(OH)₂ vitamin D₃) 10^{-8} M in culture medium + BSA 0.1% over a 48 h period and the other half of the

Table 2
Characterization of cell phenotype

	+ Vit D	- Vit D
ALP (IU/10 ⁶ cells)	110.76	80.05
OC (nM/10 ⁶ cells)	16.338	0
P1CP (pg/10 ⁶ cells)	2648	1948

Alkaline phosphatase activity (ALP) and type I procollagen (P1CP) and osteocalcin (OC) synthesis by human osteoblasts with and without stimulation with calcitriol (1,25(OH)₂ vitamin D₃) 10⁻⁸ M (vit D).

wells were treated with a control medium containing BSA 0.1% and only the vitamin D₃ vehicle, i.e. the absolute ethanol. After this delay, culture medium was harvested for osteocalcin content evaluation with the NovocalcinTM kit and for type I procollagen measurement with the Prolagen-CTM kit (Metrabiosystem, Behring, France). Cells were collected by trypsin, counted or centrifugated and sonicated for alkaline phosphatase activity evaluation with an EnzylineTM kit for ALP activity (CobasMira, Biomérieux, Marcy l'Etoile, France). These analysis confirmed the osteoblastic phenotype of the cells (Table 2). After thawing, cells were cultured in 75 cm² flasks. At confluence, the cells were harvested using trypsin-EDTA and inoculated onto samples in 24-well plates for adhesion tests. The medium was changed twice a week. The same cell line (same donor) at the same passage (2nd) was used for all the study.

2.4. Long-term adhesion measurement

To date, few studies have focused on the study of the long-term evolution of adhesion although some authors interested in interactions between osteoblasts and bone replacing materials have recently underlined that the differences found in the initial responses of cells cannot predict the cell's behaviour after longer periods [33]. To quantify the long-term adhesion, we developed several years ago an original progressive enzymatic method to detach cells and to evaluate the cell/ECM/material interface strength [24]. Briefly, samples of each surface were inoculated with 4 × 10⁴ cells/sample. In each experiment, four incubation periods were considered: 24 h, 7 days, 14 days and 21 days. The experiments were reproduced for nine to 12 samples. The cells were enzymatically detached from the samples firstly by a diluted 0.1× trypsin-EDTA (0.025% v/v) treatment and secondly by a nondiluted 1× trypsin-EDTA (0.25% v/v) treatment. Briefly, 0.5 ml of 0.1× trypsin-EDTA were added to each sample and incubated during 5 min at 37 °C under agitation. After these first 5 min of digestion (digestion time $d = 5$), the 0.5 ml of trypsin-EDTA were harvested and put in 9 ml of phosphate buffered saline + 0.5 ml of fetal calf serum in order to stop the action of the trypsin-EDTA. Fresh 0.1× trypsin-EDTA

was added again on the samples for 5 additional minutes (digestion time $d = 10$) and so on for the digestion times $d = 20, 30$ and 60 min. At the end of the 60 min of digestion with 0.1× trypsin-EDTA, nondiluted 1× trypsin-EDTA was added on the samples for two successive final 15 min treatments in order to detach all the remaining cells. At the end, the cells detached after 5, 10, 20, 30, 60 min of treatment with 0.1× trypsin-EDTA and the remaining cells detached with 1× trypsin-EDTA were counted using a Coulter Z1 (Beckman Coulter, Roissy, France). The curves of the number of released cells versus digestion time (d) were established for each time in culture T . It is considered that the cells with a low adhesion capacity are detached easily and rapidly by the first incubations with 0.1× trypsin-EDTA whereas the cells with a higher adhesion capacity need more time to detach and sometimes detach only after the final treatment with 1× trypsin-EDTA. Thus, this progressive enzymatic detachment allows a quantification of the adhesion capacity of cells in function of time in culture and in function of the surface chemistry and surface topography of the substrate as previously shown [23–26].

2.5. Scanning electron microscopy

Before culture, the samples were examined using a Hitachi S520 scanning electron microscope (SEM) at an accelerating voltage of 25 kV (Elexience, Paris, France).

The cell layers were fixed in 2% paraformaldehyde (w/v) in monosodic dipotassic 0.2 M buffer, rinsed, dehydrated in graded alcohol, critical-point dried with CO₂ (Emscope CPD 750, Elexience, Paris, France), sputter-coated (Emscope SC 500, Elexience, Paris, France) and examined using a Philips scanning electron microscope SEM 525 M (FEI, Limeil-Brevannes, France) at 15 kV.

2.6. De-correlation of long-term adhesion and proliferation

There exists an experimental bias in the measurement of long-term adhesion. The common protocol for adhesion strength measurement consists in applying a stimulus (in our case, the trypsin action) and in counting the number of detached cells at different stimulus times. The lower the number of detached cells for a given stimulus time, the higher the adhesion strength. In a large number of cases, adhesion depends on culture time. In our culture experiment, cells are deposited on substrate and will proliferate. As a consequence, the stimulus will detach both initially deposited cells and cells having proliferated. If no proliferation occurs, the measure of adhesion versus time in culture characterises the real adhesion kinetic. However a bias emerges when prolifer-

ation occurs during culture time: new proliferating cells are included in the counting process and that will diminish the real cell average time in culture. This artefact could explain a negative correlation between long-term adhesion and proliferation. We now introduce a model that allows us to de-correlate the effect of the proliferation rate on the measure of long-term adhesion. In the first time, the proliferation rate $P(T)$ versus the time in culture T is modelled by an analytical expression [24]. If we consider T_d the time needed by an individual cell to detach under a stimulus and d the duration of the stimulus, detachment could be modelled by the $H(d, T_d)$ distribution.

In mathematical terms:

$$H(d, T_d) = 0 \quad \text{if } d < T_d \quad (1)$$

$$H(d, T_d) = 1 \quad \text{if } d \geq T_d \quad (2)$$

We can postulate that when culture time T increases, long-term adhesion strength increases and so does T_d . We shall then claim that T_d is an increasing function of T . We shall suppose that it will be equal to the following power law:

$$T_d(T) = aT^b \quad \text{with } a \geq 0 \text{ and } b \geq 0 \quad (3)$$

As a consequence, this function is used to quantify the long-term adhesion of cells on substrate, without proliferation, after a time T in culture.

However, as discussed above, cells will proliferate and this proliferation must be included in our model. The expression $(\partial P(t)/\partial t)dt$ represents the number of cells which have proliferated at time t . Let us note the real number $p(T, d)$, $p(T, d) \in [0, \dots, 1]$, the ratio of detached cells after a stimulus time d having proliferated on substrate during a time T , then $(\partial P(t)/\partial t)H(d, T_d(T-t))dt$ represents the number of cells that have proliferated at time t and that will be detached by the stimulus with a time duration d .

Finally, all cells in culture from a time T and detached after the d time (stimulus time) are added up and, after normalisation by the $P(T) - P(0)$ factor, the following integro-differential equation of adhesion is obtained:

$$p(T, d) = \frac{1}{P(T) - P(0)} \int_0^T (\partial P(t)/\partial t) H(d, T_d(T-t)) dt \quad (4)$$

The main problem consists in estimating the pair coefficients a and b given by Eq. (3) by applying Eq. (4) on experimental data. Experimentally, we get s stimuli times d_1, d_2, \dots, d_s for c times in culture T_1, T_2, \dots, T_c giving $s \times c$ experimental measures of the ratio of detached cells $p(T_i, d_j)$ used to determine only the two coefficients (a, b) .

The solution consists in finding the pair a and b that minimise in L^2 the following function:

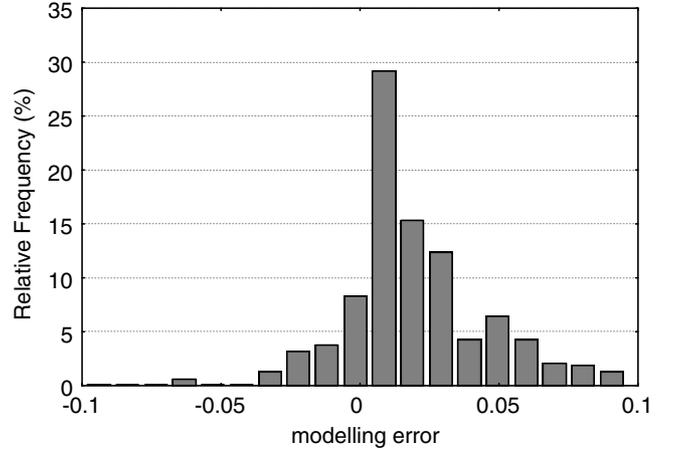


Fig. 1. Modelling error for re-calculated data obtained from Eq. (3) compared to experimental measures ($n = 1440$). Descriptive statistics are mean = 0.02 and standard deviation = 0.05.

$$\min_{a,b} \sum_{i=1}^c \sum_{j=1}^s \left\| p(T_i, d_j) - \frac{1}{P(T_i) - P(0)} \times \int_0^{T_i} (\partial P(t)/\partial t) H(d_j, T_d(T_i - t)) dt \right\|_{L^2} \quad (5)$$

For an adhesion assay, this optimisation problem is solved by the Simplex method to obtain the pair coefficient (a, b) . b represents the kinetic exponent of long-term adhesion and a a scaling factor that characterises the amplitude of long-term adhesion. We call the a coefficient the ‘‘adhesion power’’ (AP).

To check if Eq. (3) describes well the adhesion of all individual cells, an error analysis was performed on our model. The basic idea was to compare the error made in the model between experimental trypsination curves and the reconstructed curves, supposing that the detachment of all individual cells follows Eq. (3). The error could then be expressed by the following terms:

$$p(T_i, d_j) - \frac{1}{P(T_i) - P(0)} \int_0^{T_i} (\partial P(t)/\partial t) H(d_j, T_d(T_i - t)) dt \quad (6)$$

Fig. 1 represents the experimental distribution of all modelling errors for each adhesion measurement (1440 data). The standard deviation is less than 0.05, which represents less than 5% of the variation of $p(T_i, d_j)$. As a consequence, our model is validated at a high confidence level.

3. Results

3.1. Topographical description of surfaces before culture

SEM observation showed the same morphology on fine or coarse electro-eroded surfaces (TE1) consisting

of a rough heterogeneous surface with aspects of sheets with smooth edges associated with droplet-shaped or nearly spherical aspects (Fig. 2). The sandblasted surfaces (TS) presented the classical irregular rough aspect with pits, indentations and sharp ridges. The acid-etch-

ing (TA) of polished surfaces, like sandblasting, made the surface irregular and provoked the formation of pits but at a lower scale. On polished samples (TP), many residual grooves with no orientation and with various widths and depths were observed. On machine-tooled

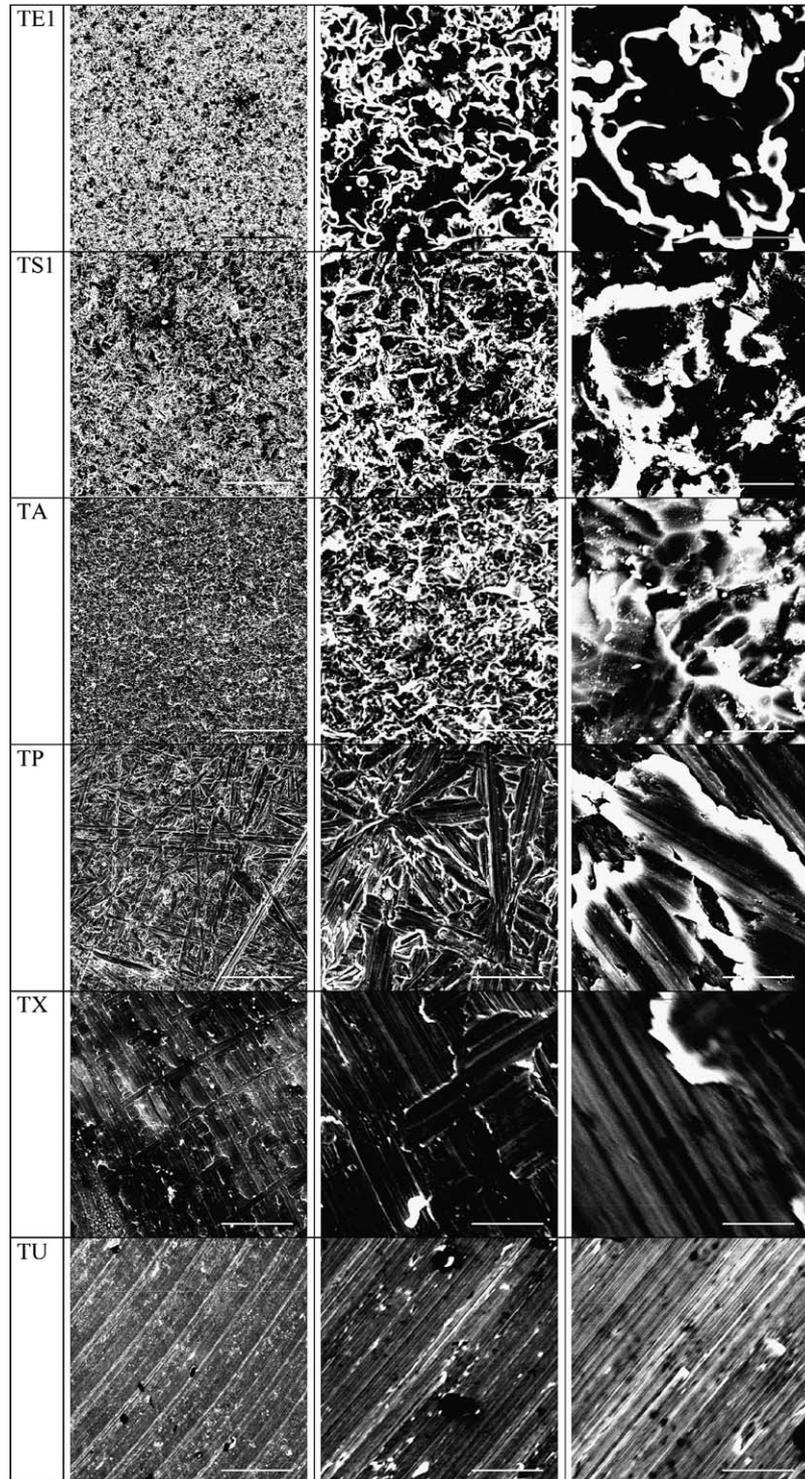


Fig. 2. Scanning electron micrographs of the tested surfaces before culture. Left column, bars = 250 μm ; middle column, bars = 50 μm ; right column, bars = 10 μm . Coarse electro-eroded surfaces (TE1), sandblasted surfaces (TS), acid-etched surfaces (TA), polished surfaces (TP), machine-tooled surfaces with parallel (TU) or crossed (TX) grooves.

Table 3
Roughness parameters (means) measured on each tested surface

Sample	Sa (μm)	Sk (μm)	St (μm)	Lac (m)	Order (%)
TU	0.79	0.676	5.28	17.9	49.0
TX	0.75	0.437	4.00	27.2	43.7
TP	0.53	0.362	4.56	21.1	27.4
TS	2.29	0.022	16.53	18.4	21.2
TE1	2.52	0.392	16.06	11.3	15.9
TA	0.71	0.710	5.33	10.9	15.7
TE0	0.76	-0.001	6.25	9.2	13.9

Sa: mean roughness amplitude, Sk: skewness, St: range amplitude, lac: autocorrelation length, order: parameter quantifying the organization of the surface topography.

surfaces either parallel (TU) or crossed grooves (TX) with a width of $200\ \mu\text{m}$ were visible (Fig. 2). As assumed, the roughness amplitude parameters (Sa, St) were higher on sandblasted and electro-eroded surfaces although the frequency parameter “order” describing the periodicity of the profile was higher on machined-tooled surfaces (Table 3).

3.2. Morphological aspect of cells on surfaces

On all surfaces, human osteoblasts showed a rather polygonal morphology with few filamentous extensions (Fig. 3). They had very intimate contact with electro-eroded surfaces and notably with TE0 ones. They look as if they were integrated in the relief. After 14 days an extracellular matrix layer was visible under the cell layer on TE0 substrates although on TE1 only a small amount of extracellular matrix was synthesized after 21 days. On sandblasted Ti surfaces (TS), the cells adhered well and displayed a polygonal morphology after 1 and 7 days. After 14 days, a quasi confluent cell layer covered the substrates with a thick underlying extracellular matrix. On the flattest surfaces like the acid-etched, the polished or the machine-tooled ones, the osteoblasts spread extensively after 1 day but without any orientation and displayed more filamentous extensions than on rough surfaces. On the polished surfaces after 7 days and 14 days, the cells appeared so flattened that the substrate’s morphology was visible through the cells. After 21 days, the cell borders of adjacent cells were fused so that the complete cell perimeter could hardly be determined. After 7 days, the cells cultured on acid-etched surfaces (TA) were elongated and displayed a parallel orientation. Extracellular matrix proteins were visible around and under the cells soon after 7 days. On machine-tooled surfaces with parallel grooves (TU), the cells oriented following the groove direction after 1 day. On cross-grooved surfaces (TX), cells appeared after 1 day mainly located in pits rather than on relief and were spread polygonally. After this delay, osteoblasts did form a multilayer of elongated cells with parallel orientation apparently not related to the under-

lying grooves organization. After 21 days, human osteoblasts formed confluent layers on all surfaces except on TE1 (Fig. 3).

3.3. Statistical analysis of cell adhesion

The trypsination curves obtained on each surface and after each culture time were drawn (Fig. 4). The detachment was slower on acid-etched (TA), electro-eroded (TE0 and TE1) and sandblasted surfaces (TS) and slowed down with culture time. The AP values were coherent with these observations. The mean of the AP number with its associated 95% confidence interval are shown on Table 4. The highest adhesion was obtained on isotropic rough surfaces like electro-eroded, acid-etched and sandblasted surfaces. We determined by a bootstrap statistical technique [36] the roughness parameter (taking from 75 roughness parameters) that better correlates with the AP. We continue to model by a power law the relation between a roughness parameter and the measure of adhesion. For each roughness parameter the standard deviation of the residual (i.e. modelled data minus experimental data) is computed. For all the relations, the lower standard deviation, the more relevant the roughness parameter. Fig. 5 represents the classification of the 35 most relevant roughness parameters according to their pertinence for cell adhesion and the measure of classification is a standard deviation of the residuals. This analysis shows that frequency parameters discriminate adhesion better than amplitude parameters. This confirms that cell adhesion is more influenced by the morphology of the surface topography than by its amplitude [26].

3.4. Relation between the topographical order of the surface and the long-term adhesion

The order parameter was the best parameter correlating to the AP. Then, we calculated by a nonlinear least square method the function that best fitted the relation between AP and the order of the surface.

We found that the equation that best represents the relation could be expressed by

$$\text{AP}(\text{order}) = \frac{16}{(\text{order} - 13.8)^{0.187}}$$

With a good regression coefficient of $r = 0.994$ (Fig. 6).

Two remarks arise from this equation. The value of 13.9 obtained by the electro-eroded surfaces with low roughness amplitude seems to be near the optimal value (which is 13.8 because $\lim_{\text{order} \rightarrow 13.8} \text{AP}(\text{order}) = +\infty$).

The lowest adhesion value could be obtained for $\text{AP}(100) = 7$ which is that of the quasi perfect periodic surface. To us, it is near the value of the tooled machined surfaces for which we obtained 7.4.

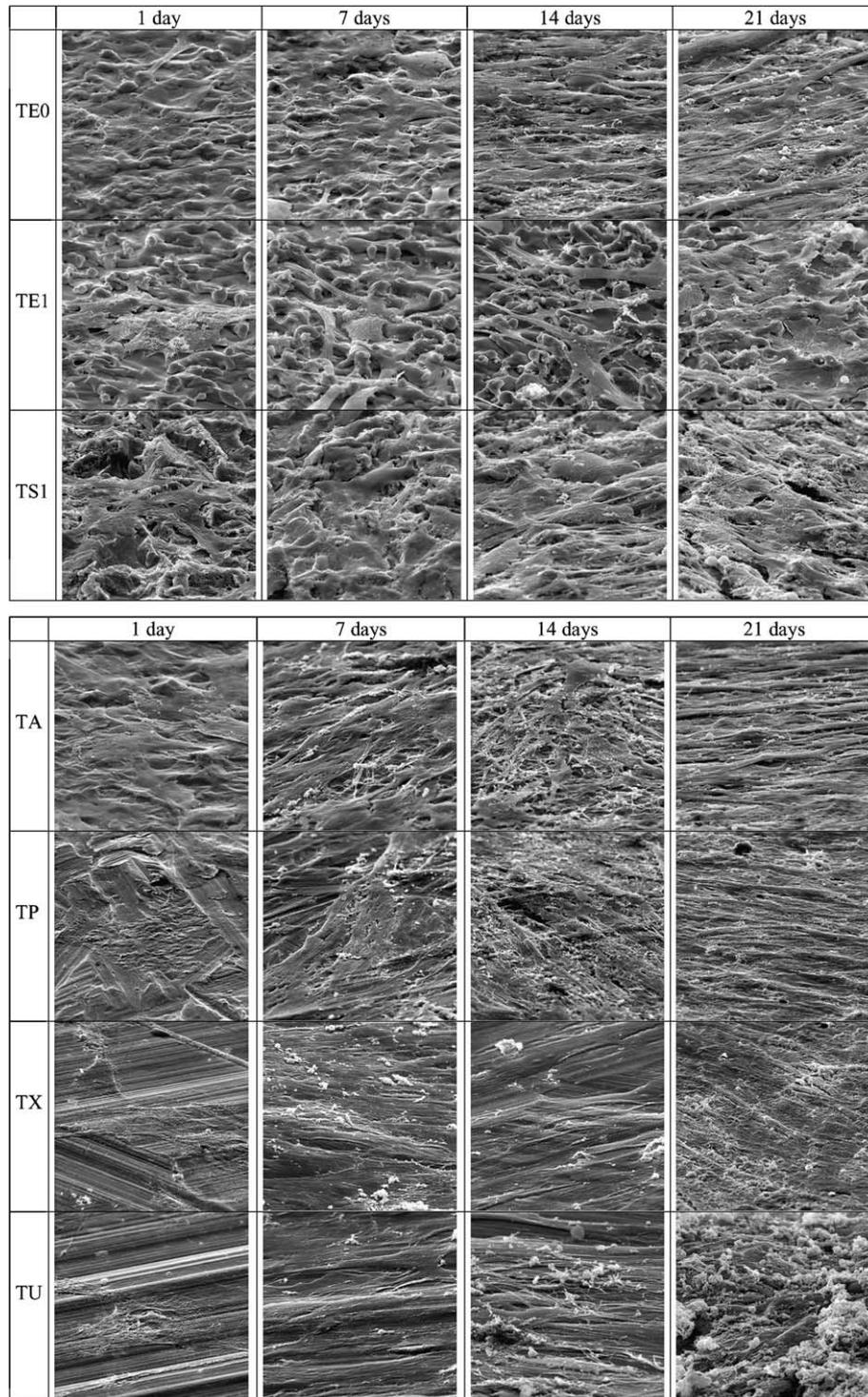


Fig. 3. Scanning electron micrographs of the tested surfaces after 1 day, 7 days, 14 days and 21 days of culture. Fine electro-eroded surfaces (TE0), coarse electro-eroded surfaces (TE1), sandblasted surfaces (TS), acid-etched surfaces (TA), polished surfaces (TP), machine-tooled surfaces with crossed (TX) or parallel (TU) grooves. Bar = 20 μm .

4. Discussion

The morphology of cells on electro-eroded surfaces was the same as previously shown [26] but interestingly, the contact with the substrates was more intimate on

fine electro-eroded surfaces (TE0) than on coarse ones (TE1). This more intimate contact was also observed on polished and machined surfaces presenting similar roughness amplitude values. As in previous work, the human osteoblasts on polished surfaces were very well

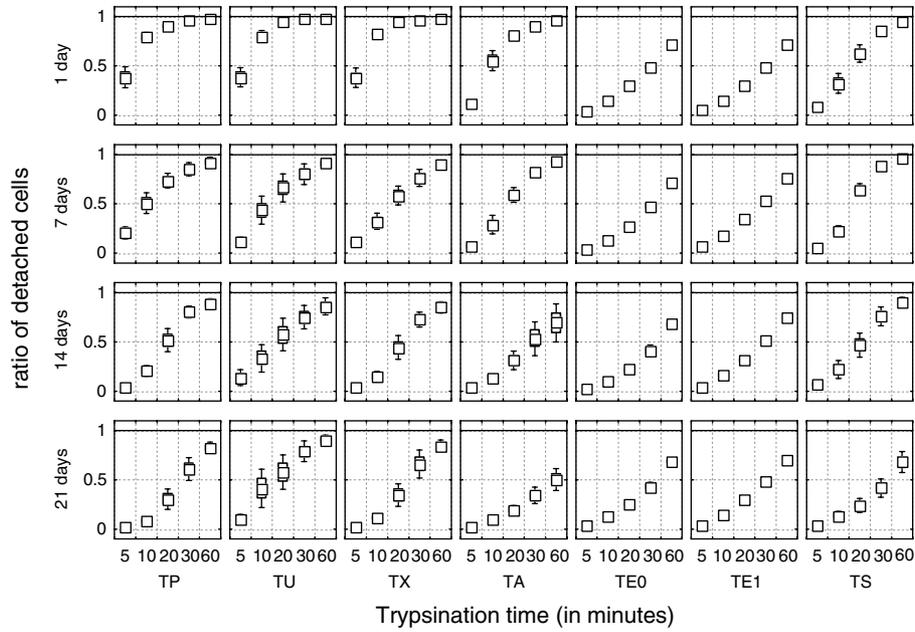


Fig. 4. Mean number (open square) of detached cells from each type of substrates in function of trypsination time. Polished surfaces (TP), machine-tooled surfaces with crossed (TX) or parallel (TU) grooves, acid-etched surfaces (TA), fine electro-eroded surfaces (TE0), coarse electro-eroded surfaces (TE1), sandblasted surfaces (TS). Open rectangle: mean value. Bars: 95% confidence interval of the mean.

Table 4
Adhesion power values (means) for each surface tested

Sample	Adhesion power	95% standard error	Number of experiments
TE0	28.1	2	9
TA	14.6	0.9	9
TE1	13.2	0.5	9
TS	12.5	0.75	9
TX	9.6	0.4	12
TP	8.5	0.5	12
TU	7.4	0.9	12

spread, since the cells were very difficult to distinguish on the substrates after 14 days and no particular orientation was observed in function of residual grooves of the polishing [24,26]. This intimate contact with the surface was also found on acid-etched surfaces but with lower spreading since the cell limits were more distinguishable than on polished surfaces. Thus, the relatively low roughness amplitude of polished, acid-etched and machine-tooled surfaces induced the maximal spreading of cells.

In this experiment, we attempted to form sorts of pits on Ti substrates by making cross-grooves using machine-tooling. In this way, our objective was to reproduce the sorts of ‘nests’ observed on coarse electro-eroded surfaces and which seemed to favour osteoblast adhesion [26]. These pits measured about 200 μm in width and 5.5 μm in depth. These dimensions and notably the width were probably too large to provoke any preferential attachment of cells in pits or on the

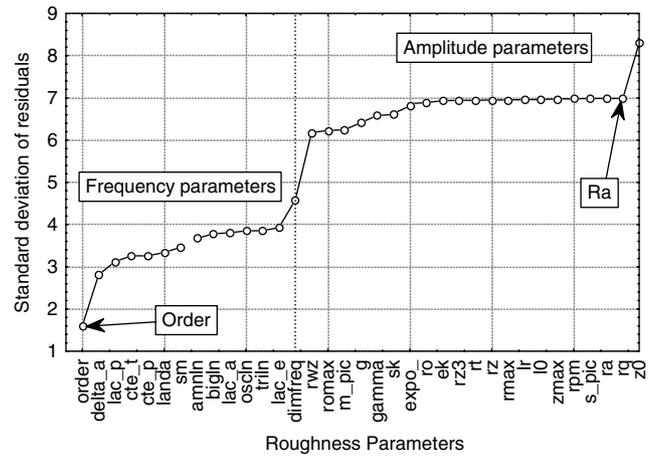


Fig. 5. Classification of the relevance of 35 roughness parameters with regard to the adhesion power (AP). x axis: roughness parameters divided in 14 frequency parameters (left of the dot line) and 21 amplitude parameters (right of the dot line). y axis: standard deviation of the residuals obtained from the statistical correlation analysis between each roughness parameter and AP. The lower the standard deviation of the residuals, the higher the correlation with AP.

ridges around. However, we previously observed that cells oriented along grooves measuring 150 or 200 μm width and 0.6 and 1.2 μm in depth, respectively, on parallel machined-tooled surfaces [25,26]. Hence, it appears that the behaviour of cells is different in regard to the morphology of the topography and that parallel and cross-grooves are not identified by the cell in the same way.

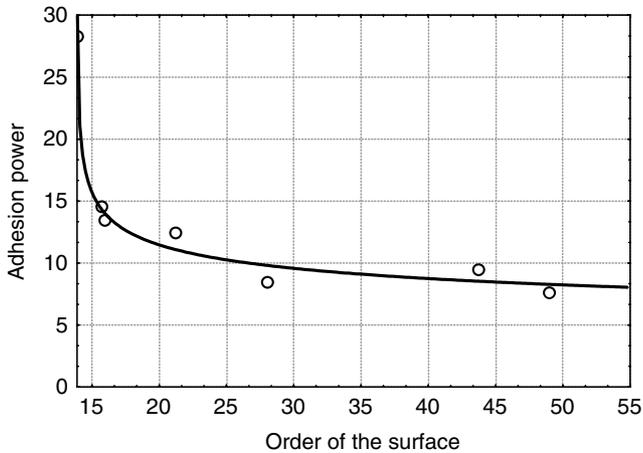


Fig. 6. Regression curve between adhesion power and order parameters on the seven tested surfaces.

Our objective in this work was to test if our new AP parameter correctly discriminated between the long-term adhesion of human osteoblasts on pure titanium substrates with various surface topographies. Firstly, we demonstrated that it fitted well with the detachment curves and was coherent with the morphological aspect of cells on surfaces. Secondly, we demonstrated that it better correlated with the order parameter among 75 other surface roughness parameters. We observed as in our previous studies on Ti6Al4V substrates that human osteoblasts adhere better on rough surfaces than on smooth ones [23,24,26]. As previously, the adhesion of human osteoblasts was high on electro-eroded surfaces [26] but surprisingly, we also obtained a high adhesion of cells on the coarse Ti sandblasted surfaces. This last result is contradictory with some of our previous results obtained on coarse sandblasted Ti6Al4V surfaces [24]. This high adhesion of human osteoblasts on sandblasted Ti surfaces is already well known [18,19,30,37]. Moreover, it is likely that the complete disturbance of the surface element composition provoked by sandblasting of Ti6Al4V that we observed in our previous experiment and which induced the formation of a cytotoxic AlO_x -enriched layer on samples surface [23], does not occur on pure titanium. These observations emphasize the influence of surface chemistry on cell growth and adhesion. It is likely that electro-erosion and acid-etching processes affect the surface chemistry of pure titanium. The surface chemistry of these substrates is currently under investigation using X-ray photoelectron spectroscopy. However, we have previously demonstrated that the human osteoblast adhesion was more influenced by surface roughness than by surface chemistry of electro-eroded Ti6Al4V and pure titanium substrates [26]. Indeed, adhesion was the same on Ti6Al4V and pure titanium electro-eroded samples whereas the surface chemistries of these two substrates were different. Moreover, the covering of these substrates by an inert nano-

metric metallic layer did not affect the human osteoblast adhesion demonstrating that the surface roughness had the most influence on human osteoblast adhesion. Concerning the acid-etched substrates, it has been shown by many authors that the sandblasting treatment followed by acid-etching induced a surface roughness favourable for cell growth and differentiation [27,28,38,39]. However, it is well known that the surface chemistry of titanium-based dental implants is modified by acid-etching [40]. Although we proceeded to a relatively short-term fluorhydric acid treatment (5 min at room temperature), the surface chemistry of our substrates are likely to be modified and residues of fluorine are expected. Prior to the complete characterization by XPS of these surfaces, we can note that cell adhesion and proliferation on the acid-etched substrates was comparable to the other substrates with the same Sa and that no cytotoxic effect was observed.

Finally, the AP confirms our previous results obtained with our previous parameters used for long-term adhesion strength measurement: adhesion index [23,24] or the detachment index percentage [25,26]. The AP has also been used to compare the adhesion of human osteoblasts cultured on 30 different materials (Ti, Ti6Al4V, 316L stainless steel) treated by various methods (polishing, sandblasting, electro-erosion, machine-tooling, acid-etching) to obtain topographies with various amplitudes and morphologies. This work confirms the validity of the AP parameter for quantification of long-term adhesion and will be published elsewhere.

5. Conclusion

For several years, our objective has been to develop experimental methods to evaluate the long-term adhesion of human osteoblasts from some hours to several weeks in order to model in vitro a tissue-like interface. We recently developed a new parameter called adhesion power to evaluate this long-term adhesion. By SEM, we observed that the human osteoblasts spread more intimately on surfaces with low roughness amplitude than on rough ones. However, they displayed a higher AP on rougher surfaces. Besides, we demonstrated that the AP was pertinent for evaluating human osteoblast long-term adhesion on pure titanium surfaces with various roughness. Its correlation with the order parameter confirmed once more that human osteoblasts are more sensitive to the organization and morphology of roughness than to its amplitude.

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