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Leading Opinion

Modelling approach in cell/material interactions studies[☆]

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Abstract

Based on our experiments, we propose a statistical modeling approach of the in vitro interactions between biological objects and materials. The objective of this paper is to provide basic principles for developing more ambitious experiments comparing the simultaneous influence of more than one or two parameters on various observations, taking advantage of convenient statistical and mathematical techniques for the treatment of measured data. Analyzing some examples of our own experiments, the essential features needed for modeling cell/material interaction studies are presented. Firstly, we describe the initial process of designing appropriate experiments that allow for comprehensive modeling. In the second part, we illustrate the different applications of a specific statistical modeling technique, the bootstrap protocol, on either the amplification of data, the elimination of correlation existing between measured parameters or, out of a set of parameters, identification of the most relevant parameter for further statistical analysis. Finally, based on recent statistical analysis tools such as the bootstrap, we illustrate the relative influence of biological and physical parameters in phenomenological studies of cell/material interactions.

Keywords: Cell adhesion; Modeling; Surface roughness; Surface analysis; Cell proliferation

1. Introduction

Recently, many efforts have been undertaken to improve bone regeneration by the use of cellular or non-cellular implants. In vitro studies of bone cell responses to artificial materials are the basic tools to determine the material surface/tissue interactions on a cellular level [1,2]. Classically, the majority of these in vitro investigations focus either on cell attachment after some hours or on proliferation during some days of culture. The effects of materials composition, as well as the effects of their surface

chemistry or surface topography on cell adhesion and proliferation have been largely studied on bone-derived cells [3–11]. The material composition always influences cell attachment [4,6,7], whereas variations of surface chemistry of titanium-based substrates following surface treatments like anodization have generally little influence on osteoblasts attachment capacity [10,11]. Likewise, Ahmad et al. [12] described a not significant difference of osteoblastic cell attachment between Grade 1 and Grade 4 pure titanium. On the contrary, 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 [4,5,13–19]. Attachment is generally increased on rough surfaces ($Ra > 1 \mu\text{m}$) produced for example by sandblasting compared to smooth ones [13,20–24] but sometimes no effects are described [9,25].

Different groups perform complementary and sometimes controversial studies to elucidate the interactions between cells and various materials (Fig. 1). One group prefers to

analyze the interaction of one isolated cell with a substrate. Then, specific methods like atomic force microscopy, magnetic twisting cytometry or micropipette aspiration [2,26] are used to quantify the cell adhesion strength. Another group focuses on the interactions of a population of cells with the material. Other specific methods like fluid shearing in flow cells, centrifugation, ultrasounds, enzymatic detachment are used to quantify adhesion strength [27–33]. However, these interactions are analyzed directly after the first minutes or first hours of cell–material contact [34,35]. The objective in this second 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. Finally, in a third approach, authors consider all together, short-term adhesive events or long-term adhesion, proliferation and differentiation phases [3,10,11,15–19,21,33,36]. We esteem this third approach as more valuable since it allows to approximate the *in vivo* situation. One of its

major inconveniences is, however, the simultaneous involvement of many parameters of which a large number cannot be fully controlled.

This is one reason why in most typical studies of cell/material interactions, only one cellular parameter (attachment, proliferation) and one surface parameter are considered (surface roughness, surface composition). In most cases, the surface parameter is poorly defined and its influence not entirely analyzed. Moreover, it has never been proven if this parameter is not correlated also with other surface parameters. For example, the surface topography is often defined only by an average roughness amplitude parameter (R_a) although several other pertinent parameters exist, which describe the surface topography, for example frequency parameters [18,37–39]. Additionally, our team has shown that, analogous to mean roughness amplitude, cell adhesion is also correlated to frequency parameters describing the organization of the surface topography [18,33].

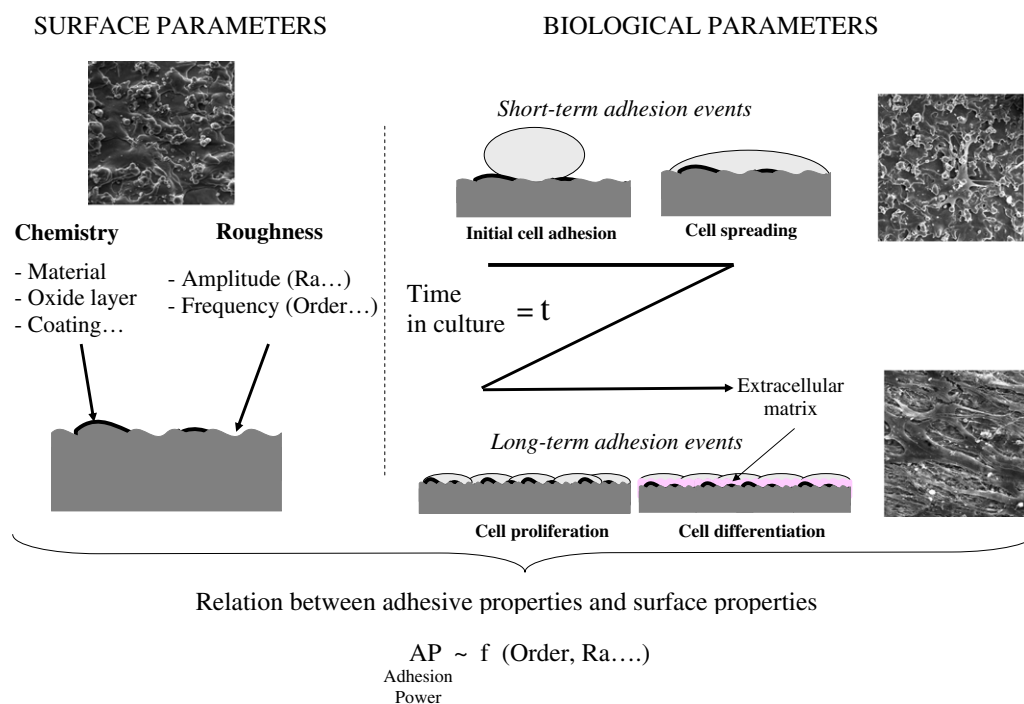


Fig. 1. Various surface and biological parameters are involved in cell/material interactions. On the material side, surface chemistry and surface roughness have together a main influence on cell behavior. The surface topography has to be characterized by more than only amplitude parameters. Frequency parameters need also to be measured in order to characterize also the organization of the topography (Table 2). In the same way, the deep analysis of surface chemistry is also essential. Indeed it has been shown that process used for increasing surface roughness can also modify surface chemistry [15] and it is necessary to take this into account when analysing the cell response to a surface. As for surface topography, we advise to measure several significant parameters describing the surface chemical state of the substrate. This could be achieved using only one technique but it must be preferable to use several techniques to fully characterize the surface chemistry. We cannot specify the techniques to use since they depend on the material studied but we want to insist on the final objective i.e. to be able, at the end, to do a quantitative measurement of one or several parameters describing the surface chemical state of the substrate for correlation with biological parameters. On the biological side, different phases can be distinguished with time in culture in the cell response to material. The first one is the initial cell adhesion phase involving non-specific electrostatic forces (e.g. Van Der Waals) and passive formation of ligand–receptor bonds, followed during the further hours by the cell spreading phase involving receptor recruitment, clustering to anchoring sites and interactions with cytoskeletal elements. The second phase concerns the proliferation and differentiation phases involving the extracellular matrix formation. In our cell culture model, this last phase belongs to what we call the long-term adhesion events. The quantification of the short-term adhesion, long-term adhesion and proliferation of cells allowed to establish relations between adhesive properties of cells (e.g. adhesion power) and surface properties (order, R_a) thanks to a specific statistical modeling approach.

For several years we have become interested in improving titanium-based implant surface topography [15–18,33]. Our objective is to understand, using *in vitro* experiments, the relation between surface properties of metallic materials and their biological activities. Similar to other authors interested in interactions between osteoblasts and bone-replacing materials, we believe that the differences found in the early stage responses of cells cannot predict the cell's behavior after longer periods [40]. For this reason, we have attempted to develop an original *in-vitro* long-term culture model permitting the formation of a cell/material interface close to the implant/tissue interface existing *in vivo* during the first days of contact. Hence, our model involves human primary bone cells cultured on metallic substrates for contact times ranging from some hours to several weeks. Our objective was to obtain during this time the *in vitro* formation of an elaborated cell/material interface. In fact, we actually were able to form a complex osteoblast/extracellular matrix/substrate interface [16]. The extracellular matrix was composed of fibronectin, type I collagen and non-collagenous proteins like osteopontin or osteonectin [16]. This extracellular matrix is comparable to that obtained during the first days of tissue–implant interface formation *in vivo* [41], demonstrating the “*in vivo-like*” character of the interface formed in our *in vitro* model.

Together with this original culture model, we developed a new progressive detachment method based on trypsin-EDTA allowing the quantification of the cell/material adhesion strength over 3 weeks of culture. Thus, we had access to an evaluation of the “enzymatic” force necessary to detach the cells from the substrate analogous to that done with a mechanical method based on fluid flow or centrifugation [27,29]. With this detachment method, we consider that a lower number of detached cells reflect a higher adhesion. The more the cells resist trypsin detachment, the lower the number of cells detached, the higher the number of adherent cells. The trypsin-based cell removal is related to maturation of cell–material interface and especially to the maturation of the extracellular matrix synthesized by cells [42] but also to cell–cell protein contacts notably as they approach confluence. Indeed, cell–cell contacts also influence cell release even after cell–surface contacts are destroyed.

Therefore, the long-term adhesion we measure with our model represents the strength of the cell–material interface formed during 3 weeks of culture, involving at once the extracellular matrix proteins synthesized by the cells themselves and the cell–cell contacts (Fig. 1). From this model we are also able to quantify the short-term adhesion after 24 h when the extracellular matrix has not yet been synthesized and the proliferation of cells by calculating the total number of cells detached at each culture time [43]. The approach, which will serve as an example throughout this paper, was developed in our laboratories during the last 2 years.

Now, an experiment design has to be built to quantify the effect of different variables on cell interactions with

materials. An experimental problem is to select the variables to design experiments and then to proceed to parametric statistical analyses. Two kinds of approaches can be performed: the first one consists in building a particular type of experimental design called “screening experimental design” [44] that allows to retain variables that plays a role without taking account the interaction between variables. This kind of experimental design allows to treat a high number of parameters with a minimal set of experiments (for example Plackett–Burman design in 12 experiments for up to 11 experiments). This kind of experimental design is not really used to quantify statistically an effect but rather to detect which variable plays a role in the statistical process. As a consequence, it will be used to select variables when nothing is known about the accuracy of the parameters. The second one consists in quantifying precisely the effect and also the interactions between variables. However, in this case, the variables that play a major role have to be known. This can be achieved from a previous study, from published review papers or from theoretical considerations with high confidence level. In this case, a classical experimental design has to be built. This last approach was used for our study. This study concerned the behavior of human osteoblasts cultured on 30 different metallic substrates presenting three different compositions (pure titanium, titanium alloy Ti6Al4V, stainless steel), two surface chemistries (with or without gold-palladium coating), six surface morphologies (obtained by sand-blasting, electro-erosion, polishing, machine-tooling, acid-etching, compared to smooth plastic) and two roughness amplitudes (0.85 and 2.35 μm) (Table 1). An analysis of three biological parameters (short-term adhesion, long-term adhesion and proliferation) was performed on these substrates and the three biological parameters were correlated with four physico-chemical parameters describing the surface chemistry of the substrates (material composition, presence or absence of a gold-palladium coating), the surface topography (roughness amplitude) and a combination of the surface chemistry and the surface topography (process used to prepare the surfaces). Each surface was tested at least 8 times, 396 curves of detachment were established and more than 2000 data were processed. For more details on the experimental procedure see [33,43].

An extensive statistical analysis of the obtained data allowed us to study for example the influence of surface topography on cell short-term adhesion, long-term adhesion or proliferation, the relative influence of surface topography and surface chemistry on the same cellular parameters, the correlation between the three biological parameters.... This approach using statistical modeling is essentially original with respect to studies of cell/material interactions field and more generally in the biology field (Fig. 1). Some comments have to be done concerning this biological model. This model concerns specifically attachment-dependent cell types. It is based on the use of human

Table 1
Experiments design of 30 different substrates used in the cell culture model

	Ra (μm)	Pure titanium (T)		Titanium alloy (V)		Stainless steel (I)	
		C	NC	C	NC	C	NC
Sand-blasting (S)	0.85		TS0N (9)		VS0N (9)		IS0N (9)
	2.35	TS1O (9)	TS1N (18)		VS1N (9)	IS1O (9)	IS1N (18)
Electro-erosion (E)	0.85	TE0O (9)	TE0N (18)	VE0O (9)	VE0N (18)	IE0O (9)	IE0N (9)
	2.35	TE1O (9)	TE1N (18)	VE1O (8)	VE1N (18)	IE1O (8)	IE1N (15)
Polishing (P)	0.7		TPN (12)	VPO (9)	VPN (21)	IPO (9)	IPN (21)
Machine-tooling (U)	0.7		TU0N (12)				
	0.7		TX0N (12)				
Acid-Etching (A)	0.7	TAO (9)	TAN (18)				
Thermanox TM	0			THER (33)			

Number in brackets represents the number of times where experiments were repeated. C means that surfaces are covered by a thin film of Au-Pd. NC: non-covered surfaces.

primary bone cells, which display a very high adhesion potential and a low proliferation rate. Moreover, these cells are able to synthesize in vitro a high amount of extracellular matrix proteins. This model could not be used with permanent cell lines characterized by a very high proliferation potential and a low adhesion capacity. However, our objective in this paper is to describe a new approach for studying cell/material interactions and this approach could be applied to other in vitro models with totally different measured parameters. The basis of this approach is to have to one's disposal quantitative measurements of parameters describing at once surface state of materials (roughness, chemistry, energy...) and biological response to material surface (adhesion, proliferation, integrin expression profiles, rates of matrix synthesis, morphology, expression of phenotypic markers...). Our objective in this paper is to describe this type of approach and to provide the reader with the basic principles for developing more ambitious experiments comparing the simultaneous influence of more than one or two parameters on various observations, taking advantage of convenient statistical and mathematical techniques for the treatment of measured data. Analyzing some examples of our own experiments, the essential features needed for the modeling of cell/material interaction studies are presented. In the first part of this article, we will describe the experimental design, which must be at the root of modeling. In the second part, we will illustrate the use of a mathematical modeling technique to eliminate correlation existing between measured parameters. As an example, we will show how we proceeded to de-correlate adhesion and proliferation occurring in our long-term culture model.

After the mathematical treatment of data, it is essential to select some essential parameters among the very large

number of identifiable biological or material parameters relevant during the cell/material interactions. The paramount objective of our statistical modeling is to accurately represent the biological and material properties by a limited number of reliable and reproducible quantitative parameters. It is useless to introduce coefficients, which cannot provide any information beyond statistical noise. For that purpose, specific analytical methods can be used to determine among all the parameters those that have the higher influence, i.e., those parameters that emerge from the noise of the measurement. In a third part of this paper, we will illustrate one of these methods, the bootstrap protocol, which is an intensive computer statistical technique allowing on one hand an increase in the amount of data available by re-sampling and on the other hand to determine the more robust parameters for further statistical analysis [45,46].

In the end, statistical analysis of the relative influence of biological and physical parameters can be performed, permitting a phenomenological approach of the cell/material interactions based on an extensive statistical analysis. Finally, an example of this type of approach will be briefly described at the end of this paper.

2. Experiment design

At the moment, since the majority of the studies in the field concern rarely more than one surface parameter and one cellular parameter, extensive statistical analysis of results is not needed. However, the need for more complex statistical analysis appears for experiments with several surface and cell parameters. When statistical correlations between physical and biological parameters like cellular adhesion strength, surface roughness and surface energy, for example, are desired [47], the need for an appropriate

experimental design becomes essential. Contrary to clinical studies, where data and parameters are statistically analyzed a posteriori using data-mining techniques like factor analysis (e.g. principal component analyses), we advocate in the biology field to rather proceed to experiment designs a priori when the influence of several parameters on several measurement variables must be studied.

Before the final design of an experiment, one needs to do some preliminary experiments in order to obtain crucial information including the variability between experiments, the physical parameters having the most influence on biological parameters or the possible correlations between parameters. When all this information has been obtained, it becomes then possible to determine the minimal number of repetitions of experiments (or runs) needed. The duration of the experiment and the definitive number of samples as well as the definitive quantity of cells can be calculated. The total number of runs needed must be calculated globally but in considering the possibility of additional experiments, which could become necessary because of experimental problems like, for example, contamination of some cell cultures.

On the materials aspect, it is essential to provide also for a sufficient number of samples for all experiment time-scales even if it will last several months or years. All samples should be stored in controlled conditions in order to limit variation with time between the samples tested at the beginning and at the end of the experimental time length. On cellular aspects, the same requirement can be applied since no variation in the cellular phenotype was acceptable over the month or years that last the whole experiment. This is one reason for the wide use of permanent cell lines for these types of experiments since they are more stable with both time and passages than primary cells. However, the use of permanent cell lines for the study of cell/material interactions is highly criticizable since these cell lines do not display all necessary phenotypic characters of cells associated with implants *in vivo*. For this reason, we chose, several years ago, to develop studies with primary human osteoblasts prepared from iliac crests of young patients undergoing orthopedic surgery. In order to have sufficient amounts of cells for all experiments, we produced extensive *in vitro* amplification of cells from the same patient during two passages. These cells were then further frozen in liquid nitrogen to be used for cell/material studies after not more than one passage after thawing.

Finally, after the decision of the experiment design, it is essential to proceed to a randomization of runs since it is difficult to avoid that cells in culture vary in their phenotype with time in culture, time in liquid nitrogen or with passages or that the surface chemistry of substrates evolves with time. Thus, such randomization has the objective to reproduce randomly the same experiment (i.e., the same levels of studied parameters) at different times during the experimental time frame and finally avoid bias of the results when they are time-correlated or when

they depend on a particular culture environment. Selection of all the samples or a random part of samples must be tested in each run in order to average the influence of time.

3. Mathematical development for eliminating experimental bias

Sometimes, various biological parameters are experimentally linked to each other. For example, the proliferation capacity of most attachment-dependent cells is a function of their adhesion capacity. This is especially true for proliferative cells like permanent cell lines or bacteria, where determination of cell adhesion strength is disturbed by their simultaneous proliferation. This was also our case when we studied the adhesion strength of cells over 21 days in culture. This may bias statistical treatment of the adhesion data. Hence, the need of means to eliminate this physical bias became crucial.

In our model, during the 3 weeks of formation of the cell/ECM/material interface, the cells will proliferate until they attain confluence. As a consequence, trypsin will detach both initially deposited cells and cells having proliferated. If no proliferation occurs, the measure of adhesion versus time in culture characterizes the real adhesion kinetic. However, a bias emerges when proliferation occurs during culture time: new proliferating cells are included in the counting process and that will diminish the real average adhesion time in culture. It was thus essential to introduce a model that allowed de-coupling of the effect of the proliferation rate on the assessment of long-term adhesion before the statistical treatment of data. Firstly, we determined several models for cell proliferation including the major types of proliferation, and secondly we developed a mathematical approach based on differential equations to get around the statistical artifact distinguishing the measure of adhesion and proliferation.

3.1. Models for proliferation

First of all, it is essential to provide an analytical expression of the osteoblast number versus the time in culture. Let us note the following parameters: t the culture time, $P(t)$: the number of cells at time t (in cells per cm^2).

Five models of proliferation can be described. We retained different kinds of models based on continuous growth model [48] that correspond to five types of proliferation met in our biological experiments. Among all mathematical models that could simulate each type of proliferation, the model with a minimum set of parameters was retained to have a good statistical robustness in parameter estimations. That allowed us to choose from experimental data, with a high confidence level, the appropriate model corresponding to each proliferation type.

Model 1: The material is not biocompatible and the cells deposited on the substrate will die i.e. will not be counted after the trypsination process. The density of cells will then

decrease with time in culture.

$$P(t) = \alpha_0 \exp(\alpha_1 t), \alpha_1 < 0, \quad (1)$$

where α_0 is the initial number of cells and α_1 is a constant term quantifying the intensity of cell death.

Model 2: The cell's proliferation is so rapid that confluence is reached within the first days. Thus, we could statistically have an increase in cellular density but this could lead to two situations. Either the cells immediately get the maximal adhesion potential and then adhesion is constant or adhesion still increases. In both situations, the proliferation rate is constant and the cells are more numerous than at the initial inoculation time.

$$P(t) = \alpha_2, \forall t \text{ with } P(t) > P(0) \forall t > 0, \quad (2)$$

where α_2 is the amount of cell at confluence.

Model 3: The cells proliferate so slowly that confluence is not attained at the end of culture. Then, we are in a classical proliferation model without any constraints and the usual model is the exponential one.

$$P(t) = \alpha_3 \exp(\alpha_4 t), \alpha_4 > 0, \quad (3)$$

where α_3 is the initial number of cells and α_4 is a positive constant term that quantifies the intensity of cell growth. It could be noticed that cell-cell crowding can retard cell proliferation even at a pre-confluence step. Then the exponential growth model is really only accurate at low cell number densities. However, including this fact in Eq. (3) we added a new delay constant t_0 with $P(t) = \alpha_3 \exp(\alpha_4(t - t_0))$ and $P(t) = \alpha_3$ if $t < t_0$. We have tested this hypothesis on 57 proliferation curves and hypothesis $t_0 = 0$ was rejected only once (with $t_0 = 3$ days).

Model 4: The cells proliferate until they attain confluence. In the beginning we obtain an exponential growth law, and when they reach the confluence the exponential law does not hold any longer and the cell density reaches an asymptote. This self-limiting process is called a logistic growth and is defined by

$$P(t) = \frac{\alpha_5}{K \exp(-\alpha_6 t) + 1} \text{ with } K = \frac{\alpha_5}{P(0)} - 1, \quad (4)$$

where α_5 is a positive constant, K the carrying capacity of the cell environment and α_6 is a positive constant that represents a measure of the rate at which stable steady state population is reached.

Model 5: The cells do not proliferate. As a consequence the number of cells is equal to the number of cells initially deposited on the substrate. The number of cells is constant and equal to the number of cells initially introduced.

$$P(t) = P(0). \quad (5)$$

Let us first notice that the value $P(0)$ in our model is not known since only the number of cells introduced in the culture is known and since this number is higher than the number of cells that will initially adhere on the substrate (very short-term adhesion). As a consequence, $P(0)$ is statistically determined from experimental data as are the coefficients $\{\alpha_0, \dots, \alpha_7\}$ used in the proliferation models. In

the particular case of models 2 or 5, it is mathematically impossible to estimate $P(0)$ (indetermination) and a rough estimation is made by descriptive statistics process on all our experimental data. In reality, a precise estimation is not required since in this case $P(0)$ becomes a threshold parameter to affirm or reject the assertion "is the number of cells more numerous than the initial number of cells?". Algebraically, the logistic equation, Eq. (4), generalizes other proliferation models (for example, Eq. (4) converges to Eq. (1) when K tends to infinity). However, in a statistical sense, this assertion is not true. As Eq. (3) requires three parameters estimated from a small finite set of experimental data, variance of the estimator will be large and then can lead to reject an appropriate model that will be accepted by a one or two parameters model. The method used to determine these coefficients is based on the non-linear least-squares method using the SASTM statistical language (SAS Institute, Cary, NC). We retain the model that minimizes the residual standard deviation if, and only if, all coefficients are statistically significant (using studentization for linear regression or asymptotical studentization in the case of non-linear regression).

3.2. De-correlation of adhesion and proliferation

We first must determine the adhesion law for an individual cell. We shall suppose that a cell possesses its own adhesion law. Two models can be proposed: a deterministic one or a probabilistic one. For example in the deterministic one, we shall suppose that after a trypsination time d , the cell is detached by the trypsin. For the stochastic one, we shall suppose that on average, the cell will be detached after d (Fig. 2).

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

$$\bar{d}(t) = at^b \text{ with } a \geq 0. \quad (6)$$

The choice of this power law is justified by some experimental and mathematical considerations. The most

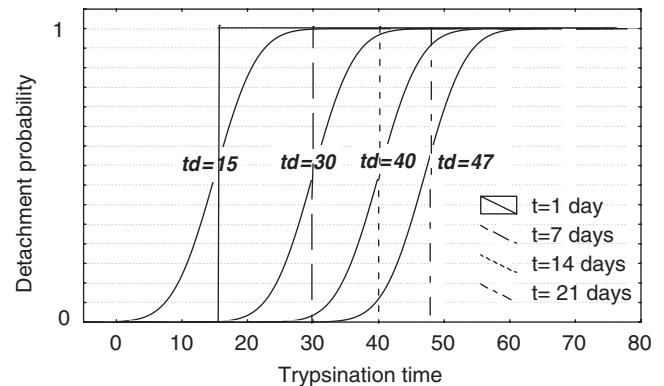


Fig. 2. Detachment probability for an individual cell versus trypsination time for different times in culture. Deterministic description is given by $Pr(d) = H(d - t_d(t))$. A stochastic form is also plotted.

important is that in our entire study we encountered neither local minima nor local maxima. In a physical and biological sense, it will be unlikely that adhesion will increase (or decrease) to reach a maximal value (or minimal) and will next decrease (or increase). Then we retain as a mathematical model an increasing ($b > 0$), constant ($b = 0$) or decreasing ($b < 0$) function. This model possesses only two parameters to be sufficiently robust in a statistical sense. Moreover, the power laws provide interesting properties to model temporal phenomena with both scaling (a) and kinetic coefficients (b). Physiologically, it is likely that adhesion is monotonic in time, but still reaches a maximum. The power law described before does not have this behavior. In fact, it is right that within the timescale of the experiments, outlined adhesion will continually increase but once full adherence has been achieved this will not be the case. However, including this fact will lead to two major problems: the first one is a statistical one. Introducing this fact leads to add a new parameter describing the asymptotic measure of final adhesion. This will statistically introduce a high noise in the a and b coefficient estimations since this asymptote was not reached in our experimental measures. This will introduce a very high variance in the parameters characterizing the asymptotic measure of final adhesion leading to a high variance of the a and b estimators. The second one is that in the biological experiments it is difficult to culture cells in static conditions more than some weeks since the results will be biased by external environment effects like limitation in culture medium volume upper the cells and in access to nutritive elements for cultured cells entrapped in extracellular matrix. Then, it would be impossible to reach in vitro the value of asymptotical maximal adhesion met surely in the in vivo process. However, as we will prove in this paragraph, our model is well posed. We will prove that under this hypothesis, adhesion will increase as a square root of time meaning that adhesion will increase only by a factor two when time increases by a factor four. This low increase will, roughly speaking, lead to reach a kind of asymptotic value in our experiments because the increase of adhesion measurement will be of the same order as experimental uncertainties. As a consequence, the variation of this function could describe the adhesion of cells on biomaterials after a time t in culture (without proliferation). To find the values of the coefficients a and b of Eq. (6) we use experimental data. We dispose of N trypsination times $\{d_1, d_2, \dots, d_N\}$ for M times in culture $\{t_1, t_2, \dots, t_M\}$, which gives $N \times M$ data per experiment and per run to determine two adhesion coefficients. The probability $\Pr(d)$ that a cell detaches after a d minute digestion with trypsin, is

$$\Pr(d) = H(d - \bar{d}(t)), \quad (7)$$

where H is the Heaviside step distribution

$$H(x) = 0 \quad \text{if } x < 0,$$

$$H(x) = 1 \quad \text{if } x \geq 0. \quad (8)$$

However, as was shown above, cells will proliferate and we have to include this fact. The value $(\partial P(\tau)/\partial \tau) d\tau$ represents the number of cells, which have proliferated during the time τ and $\tau + d\tau$. Let us note $p(t, d)$, $p(t, d) \in [0 \dots 1]$, the ratio of cells detached by the action of trypsin during d minutes and which were formed by division over a time t (the beginning being the origin $t = 0$). This number is calculated as follows $p(t, d) = \int_0^d n(t, \delta) d\delta / (\int_0^{d_{\text{sup}}} n(t, \delta) d\delta + n_f(t))$, where $n(t, \delta)$ is the number of cells detached after the action of trypsin during d minutes at time t in culture, d_{sup} is the duration of the detachment experiment, $n_f(t)$ is the number of cells detached by concentrated trypsin at the end of the diluted trypsination process. The following expression $(\partial P(\tau)/\partial \tau) d\tau H(d - \bar{d}(t - \tau))$ represents the number of cells at the time t that have proliferated at time τ and that will be detached by trypsin after a trypsination time of d minutes. Finally, we have to sum up all the cells detached for a time in culture t and to normalize this function to obtain the following integro-differential equation:

$$p(t, d) = \frac{1}{P(t) - P(0)} \int_0^t (\partial P(\tau)/\partial \tau) H(d - \bar{d}(t - \tau)) d\tau. \quad (9)$$

The main problem consists in finding the values of the coefficients a and b of Eq. (8) by using Eq. (9) and experimental data. Thus, we have five trypsination times $d_1 = 5$, $d_2 = 10$, $d_3 = 20$, $d_4 = 30$, and $d_5 = 60$ and four times in culture $t_1 = 1$ day, $t_2 = 7$ days, $t_3 = 14$ days and $t_4 = 21$ days which gives 20 experimental data per surface and per run to determine two adhesion coefficients. (note that four experimental data obtained after concentrated trypsin action are also processed to calculate the ratio of cells detached at each time in culture). In our experimental design (see Table 1), 396 experiments were processed corresponding to all surfaces and all runs (in total $396(20 + 4) \sim 9500$ measures of detachment) that leads to calculate 396 pairs of (a, b) coefficients. The solution consists in working out a and b to minimize the following functional expression:

$$\min_{a, b} \sum_{i=1}^M \sum_{j=1}^N \left(p(t_i, d_j) - \frac{1}{P(t_i) - P(0)} \int_0^{t_i} (\partial P(\tau)/\partial \tau) H(d_j - \bar{d}(t_i - \tau)) d\tau \right)^2. \quad (10)$$

The solution was obtained by the simplex method. The simplex method was first introduced by Nelder and Mead [49] for finding the minimum value of functions. The Simplex method does not require derivative function estimations that would be numerically poorly robust and stable due to experimental error. This method is based on four mechanisms of vertex movements. Vertices are moved toward the minimum point by the four mechanisms, reflection, expansion, contraction, and shrinkage. We have shown that $b = 0.5$. That means that adhesion increases

with the square root of time. To statistically minimize noise in this determination assuming that $b = 0.5$, the “ a ” coefficient was recalculated by fixing $b = 0.5$ in Eq. (7). As a consequence, the adhesion law followed $\bar{d}(t) = a\sqrt{t}$ meaning that adhesion could be characterized by only one term, a , that we have called the adhesion power (AP) [42]. We demonstrated in a previous paper that a was influenced by the surface topography of materials [33]. On the contrary, the exponent b was independent of the substrate’s characteristics meaning that the long-term adhesion increases proportionally to the square root of culture time. From this relationship, we suggested that a diffusion-based process related to the kinetic of formation of extracellular matrix should be involved in long-term adhesion on materials. Indeed, the influence of the ECM could be related either to the action of trypsin by inhibiting its enzymatic action or to an increase in the adhesion of cells by way of cell–matrix adhesions [33].

One major criticism of our model is that it is phenomenological and does not really explain the adhesion phenomenon. We totally agree with this criticism but we would prefer to put back our mathematical modeling approach in its context. Our objective is to characterize adhesion with few parameters (in our case, only one) since the critical point is that our model is highly robust in a statistical sense. If we retain a model with more than one coefficient, the modeling error would have surely decreased but the coefficients would have drastically lost their physical meaning. In our adhesion studies, 93% of our experiments were repeated three times. As a consequence, we can estimate error by calculating the standard deviation on fraction of detached cells, noted as σ^e . In our experiment, 2087 measurements of triplet values were done. We obtained an average of $\bar{\sigma}^e = 0.051_{\pm 0.0004}$, representing our experimental error. In another method, we can calculate the modeling error noted σ^m that is the standard deviation of the difference between our model and experimental data. Note that statistically, we must always have $\sigma^e \leq \sigma^m$ because modeling error cannot be less than experimental error. We obtained an average of $\bar{\sigma}^m = 0.065_{\pm 0.001}$, roughly meaning that modeling error is around $\sqrt{(\bar{\sigma}^m)^2 - (\bar{\sigma}^e)^2 - 2 \text{cov}(\bar{\sigma}^m, \bar{\sigma}^e)} \approx 0.005$, that is less than 0.5%. The empirical distribution of 2087 measures of σ^m and σ^e is represented in Fig. 3. Intrinsic experimental error and modeling error distribution are quite similar, demonstrating that our single-coefficient model is well adapted to describe adhesion (whatever the substrate nature), and that proliferation is also well modeled.

Some other criticisms of our model include the risk of a wrong choice among the five models proposed for proliferation, or over-simplification of reality given by Eq. (6). However, the low percentage of error between experimental and modeling error we found demonstrates that these criticisms could be theoretically stated but would not lead to better results than our analysis. We think that it is impossible, due to the high experimental variation, to

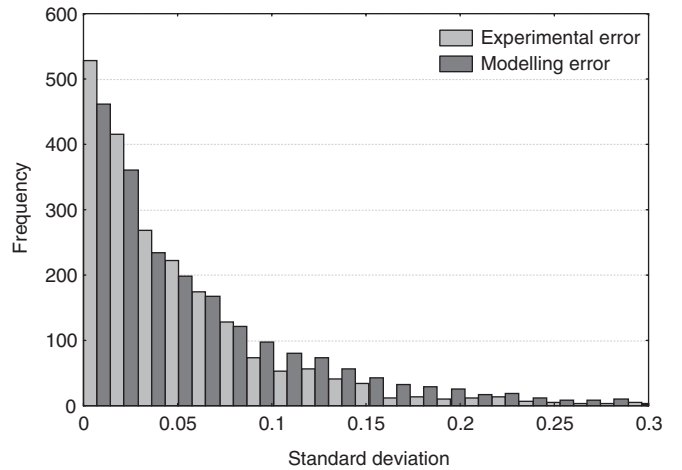


Fig. 3. Empirical probability density function (PDF) of error in adhesion measurement σ^e and modeling error σ^m of the adhesion model.

propose a model with more than one coefficient that will better describe a so-complex phenomenon including the influence of numerous uncontrolled parameters like surface chemistry, surface roughness, cell–cell interactions.... In fact, the major question we should ask is: “Is it really possible to model based on biophysics laws, such a complex biological system by taking into account all the rules of the cellular adhesion?” If the entire biophysical system was deterministic, we could surely answer positively but viewing the large dispersion in repeated experiments, we are more convinced that a deterministic position is utopic and we thus prefer to treat these problems in a statistical way. In fact, having (a) robust parameter(s) of adhesion allows us to analyze the relation between one of these coefficients with some properties of the surface to quantify surface effects on adhesion, thanks to appropriate statistical tools.

To illustrate the efficiency of our mathematical modeling, we shall analyze the results for cell adhesion on four surfaces, which have shown very typical different proliferation rates in previous studies [43,46]: ThermanoxTM (high proliferation rate), IE0N (middle proliferation rate), TE00 (low proliferation rate) and VE00 (very low proliferation rate) surfaces. According to our model, for low proliferation rate, the macroscopic measure of adhesion we perform will be on the same order as individual cell adhesion given by Eq. (10). On the contrary, when proliferation rate increases, the macroscopic adhesion measure includes newly formed cells. Then, the macroscopic measure will be more and more different than individual measure and will be always lower than individual measure taken at the same time in culture. Fig. 4 represents the normalized experimental mean trypsination curves obtained on these selected surfaces. Then we applied our model to calculate values of a and b for each experiment: each value of a and b allowed us to calculate four trypsination curves for the four times in culture (1, 7, 14, 21 days). These curves are Heaviside

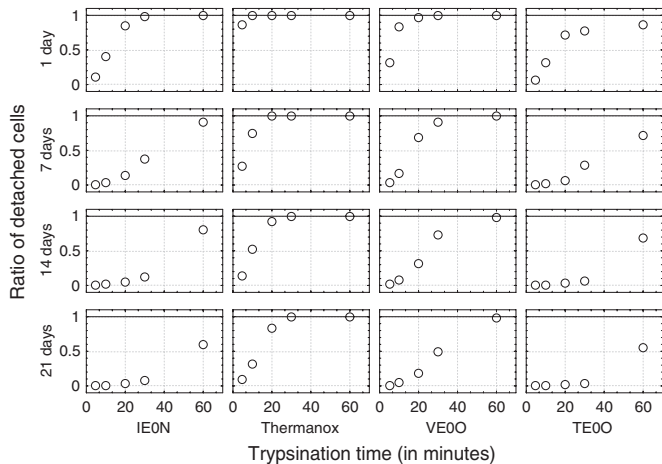


Fig. 4. Experimental ratio of detached cells versus trypsination time for different times in culture and four different substrates. Ratio is given by the number of cells detached after time t of trypsination divided by the total number of cells (i.e. the sum of all detached cells at all times t with diluted trypsin + the number of cells detached with concentrated trypsin). IEON: uncoated electro-eroded stainless steel substrates, ThermanoxTM: cell culture-treated plastic coverslips, VE00: gold-palladium coated electro-eroded Ti6Al4V alloy substrates, TE00: gold-palladium coated electro-eroded pure titanium substrates. For more details on substrates and detachment procedure see Table 1 and Ref. [43].

functions. However, as many experiments were done for a given sample and the same time in culture, many Heaviside curves were obtained. To include the statistical variation between experiments, we summed up all Heaviside functions and divided them by the number of experiments to obtain a real cumulative probability density function. By repeating this normalization for each time in culture and each type of samples, we obtained the modeled trypsination curves in Fig. 5. After optimization, Fig. 5 represents the trypsination curves without the contribution of proliferation on the four model surfaces. As can be observed, modeled curves look like theoretical cumulative curves in Fig. 2, meaning that the hypothesis that cell detachment time for a given time in culture follows a probabilistic model is justified. As it can be stated in our model, for low proliferation rate (VE00 sample), the macroscopic measures of adhesion plotted on Fig. 4 are identical to modeled curves (Fig. 5) because the bias introduced by newly formed cells in the number of detached cells is relatively low. On the contrary, for high proliferation rate (ThermanoxTM, IEON), because of the increase of number of detached cells (since newly formed cells can be also detached), the macroscopic measure underestimates adhesion and curves are more different than modeled curves obtained by retrieving of proliferation effect. These curves allow us to validate the efficiency of our model.

4. Bootstrap protocol

As claimed before, we have characterized cell adhesion, thanks to an appropriate mathematical model, by only one

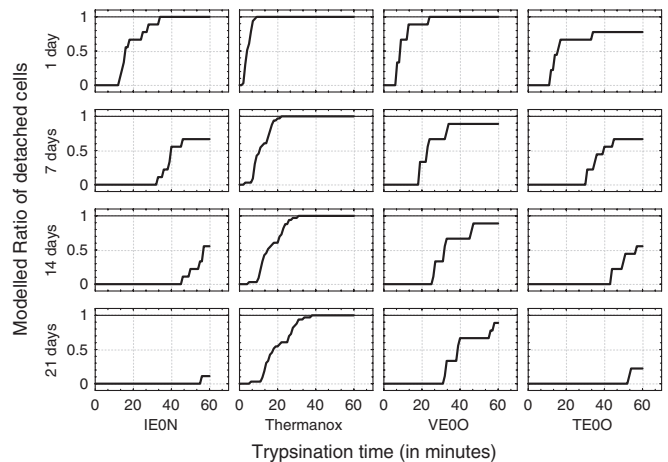


Fig. 5. Modeled ratio of individual detachment probability versus trypsination time for different times in culture and four different substrates: IEON: uncoated electro-eroded stainless steel substrates, ThermanoxTM: cell culture-treated plastic coverslips, VE00: gold-palladium coated electro-eroded Ti6Al4V alloy substrates, TE00: gold-palladium coated electro-eroded pure titanium substrates.

robust coefficient called the AP. However, we have not introduced statistical aspects as, for example, the confidence intervals for the adhesion coefficient. Further, if we want to correlate this coefficient with physical properties of the surface, some statistical tools must be used to quantify the reliability of this correlation. If our data obeys some known density function, it is possible using the statistical inference to construct confidence intervals. Most statistical procedures require some knowledge of the sampling distribution of the statistics used for analyses in order to estimate or approximate the accuracy of measures for a given statistic (estimator). Sometimes it requires a very large sample size in order to have accurate measure of the estimator [50]. However, the number of experimental data in the biological experiments are generally too few to verify the assumption of the statistical inference [51]. Surely, other techniques could be used such as non-parametric or Bayesian approaches to minimize the statistical properties of the data, but these techniques require a large number of data to be efficient. Another possibility is to use a recent statistical methodology called the Bootstrap [45]. The application of this method will be illustrated with two studies: the calculation of confidence intervals of the AP coefficient and the determination of the more appropriate morphological roughness parameters to characterize the effect of roughness on cell adhesion. The bootstrap is an intensive computer statistical technique from Efron and Tibshirani based on the re-sampling of data [45,46]. The introduction of the Bootstrap theory allows considering experimental variations on the mean's estimation of independent measurements. A data-based simulation method for statistical inference is used to study the variability of values of a set of observations and provide confidence intervals for parameters in situations where these are difficult or impossible to derive analytically. The

basic idea involves re-sampling with replacement of data to produce random samples of size n from the original data. Each of these samples is known as a bootstrap sample and each provides an estimate of the parameter of interest. Repeating the sampling a large number of times provides information on the variability of the estimator. The major advantage of this method is to have no preconceived opinion on the probability density followed by the data. The growing stream of scientific articles using re-sampling techniques, both as a basic tool as well as for difficult applications, testifies to value of re-sampling [52]. Additionally, the increasing literature in mathematical statistics shows its acceptance on a theoretical basis, after many years of doubts. A major disadvantage of the bootstrap is that it is necessary to develop an appropriate software. The elementary calculus has to be performed a high number of times. Thus, an automatic process with computation procedure optimization is required. In our case, we have written our own code in the SAS language that contains a real computer language (not classical macro language that can be met in some statistical softwares) and that allows to optimize the basic or extended statistical procedures.

4.1. Calculation of confidence intervals

Let us now analyze an example to understand the principle of this method. We will try to find the confidence interval of the mean a value given in Eq. (6). As Eq. (7) is highly non-linear, it becomes very difficult to determine analytically without gross simplification an estimation of the confidence interval of a . Here, the bootstrap method is a good alternative to estimate the probability density function for a and the associated confidence intervals. Twenty data points were used to calculate the coefficient a for each run in order to quantify adhesion thanks to our Eq. (6). However, in our case, data were very scattered and we had not a sufficient number of samples to validate the statistical hypothesis allowing the use of statistical tests. The aim of the bootstrap in our case was to create a data bank by sampling randomly with replacement of 20 data in the original data set (t_i, d_i) that constituted the first bootstrap sample from which the adhesion power was noted $a_1^B(1)$. Further, from the second run, another bootstrap sample was created and gives $a_1^B(2)$. If n runs are made, then from the first bootstrap, we get n evaluations of a values that gives $\{a_1^B(1), a_1^B(2), \dots, a_1^B(n)\}$ to calculate a first bootstrap estimation of the mean values: $\bar{a}_1^B = (1/n)\sum_{i=1}^n a_1^B(i)$. Finally, this procedure was repeated a high number of times: 10,000 in our case. This is a good compromise to have a good estimation of the probability density function of a and an acceptable time of computing that was of 1 week for the 396 experiments. This allowed building statistics $\{\bar{a}_1^B, \bar{a}_2^B, \dots, \bar{a}_{10000}^B\}$ on which a probability density function (PDF) can be drawn allowing the further calculation of descriptive statistics. Thanks to this bootstrap technique, the mean histograms of the adhesion power of all samples were established. Three sampled

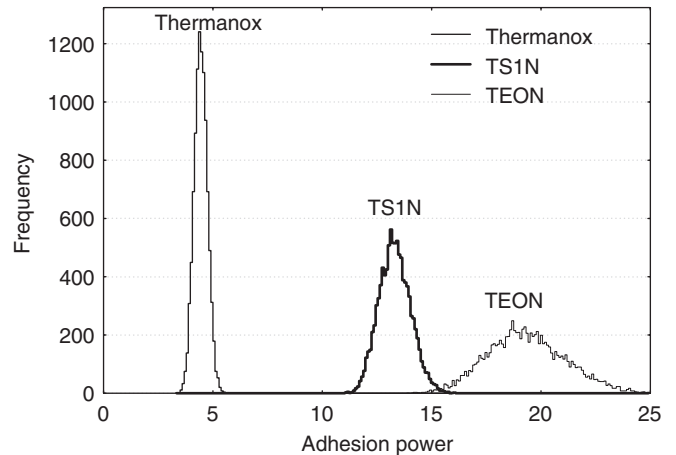


Fig. 6. Bootstrapped probability density function of the Adhesion power coefficient characterizing cell adhesion on three substrates: ThermanoxTM, sandblasted pure titanium substrates (TS1N) and electro-eroded pure titanium substrates (TEON). For more details on the culture see Ref. [43].

histograms are plotted in Fig. 6. As shown in this figure, our original adhesion parameter a allowed good discriminating of cell adhesion strength on these three different surfaces. For the demonstration of the pertinence of adhesion power on all the other surfaces tested, see Ref. [33].

4.2. Estimation of the influence of surface morphology on cell adhesion

We now apply the bootstrap methodology to estimate the effect of surface morphology on cell adhesion. The bootstrap theory was also the basis of a new methodology we developed in order to determine the relative influence of the physical tested parameters on cell response. This methodology aims at the construction and the calculation of a performance index as well as a confidence interval in order to account for the relevance of parameters. We model by a power law the relation between a surface roughness parameter and the measure of cell adhesion. By taking for each surface studied a bootstrap value for the adhesion power parameter and for a given roughness parameter, a first estimation of the standard deviation of the residual (i.e. modeled data minus experimental data) can be computed. This procedure is repeated 100,000 times (since this bootstrap procedure is less costly in computing time and since all variance estimators converge to this number of bootstrap) and descriptive statistics can be constructed. For each roughness parameter, the bootstrap protocol is applied. As an example, we previously published the classification of the 35 most relevant surface roughness parameters according to their influence on cell adhesion [33]. Fig. 7 represents this classification by using the standard deviation of the residuals. For all the relations, the lower the standard deviation, the more relevant the parameter. Then, as confidence intervals

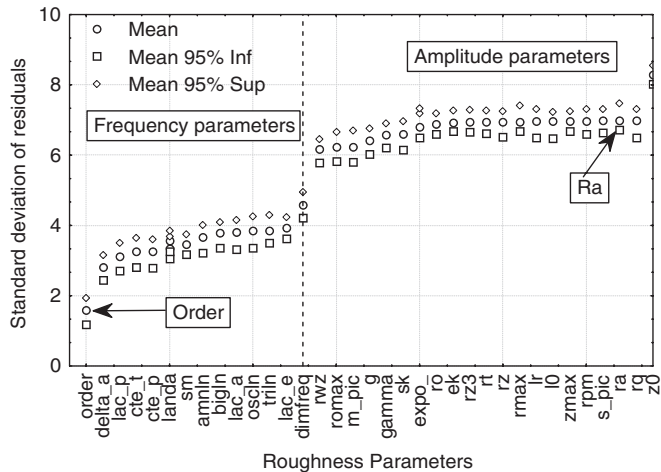


Fig. 7. Classification of the relevance of 35 roughness parameters with regard to cell adhesion power (AP). *x*-axis: roughness parameters divided into 14 frequency parameters (left of the dotted line) and 21 amplitude parameters (right of the dotted line). *y*-axis: standard deviation of the residuals 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. Adapted from [33]. For the meaning of the roughness parameters see Table 2.

obtained by bootstrap do not overlap with others, it could be concluded that the ‘order’ parameter was the most influential parameter on AP. The ‘order’ parameter is an original parameter defined in our laboratory [33]. As the literature did not provide us with any other parameter to quantify the order of a surface with scaling invariance both in amplitude and position, we defined a new “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 a sin–cosine basis is not always appropriate to characterize surface roughness), which gave an upper limit value (100%) if surfaces were periodic, a medium value if surfaces get a non-negligible first-order autocorrelation, and zero as the lower limit value for uncorrelated random surfaces (white noise). This parameter is now used in our laboratory to quantify the impact of different processes on the topography of the surface [17,18,37]. The most important quality of this parameter is to be mathematically independent of the amplitude parameters. Thus, the effect of the order of the surface and also the “scaled amplitude parameter” can be analyzed without any correlation bias. The analysis of Fig. 7 shows that frequency parameters discriminate cell adhesion better than amplitude parameters. This confirmed that cell adhesion is more influenced by the morphology of the surface topography than by its amplitude [18].

5. Conclusion

Using examples derived from our experiments, we presented the essential features needed for the modeling of cell/material interactions studies. Firstly, we described

Table 2
Meaning of the roughness parameters met in Fig. 7

Parameter	Meaning	Reference
Order	Order of the surface	[33]
Delta_a	Profiles slope	[53]
Lac_p	Auto correlation length	[33]
Cte_p	Number of peaks	[53]
Cte_t	Number of peaks following a valley	[53]
Lambda	Peak width	[53]
SM	Mean width of a peak	[53]
AMNLN	Fractal dimension (Bigerelle method)	[54]
BIGLN	Fractal dimension (Bigerelle method)	[54]
LAC	Integral auto correlation length	[33]
OSCLN	Fractal dimension (Tricot method)	[54]
TRILN	Fractal dimension (Dubuc method)	[54]
LAC_E	Exponential auto correlation length	[53]
DIMFREQ	Fractal dimension (spectrum method)	[54]
RWZ	Mean peak radii curvature	[53]
Romax	Maximum peak radii curvature	[53]
M_pic	Maximal mean amplitude	[53]
g	Amplitude of the profile curvature	[53]
Gamma	Amplitude of the profile curvature	[53]
Sk	Amplitude skewness	[53]
Ro	Amplitude of mean peaks	[53]
Ek	Amplitude kurtosis	[53]
RZ3	A mean average amplitude	[53]
RZ	A mean maximal amplitude	[53]
Rmax	Maximal amplitude	[53]
Rt	Amplitude range	[53]
Lr	Relative developed profile length	[53]
L0	Absolute developed profile length	[53]
Zmax	A range amplitude parameter	[53]
Ra	Average roughness	[53]
Rq	Root mean square amplitude roughness	[53]
Z0	Amplitude with sign	[53]

For the meanings of the roughness parameters, reader could refer to {2615}, for the meanings of fractal dimension parameters, refer to {2614}.

the way to carry out the experimental design, which must be at the root of modeling. Secondly, we illustrated the use of a statistical modeling technique based on a bootstrap protocol, used either to eliminate correlation existing between measured parameters, to increase the number of data available by re-sampling or to determine among all the parameters which ones have the higher significance. In a third part of this paper, examples of statistical analysis of the relative influence of biological and physical parameters were given, illustrating a phenomenological approach to cell/material interactions based on an extensive statistical analysis. As we dispose now of robust models describing macroscopic tendencies of cell/material interactions, the future objective of this type of modeling approach must be the development of more efficient and discriminating models allowing to explain this phenomenon also at the mesoscopic level (Table 2).

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