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On the Relation Between Surface Roughness of Metallic Substrates and Adhesion of Human Primary Bone Cells

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Summary: Surface characteristics of materials, whether their topography, chemistry, or surface energy, play an essential part in osteoblast adhesion on biomaterials. Thus, the quality of cell adhesion will influence the cell's capacity to proliferate and differentiate in contact with a biomaterial. We have developed for more than ten years numerous studies on the influence of topography and chemistry of metallic substrates on the response of primary human bone cells. The originality of our approach is that contrary to most of other authors, we quantified the adhesion of primary human bone cells on metallic substrates with perfectly characterized surface topography after some hours but also over 21 days. Moreover, we have developed original statistical approaches for characterizing the relation between surface roughness and cell-adhesion parameters. In this article, we will illustrate different studies we did these last ten years concerning the development of a new adhesion parameter, the adhesion power; the correlation between short-term adhesion, long-term adhesion, and proliferation; the influence of roughness organization on cell adhesion and the development of the order parameter; our modeling approach of cell adhesion on surface topography; the relative influence of surface chemistry and topography on cell adhesion and contact angle; the relation between surface features dimensions and cell adhesion. Further, some considerations will be given on the methods for scanning surface topography for cell-adhesion studies. Finally, perspectives will be given to elucidate these intracellular mechanotransduction mechanisms in-

duced by the deformation of cells on model sinusoidal peaks-or-valleys surfaces. SCANNING 36: 11–20, 2014. © 2012 Wiley Periodicals, Inc.

Key words: surface analysis, metrology, computer simulation, life sciences

Introduction

Cell adhesion is involved in various natural phenomena such as embryogenesis, maintenance of tissue structure, wound healing, immune response, and metastasis as well as tissue integration of biomaterial. The biocompatibility of biomaterials is very closely related to cell behavior in contact with them and particularly to cell adhesion to their surface. Surface characteristics of materials, whether their topography, chemistry, or surface energy, play an essential part in osteoblast adhesion on biomaterials (Anselme, 2000). Thus, the quality of the cell adhesion will influence the cell's capacity to proliferate and to differentiate itself on contact with the implant. We have developed for more than ten years numerous studies on the influence of topography and chemistry of metallic substrates on the response of primary human bone cells. The originality of our approach is that, contrary to most of other authors, we studied the adhesion of primary human bone cells over 21 days (long-term adhesion or LTA) on metallic substrates presenting perfectly characterized surface topography and did proceed to statistical approaches for characterizing the relation between surface roughness and cell-adhesion parameters. In this article, we will illustrate the studies we developed last ten years: first to define a new parameter (adhesion power or AP) able to quantify the LTA (Anselme *et al.*, 2000a; Bigerelle *et al.*, 2002b; Anselme and Bigerelle, 2005; Bigerelle and Anselme, 2005a); second, to analyze the

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sensitivity to surface roughness of various biological parameters such as short-term adhesion (STA), LTA, and proliferation (Bigerelle and Anselme, 2005b; Bigerelle and Anselme, 2005c); third, to demonstrate that the response of cells to topography is more related to the roughness organization of cells' substrates than to their roughness amplitude (Anselme *et al.*, 2000a; Bigerelle *et al.*, 2002a, 2002b; Anselme and Bigerelle, 2005); fourth, to develop a statistical approach to model cell/material interactions; fifth, to elucidate the relative influence of surface chemistry and topography on cell adhesion and on contact angle (Anselme *et al.*, 2000b; Anselme and Bigerelle, 2006a, 2006c); sixth, to demonstrate the twofold reduction of the adhesion of cells on peaks-or-valleys that are approximately the size of cells suggesting a negative mechanotransduction process inside deformed cells (Bigerelle *et al.*, 2011a). Finally, some surface metrology considerations for cell/surface interaction studies will be developed.

Development of a New Cell-Adhesion Parameter: The AP

The objective of our initial paper was to evaluate quantitatively the adhesion of cells on Ti6Al4V surfaces presenting various surface roughness, obtained either by polishing or sandblasting, and to correlate these values with parameters describing surface topography (Anselme *et al.*, 2000a). In parallel, the adhesion of cells was evaluated qualitatively in term of expression of proteins from extracellular matrix (ECM) and focal adhesions to correlate with quantitative values of cell adhesion. Moreover, we developed an original method to evaluate the adhesion of cells over a period of 21 days that we called "LTA." This approach was derived from a method initially developed for quantifying the adhesion of a cell layer formed around tissue explants in an organotypic culture model (Anselme *et al.*, '94). Briefly, this technique is based on a progressive detachment of cells because of a solution of trypsin-EDTA (classically used to detach cells) that is diluted ten times at 0.025%. The cells detached after 5, 10, 20, 30, and 60 min are counted. At the end of this digestion, an undiluted solution of trypsin-EDTA is added to detach all the undetached cells. Then, a cumulative curve of the number of detached cells with time of digestion with trypsin-EDTA solution can be drawn. The area under the detachment curve was considered as a detachment index that was inversely proportional to the cell adhesion on the biomaterial. Cells with a low adhesion capacity were detached easily and rapidly by incubations with the diluted trypsin-EDTA whereas the cells with a higher adhesion capacity needed more time to detach and sometimes detached only after the final

treatment with undiluted trypsin-EDTA. This index was initially called "adhesion index" (Anselme *et al.*, 2000a), and then "detachment index" (Bigerelle *et al.*, 2002b).

One limit of this detachment method was that contrary to the classical methods that evaluate the detachment of cells only some hours after inoculation, we did this quantification over 21 days. During these 21 days of culture, cells did proliferate and synthesize proteins from ECM. In order to remove this experimental bias, we introduced the following model (Anselme and Bigerelle, 2005):

$$\text{Td}(T) = aT^b \text{ with } a \geq 0 \text{ and } b \geq 0,$$

where Td is the time-dependent LTA index.

As the cells did proliferate during the 21 days, the proliferation was included in the model. After resolution by the simplex method, a and b coefficients were determined. b did represent the kinetic exponent of LTA and a was a scaling factor that characterized the amplitude of the LTA. We call a coefficient the "AP."

Later, the exponent b was determined to be equal to 0.5 ± 0.03 , and to be independent of the substrate's characteristics meaning, the LTA increased proportionally to the square root of culture time. On the contrary, the a parameter did depend on the substrate's characteristics. It was demonstrated that the a parameter was sufficient to characterize cell adhesion (Bigerelle and Anselme, 2005a). Interestingly, it was suggested from this relationship that a diffusion-based process related to the kinetics of formation of ECM may be involved in LTA on materials. Indeed, the used detachment method based on trypsin-EDTA would be dependent on the diffusion of the enzyme inside the dense interface separating cells from their substrates and composed of more and more ECM macromolecules with culture time. Thus, the enzymatic activity of the trypsin-EDTA would be probably more and more inhibited by the increase of ECM macromolecules with time following a square root law (Bigerelle and Anselme, 2005a). Finally, the AP was systematically used as a quantitative parameter for LTA measurement in all our next papers.

Correlation Between STA, LTA, and Cell Proliferation on Substrate

Classically, the evaluation of cellular adhesion on substrates is limited to the evaluation of cell attachment after some hours. We have claimed for several years that this evaluation is incomplete to evaluate cell adhesion or more precisely to evaluate the quality of the *in vitro* cell/biomaterial interface. With a view to demonstrate this assertion, we developed statistical correlations between STA evaluating the attachment after 24 h (IA: initial attachment) and LTA

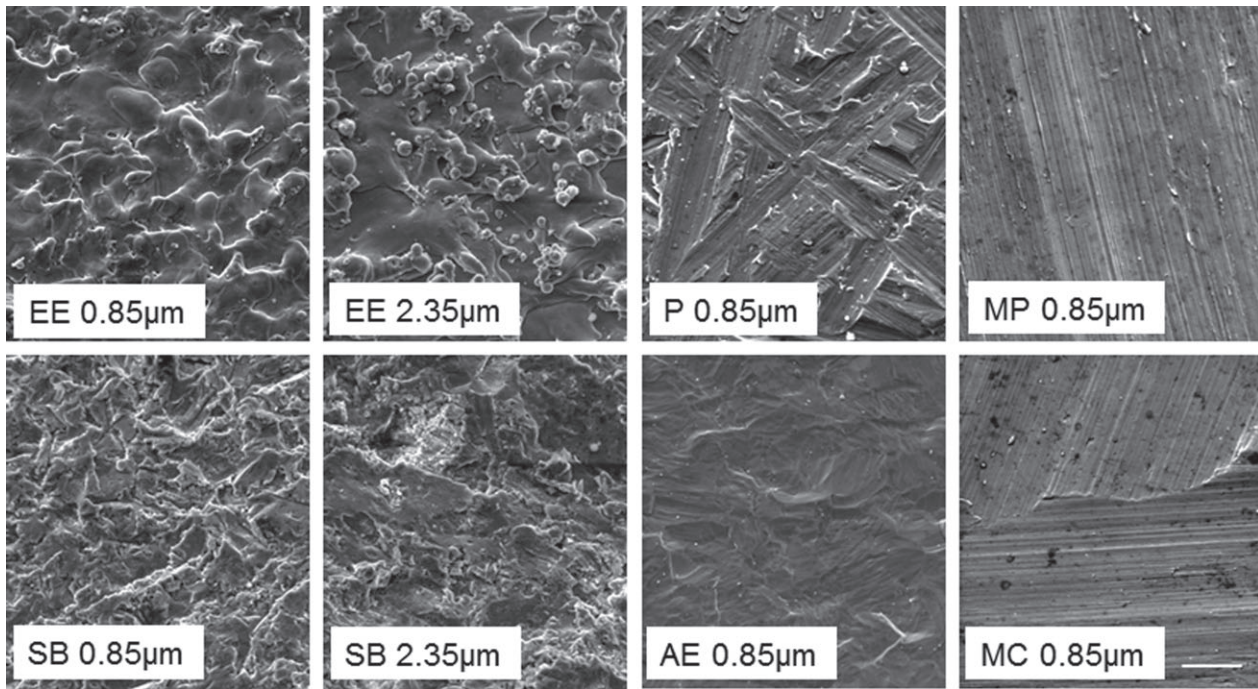


Fig 1. Morphologies of surfaces made by sandblasting (SB), electroerosion (EE), acid-etching (AE), polishing (P), and machine tooling in one direction (MP) and two directions (MC). Six surfaces with different morphologies but same roughness amplitude ($R_a = 0.85 \mu\text{m}$) were produced to test the influence of morphology. Two different amplitudes were obtained by electroerosion (EE 0.85 and $2.35 \mu\text{m}$) and sandblasting (SB 0.85 $2.35 \mu\text{m}$) to test the influence of roughness amplitude but by keeping the same morphology. Bar = $20 \mu\text{m}$.

evaluating the strength of the cell/matrix substrate interface over 21 days of culture (AP). Additionally, as the adhesion phase is known to influence further growth of cells, we proceeded to the correlation of STA with the final number of cells after 21 days (FNC). We demonstrated statistically the expected positive relation existing between STA and cell growth and showed that this relation was totally independent of the substrate's surface topography or chemistry. More surprisingly, we demonstrated the absence of correlation between IA and AP. This illustrates the fact that different mechanisms underlie STA and LTA. Moreover, this study demonstrated that the evaluation of the number of attached cells after some hours cannot let us presume either that cells will survive or that they will adhere at later times by forming a complex cell/substrate interface with synthesis of ECM proteins. Finally, the originality of this work lied in the extensive statistical correlation analysis performed between biological parameters describing the cell behavior on a substrate and parameters describing surface topography (Bigerelle and Anselme, 2005b).

Seemingly, we correlated LTA with proliferation of human osteoblasts cultured from 24 h to 21 days on pure titanium, titanium alloy, and stainless-steel substrates presenting six different surface morphologies and two different roughness amplitude (Fig. 1).

Hence, we did proceed to the statistical correlation of cell adhesion and cell proliferation on these 30 different substrates. Additionally, we described surface topography not only by the roughness amplitude but also by the roughness morphology using new specific parameters. By multiple analysis of variance, we demonstrated that neither material composition nor surface roughness amplitude did influence cell proliferation, whereas a very significant influence of the process used to produce the surface was systematically observed meaning that the main influent factor on cell proliferation was the surface morphology (Table I). The LTA and proliferation capacity of cells were positively correlated on 13 substrates among 30. This study was the first demonstration of the existence of a statistical correlation between LTA and proliferation capacity of human bone cells on substrates with various chemical composition, surface chemistry, and surface topography (Bigerelle and Anselme, 2005c).

Influence of Roughness Organization on Cell Adhesion

Development of a New Parameter: The Order Parameter

In our initial paper, usual and original roughness parameters were calculated from measures made

TABLE I Summary of results obtained from our third experiment where three materials were compared (pure titanium, Ti6Al4V alloy, 316L stainless steel: Material), two roughness amplitudes were produced (Roughness) because of sandblasting and electroerosion

	Material Material nature	Roughness Roughness amplitude	Process Surface morphology	Coating Surface chemistry
Short-term adhesion	Yes, with process	No	Yes	Yes
Long-term adhesion	No	Yes, with process	Yes	No
Proliferation	No	No	Yes	Yes

Note: Six processes were used to produce either isotropic or anisotropic roughness (Process). Half of the samples were sputter-coated with a nanometric layer of gold-palladium to assure a homogenous surface chemistry (Coating). Multivariate analyses of variance were done in order to determine what were the most influencing surface parameters on short-term adhesion, long-term adhesion, or proliferation. In this table “yes” means that the parameter has a significant influence on cell response when “no” means no influence. When it is specified “yes, with process,” it means that the effect is function of the underlying surface morphology. Finally, it appears that whatever the biological parameter analyzed, the surface morphology will systematically influence the cell response.

using a confocal laser microscope (Anselme *et al.*, 2000a). Notably, the fractal dimension parameter (Δ) was used to quantify the roughness organization of the surface. The greater the fractal dimension, the more chaotic the surface. When the surface was very ordered $\Delta = 2$, whereas when the surface was chaotic $\Delta = 3$. We demonstrated in this article that the fractal dimension parameter correlated better with the proliferation than the roughness amplitude parameter R_a . Another roughness parameter describing the surface morphology was defined, i.e. the developed surface (Surf). The Surf parameter was the one that best correlated with the detachment index (Anselme *et al.*, 2000a).

In a second experiment, a new parameter was developed to quantify the order of a surface. This parameter was scale-independent and could be used to compare the organization of a surface at different scales (Bigerelle *et al.*, 2002a). In order to assess the physical relevance of this new parameter, we elaborated surfaces presenting different morphologies because of different machining process on pure titanium and titanium Ti6Al4V alloy (Bigerelle *et al.*, 2002b). Isotropic rough morphologies were obtained by electroerosion while isotropic smooth morphologies were obtained by polishing with paper grade 80. Machine tooling produced surfaces with an anisotropic morphology made of concentric grooves. Then we studied the influence of the roughness on *in vitro* adhesion and proliferation of human osteoblasts. It was then shown that our new order parameter better discriminated among the cell-adhesion phenomena than other parameters ($R_a \dots$). Cells adhered better on isotropic surfaces with a low order, provided this order was quantified on a scale that was more important than that of the cells. This approach allowed us to demonstrate that the surfaces produced by electroerosion were particularly interesting for implant preparation and to improve bone implant integration.

In a third experiment, we produced surfaces presenting different morphologies because of different machining process (Bigerelle *et al.*, 2002b). Isotropic

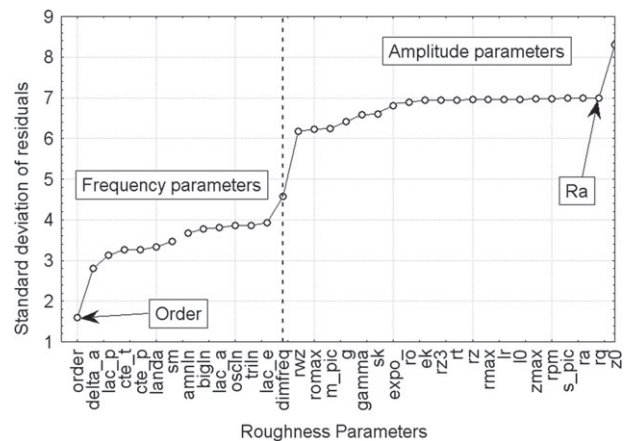


Fig 2. Classification of the relevance of 35 roughness parameters with regard to the adhesion power (AP). *x*-axis: roughness parameters divided in 14 frequency parameters (left of the dotted line) and 21 amplitude parameters (right of the dotted line). *y*-axis: standard deviation of the residuals obtained 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. Copy with permission from Anselme and Bigerelle (2006b). For more details on the roughness parameters refer to Najjar *et al.* (2003).

rough morphologies were obtained by sandblasting, electroerosion, and acid etching of three different materials (pure titanium, Ti6Al4V alloy, 316L stainless steel). Machine tooling in one or two directions and polishing with paper grade 40 produced surfaces with anisotropic morphology (Fig. 1) (Bigerelle and Anselme, 2005c).

Again, the AP parameter was calculated on these different surfaces. We determined by a bootstrap statistical technique (Najjar *et al.*, 2003) the roughness parameter (taken from 75 roughness parameters) that better correlated with AP. For each roughness parameter, the standard deviation of the residual (i.e., modeled data minus experimental data) was computed. For all the relations, the lower standard deviation was, the more relevant the roughness parameter was. Figure 2 represents the classification

of the 35 most relevant roughness parameters according to their pertinence for LTA. 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 LTA is more influenced by the morphology of the roughness than by its amplitude (Anselme and Bigerelle, 2005).

Modeling Approach of Cell Response to Surface Topography

Following these studies, the *Biomaterials* journal gave us the opportunity to publish a leading opinion paper to explain our modeling approach of cell/material interactions to the researchers of the field (Anselme and Bigerelle, 2006b). Indeed, these interactions are generally studied only after the first minutes or first hours of cell–material contact. The objective with this approach is to exclusively consider short-term adhesive events occurring between cells and a given surface before cell proliferation begins and before cell/cell interactions are established. However, it exists another approach where authors consider all together, STA and LTA, proliferation and differentiation phase. We esteem this second approach as more valuable since it better approximates the *in vivo* situation. One of its major drawbacks is the simultaneous involvement of many parameters of which a large number cannot be fully controlled. This is the reason why in most typical studies of cell/material interactions, only one cellular parameter (attachment, proliferation) and one surface parameter were considered (surface roughness, surface composition). In most cases, the surface roughness parameter was poorly defined and its influence not entirely analyzed. Moreover, it was generally not considered if this parameter was also correlated with other surface parameters. For example, the surface topography was often only defined by the average roughness amplitude parameter (R_a) although our team has shown that cell adhesion was more correlated to frequency parameters describing the organization of the surface topography (Bigerelle *et al.*, 2002b; Anselme and Bigerelle, 2005). Moreover, very few teams attempted to use statistical tools to analyze their results (Nebe *et al.*, 2007). This modeling approach allowed us also to decorrelate the proliferation and the adhesion contribution in the AP measurement. Thus, we were able to show that LTA increased with proliferation contrary to what is generally admitted in the community.

In this leading opinion article, we also provided basic principles for developing more ambitious experiments comparing the simultaneous influence of more than one or two parameters on measured data, taking

advantage of existing convenient statistical and mathematical techniques for the treatment of data. Analyzing some examples of our own experiments, the essential features needed for modeling cell/material interaction studies were presented. First, the initial process for designing appropriate experiments allowing for comprehensive modeling was described. In the second part, the different applications of a specific statistical modeling technique, the bootstrap protocol, on the amplification of data, the elimination of correlation existing between measured parameters or the identification of the most relevant parameter out of a set of parameters were illustrated. Finally, based on recent statistical analysis tools such as the bootstrap, the relative influence of biological and physical parameters in phenomenological studies of cell/material interactions was illustrated (Anselme and Bigerelle, 2006b).

Relative Influence of Surface Chemistry and Surface Topography

Cell Adhesion

In our first experiment, the adhesion and proliferation of cells were reduced on Ti6Al4V surfaces sandblasted by 3 mm in diameter alumina particles (Anselme *et al.*, 2000a). However, we wondered if this low biocompatibility was relative to increased surface roughness amplitude, lowest surface organization or to eventual surface chemistry modifications due to the sandblasting process. In order to answer this question, we did proceed to a chemical composition analysis of surfaces and sections of samples with the help of an electron microprobe microanalysis (Anselme *et al.*, 2000b). The wave dispersion scanning technique was applied to determine the $K\alpha$ characteristic lines of O, Al, Ti, and the $K\beta$ characteristic line of V. The topographical distribution of Al, Ti, and V was not disturbed on surface of polished samples although it was completely disturbed on surfaces sandblasted with 3-mm alumina particles. The observation of sections confirmed enrichment in an oxidized aluminum compound that was not single-phase Al_2O_3 (Fig. 3). Moreover, given the thickness of this oxidized layer, sometimes more than 10 μm , the formation of this layer undoubtedly originated from an important chemical transformation of the alloy due to stresses generated by sandblasting and not from contamination by aluminum oxide originating from the alumina particles used for sandblasting. To confirm such assertion, section analysis was carried out on control samples sandblasted with silicon carbide (SiC) particles. Both of the quantitative section analyses performed on alumina or SiC sandblasted samples revealed the Al enrichment and the V impoverishment at the outermost surface of the alloy (Fig. 3).

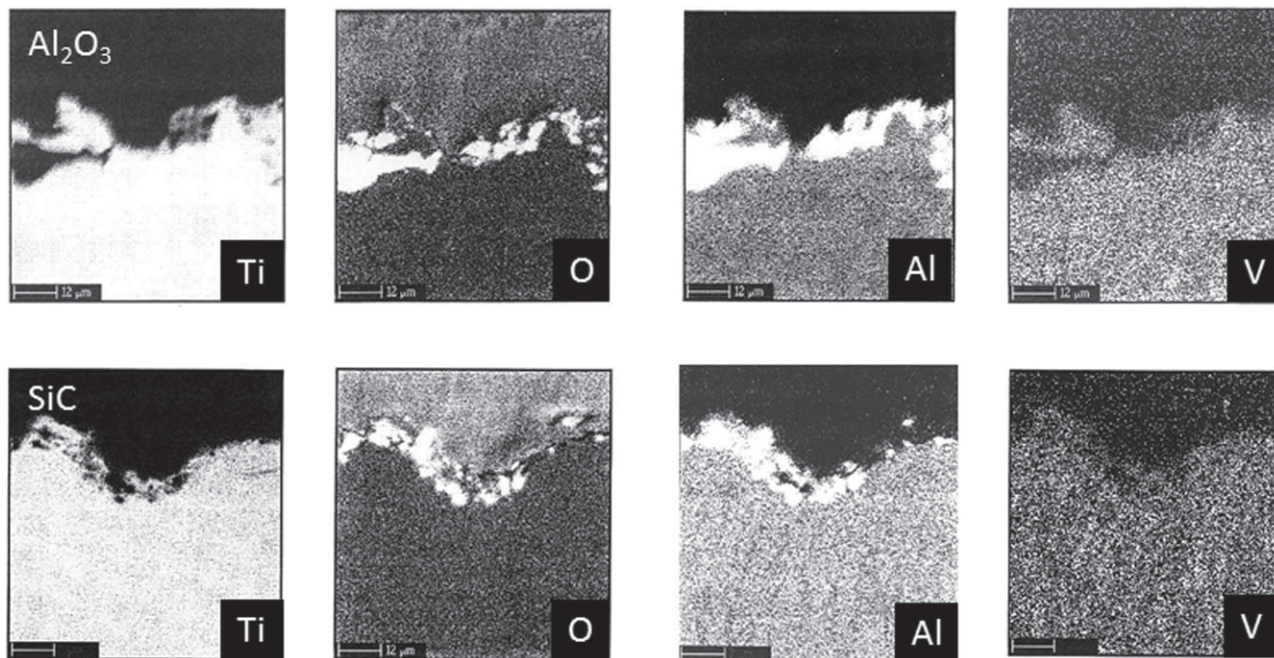


Fig 3. Wavelength dispersive spectrometry (WDS) section analysis of the 500 μm alumina (Al_2O_3) particles sandblasted Ti6Al4V samples revealed an enrichment (white area) of surface with an oxidized aluminum compound (AlO_x ; top row). WDS section analysis of silicon carbide (SiC) particles sandblasted samples confirmed that the surface chemistry modification was related to the process and not to the particles used (bottom row; bar = 12 μm).

In our further experiments, in order to discriminate directly the relative influence of surface topography and surface chemistry on cell response, we sputter-coated our surfaces with a 50-nm-thick gold-palladium layer. Because of this simple approach, we showed that the STA was mainly influenced by surface chemistry (Anselme and Bigerelle, 2006c) while LTA could be negatively, positively, or not influenced by surface chemistry depending of the initial composition of the substrates. However, this influence was significantly demonstrated only three times and in all three cases, the LTA was significantly improved by the coating (Anselme and Bigerelle, 2006a).

Contact Angle

The influence of surface chemistry on cells is sometimes dependent of modified surface energy. The surface energy is influenced by surface chemistry but also by surface topography. One of our objectives in the last experiment we developed in this field was to elucidate how surface chemistry was related to surface topography using isotropic rough surfaces produced by the electroerosion process also called electrical discharge machining (EDM). Indeed, this process produces perfectly isotropic and self-affine surfaces. The term “self-affine” means that the arithmetic roughness amplitude R_a is linearly related to the mean dis-

tance between asperities S_m and that the height of surface features (peaks and valleys) increases concomitantly with their width. Thus, when the roughness increases, the morphology of the surface remains constant; only the size of peaks and valleys changes. Consequently, on these surfaces, eventual modification of contact angle or cell response will be only related to roughness amplitude and not to roughness morphology. Moreover, the range of roughness we were able to produce by EDM was very wide, starting from an $R_a \sim 1.2 \mu\text{m}$ to $\sim 21 \mu\text{m}$. We used these surfaces to study the influence of roughness amplitude on contact angle (Giljean *et al.*, 2011). Indeed, the wetting mechanisms on rough (topographically structured) substrates have been essentially discussed and modeled on materials with low surface energy. On the contrary, the wetting behavior of rough substrates with high surface energy such as metallic materials still remained sparsely investigated and thus misunderstood. In order to be able to measure the contact angles on these EDM-treated titanium materials with high surface energy, we did proceed to the wetting of surfaces with water in liquid alkane medium by the so-called two liquid phase method. It was shown that the contact angle first increased with the roughness parameter, until a threshold value from which it leveled off (plateau; Fig. 4). Interestingly, this increase was visible only on perfectly cleaned surfaces because of soakings in ultrasonic baths of acetone, cyclohexane and acetone and argon plasma cleaning. Indeed,

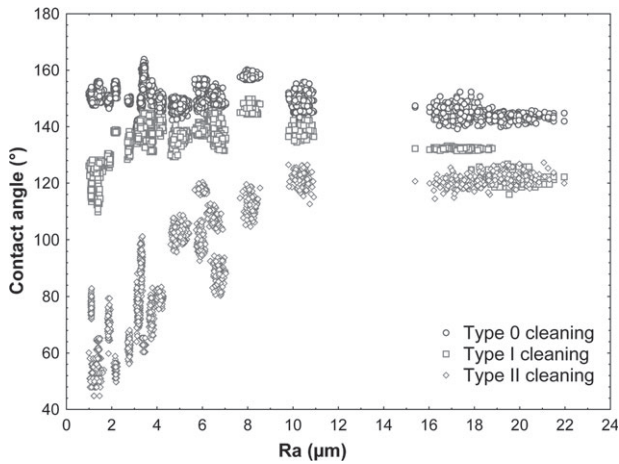


Fig 4. Evolution of the contact angle versus the roughness for three cleaning methods. First, a water rinsing followed by nitrogen drying was called “type 0” cleaning. “Type I” cleaning was defined as successive soakings in ultrasonic baths of acetone, cyclohexane and acetone, followed by water rinsing, nitrogen drying. Finally, “type II” cleaning was defined as “type I” cleaning followed by an argon plasma cleaning. After cleaning, samples were immediately immersed in octane medium. Only type II cleaning allowed to observe an evolution of the contact angle with roughness amplitude. Adapted from Giljean *et al.* (2011).

because their high surface energy, metallic surfaces are immediately contaminated by carbon when they are in contact with atmosphere. This large carbon contamination impedes correct contact angle measurement.

Instead of the jump between distinct wettings regimes that is often observed on patterned surfaces for some critical roughness, the increase of the contact angle toward the plateau was here gradual. This was explained by the heterogeneous distribution of the peaks and valleys within the isotropic roughness produced by EDM. The structure of the contact line and wetting mechanism were described by a composite regime (hemiwicking model) involving the Wenzel and Cassie–Baxter models (Giljean *et al.*, 2011).

Relation Cell Adhesion/Surface Feature Dimensions

Because of the wide range of roughness previously described, we attempted to determine whether a threshold in sensitivity of human mesenchymal stem cells (hMSCs) to isotropic roughness exists (Bigerelle *et al.*, 2011a). The curve of the number of adherent hMSCs after two-day culture as a function of roughness showed a U-shape with a minimum number of attached cells for a roughness amplitude $R_a = 4.5 \mu\text{m}$ and a distance between surface features (width of peaks and valleys) $S_m = 110 \mu\text{m}$. The maximum cell number was observed at the lowest (1.2–4.5 μm) and

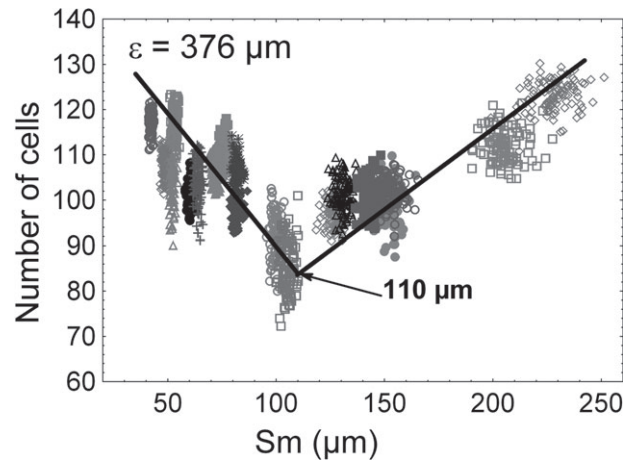


Fig 5. Evolution of the number of attached cells as a function of the S_m parameter at the best evaluation length (376 μm). Each point in each group is the mean obtained from the experimental data after drawing and replacement during the bootstrap process. Adapted from Bigerelle *et al.* (2011a).

highest roughness (4.5–21 μm). Due to this very wide range of roughness, it was possible to demonstrate that the response of hMSCs to roughness varies with the dimensions of the surface features relative to the cell size ($\sim 100 \mu\text{m}$). Above or below their own size, hMSCs essentially adhered to the nano- and submicron features. When the surface displayed features about the same size as hMSCs, the curvature of these surface features will reduce twofold the number of attached cells (Fig. 5). A modeling approach was proposed to help the interpretation of these results. It was hypothesized that this minimal adhesion was a consequence of an unfavorable stress imposed on the cell cytoskeleton.

Consideration on Scanning of Surface Topography for Cell Response Studies

The choice of a scanning apparatus to record surface topography is a hard task. In the literature, various apparatus are employed such as atomic force microscopy (AFM), tactile profilometer, confocal microscopy, interferometry microscope, etc. We have proceeded to an exhaustive review of the apparatus used in the field of cell/surface interactions studies (Anselme and Bigerelle, 2011) and discussed the relevance of these apparatus (Jouini *et al.*, 2009). From these reviews, it appears that the choice of the apparatus is never justified. In a high number of circumstances, authors use the apparatus accessible in their laboratory. For example, if authors use AFM to quantify the mechanical properties of cell adhesion, they also employ it to quantify topography. In a same order, if they employ confocal microscopy for imaging living cells by fluorescence techniques, they also

use this microscope to quantify roughness. Finally, if authors work in the field of engineering science (materials, machining, etc.), they will prefer tactile profilometry or interferometry microscope. However, the choice of a relevant apparatus must include the surface functionalities that must be studied further (Bigerelle *et al.*, 2003; Najjar *et al.*, 2003; Bigerelle *et al.*, 2005), such as cell behavior in this article. To analyze these surface functionalities, two basic questions can be stated:

- Is it needed to test the effect of the surface anisotropy on the cell response?
- What is the scale range on which the cell response must be tested?

If the surface is adapted for measurement (e.g., enough stiff to be recorded by a tactile recorder), then only answers to these two questions are necessary to choose the relevant apparatus. These questions are fundamental. If surface anisotropy parameters are required, then only 3D measurements must be performed. However, basically speaking, the 3D scale range is narrow and the scale of relevance of cell behavior must be known. Even if this size can be increased by stitching methods (assembling of successive elementary 3D topographies), the defaults of the stitching method and the time of scanning does not allow to drastically increase the resolution (Bigerelle *et al.*, 2007). Then the first approach consists in searching if the surface is isotropic. In 3D measurements, some parameters allow to verify if surface is isotropic at the scale of the scanning length. However, this clearly means that the scale of relevance must be first known to quantify anisotropy of the surface (Van Gorp *et al.*, 2007).

The choice of the scale length is of major importance (Van Gorp *et al.*, 2010). If we suppose that such biological feature (cell adhesion, proliferation, etc.) can be influenced by the roughness, the scale length must be determined. If the scale of relevance is not well known, it is possible to estimate that the roughness influence lies in the $[s_{\min}, s_{\max}]$ interval (Bigerelle *et al.*, 2008a, 2008b, 2011b). Then, the following question comes: what will be the more relevant apparatus to use? Let us analyze the case of the study presented in this article about the relation found in Figure 5. Initially, we have claimed that adhesion can be governed by roughness under and over the cell size (100 μm). So, it was decided to choose as $[s_{\min}, s_{\max}]$ interval, the interval [100 μm , 1 mm]. Basically, roughness must be well defined under 100 μm , i.e. to well-defined peaks of the roughness. As a consequence, a minimum of 1,000 discretized altitudes is needed under this size. To have a respectable definition of the waviness evaluated at 1 mm, a minimum of 10 mm is necessary. To study roughness in the interval [100 μm , 1 mm], the scanning length is defined on [0.1 μm , 10 mm] inter-

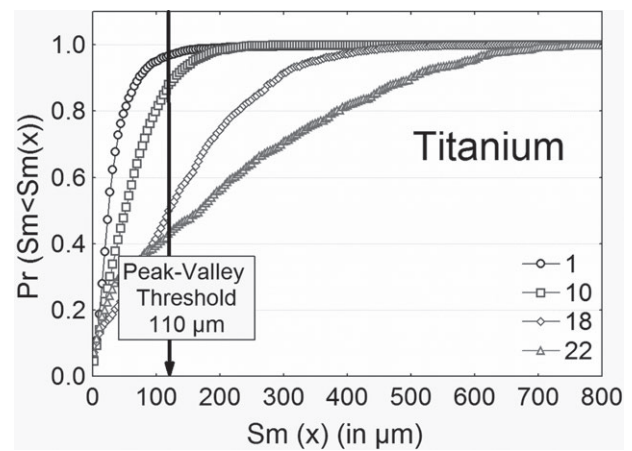


Fig 6. Cumulative density functions of peaks and valleys for surfaces tooled with four EDM intensities.

vals that require a resolution of 1,000,000 data points. Such resolution is impossible to reach with 3D measurements: 1,000,000 recording lines must be done with 3D profilometry and more than $1,000^2$ images must be stitched with 3D imaging methods. Consequently, only 2D techniques could be used. We have first verified by an original technique that the images were homogenous at all scales (Dalla Costa *et al.*, 2007). Then, 2D measurements were processed with a tactile profilometer with low radii curvature of the tip (50 nm). Our multiscale analysis has shown that the roughness must be computed at the scale of 400 μm and that the parameter that discriminated the best the adhesion was the S_m (mean distance between asperities) lying from 50 and 250 μm . Let us now analyze more precisely the peaks distribution. Figure 6 represents the individual distance between asperities on four roughness degrees. As it can be observed, distribution is quite wide and S_m varies from 5 to 600 μm . This proves that a wide range of S_m is required to compute the average S_m value. If surfaces are recorded by AFM (resolution around 20 μm) and used for correlation of roughness with cell adhesion, no relation will emerge (Fig. 7). As it can be seen, the spectra of adhesion can be wide. These results were confirmed by Brown *et al.* by analyzing adhesion on four substrates. Roughness area from 2 μm^2 to 120,000 μm^2 was required to find the most relevant scale of adhesion (Brown and Siegmann, 2001). This was also confirmed in a study of adhesive strength of staphylococcus epidermidis bacteria (Emerson *et al.*, 2006).

Conclusions and Short Perspectives

For more than ten years, we have developed original approaches to study the influence of topography and chemistry of metallic substrates on the

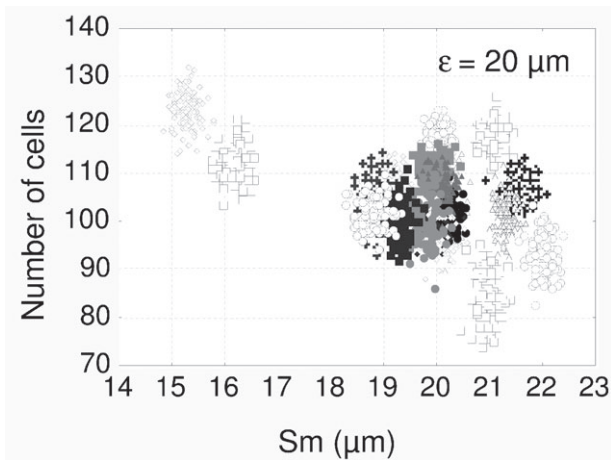


Fig 7. Evolution of the number of attached cells as a function of the S_m parameter at the evaluation length of $25 \mu\text{m}$. Each point in each group is the mean obtained from the experimental data after drawing and replacement during the bootstrap process.

response of primary human bone cells. Notably, we developed original and new parameters to quantify surface roughness and cell behavior and original statistical modeling approaches to analyze the correlations existing between these parameters. Because of this approach, we were the first to demonstrate that finally the human primary bone cells are more sensitive to surface morphology than to surface roughness amplitude, whatever the biological parameter analyzed (Table I). This was further confirmed by other authors at the nanoscale (Dalby *et al.*, 2007; McMurray *et al.*, 2011).

From these studies, it was also clear that the analysis of cell/material interface at short-time periods (less than 24 h) is not representative of the future evolution of the interface and cannot let presume either that cells will survive or that they will adhere at later times by forming a complex cell/substrate interface by synthesis of ECM proteins.

Finally, because of a very wide range of isotropic roughness obtained by EDM, we obtained original results on one hand on the relation between contact angle and roughness on high surface energy materials and on the other hand on cell response to surface features with dimensions relative to their own size.

Because of this last study, we hypothesized that cells could be stressed by the deformation imposed to their cytoskeleton by convex or concave surface features at their own dimension. In order to check the validity of this hypothesis, we will develop, in a next future new model, sinusoidal surfaces with peaks-or-valleys measuring about $100 \mu\text{m}$ to follow the deformation of cell's cytoskeleton on these surfaces features.

These new sinusoidal standards with different amplitude and frequencies will be usable for roughness

measurements in biomaterials field but also in other fields not directly related to the project (e.g., fluid mechanics, wear, adhesion, etc.).

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