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Bootstrap analysis of the relation between initial adhesive events and long-term cellular functions of human osteoblasts cultured on biocompatible metallic substrates

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Abstract

Classically, the evaluation of cellular adhesion of cells on substrates is limited to the evaluation of cell attachment after some hours. We have claimed for several years that this evaluation is incomplete concerning the evaluation of cell adhesion and more precisely of the quality of the in vitro cell/biomaterial interface. With a view to demonstrating this assertion, we develop in this paper statistical correlations between short-term adhesion (STA) evaluating the attachment after 24 h (IA: initial attachment) and long-term adhesion (LTA) evaluating the strength of the cell/matrix substrate interface over 21 days of culture (AP: adhesion power). Additionally, as the adhesion phase is known to influence further growth of cells we proceed to the correlation of STA with the number of cells after 21 days. We demonstrate statistically the expected positive relation existing between STA and cell growth and we show that this relation is totally independent of the substrate's surface topography or chemistry. More surprisingly, we demonstrate the absence of correlation between IA and AP. This illustrates the fact that different mechanisms underlie STA and LTA. Moreover, this study demonstrates 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 by synthesis of extracellular matrix proteins. Finally, the originality of this work lies in the extensive statistical correlation analysis performed between biological parameters describing the cell behaviour on a substrate.

Keywords: Attachment; Adhesion; Proliferation; Statistics; Metallic substrates

1. Introduction

Attachment and adhesion are two terms which are used equally by people who are interested in studying the interactions between adherent cells and substrates. It is known that adhesion is principally governed during its first flattening phase by non-specific forces like van der Waals and by passive formation of ligand–receptor

bonds. This phase, which is achieved in a fraction of a second, is followed during the next 10 min or so by an alignment phenomenon resulting in the widening of the contact area, which can take place without active cell participation. Thereafter, the spreading phase going on over a few hours involves active cell reorganisation and is much more dependent on cell metabolism [1]. It is during this phase that a receptor recruitment and clustering to anchoring sites occurs and that interactions with cytoskeletal elements are formed. The flattening, alignment and spreading phases have been defined by

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Pierres et al. as forming the fitting phase, the prerequisite to firm attachment and subsequent events [1]. Other authors have proposed that adhesion is composed of two phases: a stick phase, where initial adhesion events occur like the formation and the multiplication of integrin–ligand bindings, and a grip phase, defined by cell spreading, receptor clustering and focal adhesion assembly [2]. It is during the initial adhesion phase that proteins adsorb from biological fluids onto surfaces. This adsorption is directly correlated with surface charges and the adsorbed proteins have a direct influence on cell adhesion [3–6].

We previously defined the attachment phase as a short-term event like physico-chemical linkages between cells and materials involving ionic forces, van der Waals forces, and so on, although the adhesion phase occurs in a longer term and involves various biological molecules: extracellular matrix proteins, cell membrane proteins and cytoskeleton proteins which interact to induce signal transduction, promoting the action of transcription factors and consequently regulating gene expression [7]. Moreover, we recently defined a new adhesion parameter evaluating the adhesive efficiency of cells on substrates at times longer than one day: the cell/matrix/substrate adhesion parameter or CMS adhesion [8]. This parameter takes into account the fact that after some days of contact with a substrate, cells develop a complex interface close to the *in vivo* situation. Indeed, we have been interested for several years in developing an *in vitro* culture model of “a tissue-like interface”. In a recent work, Meyer et al. described the first days of formation of the tissue–implant interface *in vivo*. They observed the formation of an extracellular matrix composed of fibronectin, type I collagen and non-collagenous proteins like osteonectin [9]. We previously analysed the extracellular matrix synthesized by the human osteoblastic cells in our model and we demonstrated that it was also composed of type I collagen, fibronectin and osteopontin [10]. Additionally, we showed the expression of the $\alpha 3 \beta 1$ integrin which is a specific receptor for collagen and fibronectin. This demonstrates that our culture model is really close to the *in vivo* situation since a complex osteoblasts/extracellular matrix/substrate interface composed of the same molecules as *in vivo* forms during the 3 weeks of culture. Therefore, the long-term adhesion we measure represents the adhesive efficiency of the cell–material interface formed during the 3 weeks of culture involving the extracellular matrix proteins synthesized by the cells themselves. In order to take into account the fact that cells proliferate during this CMS adhesion phase and to suppress the experimental bias thus existing when measuring the adhesion of a cell population cultured on a substrate for 21 days, we proceeded to the de-correlation of adhesion and proliferation. At the end of this mathematical treatment, we now have at our disposal a

quantitative parameter representative of the cell/matrix/substrate adhesive efficiency on the various substrates that we have called the adhesion power (AP) [11,12].

In our opinion, the evaluation of cell adhesion after some minutes or some hours of contact only is insufficient to anticipate the future integration of a material after implantation and it is preferable to evaluate the adhesion throughout the culture time. However, this assertion is based on intuition rather than on scientific demonstration. With a view to demonstrating this assertion, we shall develop in this paper statistical correlations between short-term adhesion (STA) after 24 h (IA: initial attachment) and long-term adhesion (LTA) calculated on the 21 days of culture. Additionally, as the adhesion phase is known to influence further growth of cells we shall proceed to the correlation of STA with the number of cells after 21 days.

2. Materials and methods

2.1. Surface preparation

Ti6Al4V titanium alloy (V), pure titanium (T) or stainless steel samples (I) were machine-tooled to obtain various surface morphologies using different techniques. The three different materials were electro-eroded (E) or sandblasted (S) under two different conditions to produce surfaces with two different roughness amplitudes ($S_a = 0.85 \mu\text{m}$ and $S_a = 2.35 \mu\text{m}$). To obtain various surface morphologies, Ti samples were also polished (TPN), acid-etched (TAN) or machine-tooled under different conditions to produce parallel (TU0N) or cross grooves (TUXN) with a S_a of $0.7 \mu\text{m}$. Some samples were coated with a layer of gold–palladium. Finally 30 different substrates were compared with the same cellular model and the STA, the LTA and the growth of cells was quantified on these substrates. The plastic Thermanox[®] was systematically used as a control in each experiment with a S_a being near 0 ($S_a = 42 \text{ nm}$) (Table 1). The complete description of the surfaces used in this work was previously published [11,12].

2.2. Cell culture

Human osteoblasts were obtained from trabecular bone taken from the iliac crest of young patients. Briefly, bone was minced in about 5 mm^3 pieces, extensively rinsed under stirring in phosphate buffered saline (PBS), distributed in 100 mm dishes (about 30 explants/dish) and recovered by a Dulbecco’s modified Eagle’s medium (DMEM, Eurobio, Les Ulis, France) containing 10% foetal bovine serum (FBS) + 2 mM L-glutamine + 50 IU/ml penicillin + 50 $\mu\text{g/ml}$ streptomycin. The culture medium was changed twice a week and cultures were maintained until confluence (about 3 weeks).

Table 1

Details of the experiments for preparing the 30 different substrates used in our culture model

	S_a (μm)	Pure titanium (T)		Titanium alloy Ti6Al4V (V)		Stainless steel 316L (I)	
		C	NC	C	NC	C	NC
Sandblasting (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) TUXN (12)				
Etching (A)	0.7	TAO (9)	TAN (18)				
Thermanox [®]	–			Thermanox [®] (33)			

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

Then, cells were used immediately for in vitro characterisation of phenotype and frozen in liquid nitrogen by holding for 2 h in vapour before immersion.

For characterisation of the cell phenotype, cells were inoculated at 2×10^4 cells/well in 24-multiwells plates. After 28 days the wells were rinsed in PBS and recovered by DMEM + bovine serum albumin (BSA) 0.1% over 24 h. The half of the wells was then treated with calcitriol ($1.25(\text{OH})_2$ vitamin D_3) 10^{-8} M in culture medium + BSA 0.1% for 48 h although the other half of the wells was treated with a control medium containing BSA 0.1% and only the vitamin D_3 vehicle, i.e. the absolute ethanol. After this delay, the culture medium was harvested for osteocalcin content evaluation with the Novocalcin[™] kit and for type I procollagen measurement with the Prolagen-C[™] kit (Metrabiosystem, Behring, France). Cells were collected by trypsin, counted or centrifuged and sonicated for alkaline phosphatase activity evaluation with an Enzyline[™] kit for ALP activity (CobasMira, Biomérieux, Marcy l'Etoile, France). These analyses confirmed the osteoblastic phenotype of the cells [12]. After thawing, cells were cultured in 75 cm² flasks. At confluence, the cells were harvested using trypsin–EDTA and inoculated onto samples in 24-well plates for adhesion tests. The medium was changed twice a week. The same cell line (same donor) at the same passage (2nd) was used for the whole of the study.

2.3. STA and LTA measurements

Samples of each surface were inoculated with 4×10^4 cells/sample. In each experiment, four incubation periods were considered: 24 h, 7 days, 14 days and 21 days. The experiments were reproduced on 8–33 samples. A progressive enzymatic detachment of cells was used in order to quantify the adhesion of cells. The cells were enzymatically detached from the samples firstly by a diluted 0.1× trypsin–EDTA (0.025% v/v) treatment and

secondly by a non-diluted 1× trypsin–EDTA (0.25% v/v) treatment, as previously described [11]. The curves of the number of released cells versus trypsination time (T_d) were established for each time in culture T . These experiments were done at least in triplicate.

It is considered that the cells with a low adhesion capacity are detached easily and rapidly by the first incubation with 0.1× trypsin–EDTA although the cells with an higher adhesion capacity need more time to detach and sometimes detach only after the final treatment with 1× trypsin–EDTA. The lower the number of detached cells for a given digestion time, the higher the adhesion is. Thus, this progressive enzymatic detachment allows a quantification of the adhesion capacity of cells as a function of time in culture and as a function of the surface chemistry and surface topography of the substrate as previously shown [8,10,13,14]. The total number of cells released after 24 days was considered as the initial attachment (IA) parameter.

In order to differentiate the adhesion of cells throughout the culture time from 24 h to 21 days, from the growth occurring during the same period, we proceeded to the de-correlation of adhesion and proliferation. Indeed, there exists an experimental bias when measuring the adhesion of a cell population cultured on a substrate over 21 days. In a high number of cases, adhesion depends on culture time. In our culture experiment, cells are deposited on a substrate and will proliferate. As a consequence, the stimulus will detach both initially deposited cells and cells that have proliferated. If no cell growth occurs, the measurement of adhesion versus time in culture characterises the real adhesion kinetics. However, a bias emerges when cell growth occurs during culture time: new proliferating cells are included in the counting process and that will diminish the real cell average time in culture. This artefact could give a negative correlation between adhesion and proliferation. We introduced recently a model that allowed us to

de-correlate the effect of cell growth rate on the measure of adhesion and we defined the relation existing between T the time in culture and T_d the time needed by an individual cell to detach under trypsination: $T_d(T) = aT^b$ where b represents the kinetic exponent of long-term adhesion and a is a scaling factor that characterises the amplitude of long-term adhesion. We call the a coefficient the adhesion power [11].

2.4. Cell growth measurement

From the total number of cells detached at each time during the adhesion measurement we were able to establish the growth curves of cells. The final number of cells after 21 days of culture was used for most of the statistical analysis. Alternatively, the growth rate (number of cells after 21 days/number of cells after 1 day) was considered.

2.5. Bootstrap protocol

The bootstrap is an intensive computer statistical technique devised by Efron and Tibshirani based on the over-sampling of data [15]. Let us now analyse a simple example to understand the basis of this method. We shall try to find the confidence interval of the means for initial attachment or STA (I) and final adhesion or LTA (A) for the TU0N samples. For these samples,

we did 12 experiments (Table 1) meaning that we obtained 12 pairs (I, A). However, the trypsination process was used to evaluate both STA and LTA. As a consequence, due to the hypothetical interaction between the STA and LTA measures, a value of A and I for a given experiment could not be dissociated. How does the bootstrap method work in this case? Consider (A_i, I_i) the pair of i th experimental data ($i = 1, \dots, N$). The aim of the bootstrap is to create a data bank by taking randomly replacement N pairs from the original data (A_i, I_i) that constitute the first bootstrap sample denoted $(A, I)_1^B$. Then a statistic S_1^B (a scalar or a vector) can be built on these data. The procedure is repeated a high number of times (n) to obtain a data bank $\{(A, I)_1^B, (A, I)_2^B, \dots, (A, I)_n^B\}$ that allows us to build statistics $\{S_1^B, S_2^B, \dots, S_n^B\}$ on which a probability density function (PDF) will be drawn, allowing the calculation of descriptive statistics.

3. Results

3.1. Relation between short-term adhesion and long-term adhesion

Our aim in this part is to determine if a relation exists between the STA (I) and LTA characterised by the adhesion power (A) coefficient. We shall distinguish

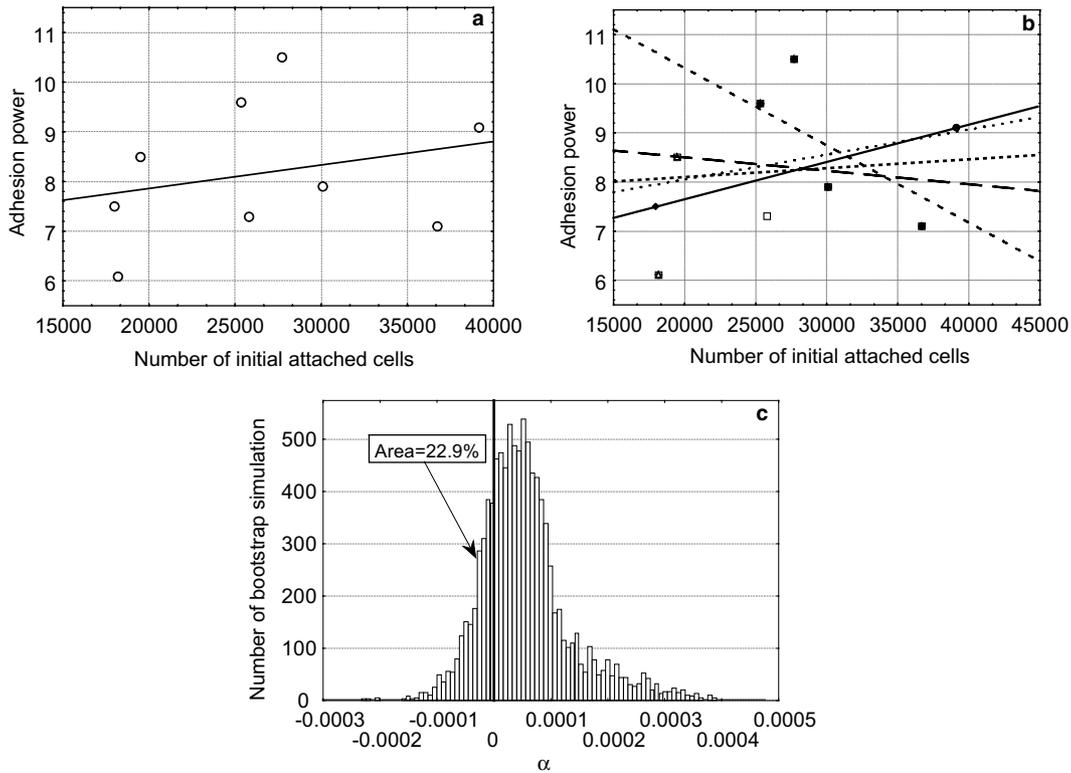


Fig. 1. (a) Example of an original plot of $A = \alpha I + \beta$ for the IS0N sample. (b) Five bootstrap simulated data with their regression line. (c) Histogram for 10,000 simulated bootstrap samples.

between the intra-relation for each sample and inter-relation for all samples.

3.1.1. First bootstrap analysis: intra-relation between STA and LTA for a given sample

The first question we have to answer is the following: is there a relation between STA and LTA for each sample described in Table 1? For example, for one IS0N sample, if STA is higher, is LTA higher too? In statistical terms, this means that there is a positive correlation between A and I characterised by a linear relation $A = \alpha I + \beta$ where three cases can occur: $\alpha > 0$ (positive correlation), $\alpha = 0$ (no correlation) and finally $\alpha < 0$ (negative correlation), the relation being given at a p confidence interval (in our case, $p = 0.05$). To build the statistical test by the bootstrap protocol, we first created the bootstrap samples $\{(A, I)_1^B, (A, I)_2^B, \dots, (A, I)_n^B\}$ described in Section 2. Then, for each sample $(A, I)_j^B$, α was estimated by the linear least squares method to obtain α_j^B . Fig. 1a represents the original plot $A = \alpha I + \beta$ for the IS0N samples. Fig. 1b represents five bootstrap simulated data with their regression lines. Fig. 1c represents the histogram of the slope α for 10,000 simulated

Table 2

Values of bootstrap probabilities to have a positive slope (P_{sup}) or a negative one (P_{inf}) between the adhesion power and the number of initial attached cells on each substrate. The correlation is statistically relevant (positive or negative) at the usual 5% critical value

Material	P_{inf}	P_{sup}	Correlation
VPO	100	0	Positive
TAO	99.97	0.03	Positive
VE1O	94.49	5.51	Null
TE0O	93.99	6.01	Null
Thermanox	92.25	7.75	Null
VE0O	89.8	10.2	Null
TS0N	88.83	11.17	Null
TU0N	86.32	13.68	Null
IS0N	77.03	22.97	Null
VPN	71.63	28.37	Null
TS1N	59.4	40.6	Null
IE0N	59.19	40.81	Null
VS0N	53.26	46.74	Null
IPO	49.76	50.24	Null
TUXN	34.48	65.52	Null
IE1O	34.06	65.94	Null
VE0N	29.76	70.24	Null
IE0O	24.09	75.91	Null
IS1O	20	80	Null
TAN	19.54	80.46	Null
VE1N	18.02	81.98	Null
IPN	15.54	84.46	Null
IE1N	6.58	93.42	Null
VS1N	6.36	93.64	Null
TPN	5.33	94.67	Null
TS1O	5.10	95.90	Null
IS1N	0.58	99.42	Negative
TE1O	0.5	99.5	Negative
TE1N	0.01	99.99	Negative
TE0N	0	100	Negative

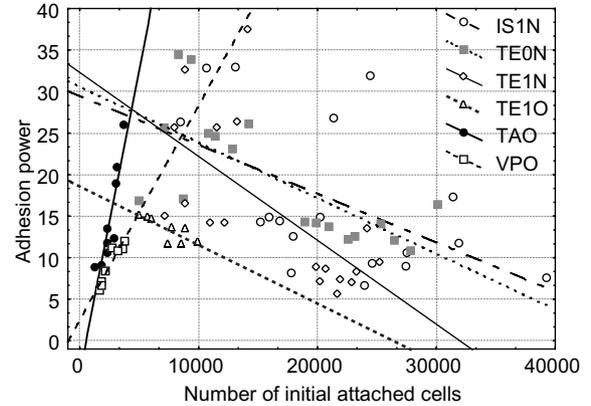


Fig. 2. Plot of the correlation with regression lines between number of initial attached cells and adhesion power for four samples, TE0N, TE1N, VPO and TAO, with a positive significant correlation for VPO and TAO and a negative one for TE0N and TE1N.

bootstrap samples. Confidence intervals were built. For this sample, 22.9% of the slopes get negative values; therefore 77.1% have positive ones. This means that if we concluded that a positive value exists between STA and LTA (i.e. I and A), then this assertion could be false in 22.9% of cases. At the usual critical values of $p = 0.05$, correlation between A and I must be rejected. This protocol is repeated on the 30 samples tested and Table 2 allowed us to quantify these correlations. Among them, only six get a significant correlation, negative for both TE1O, TE1N, TE0N and IS1N and positive for both VPO and TAO (Fig. 2). However, according to their variation amplitude, these positive correlations seem not to have any real physical meaning since for example for TAO or VPO a variation of fewer than 2000 attached cells after 24 h would change the LTA by a 20-fold factor. In Table 2, the IPO sample has approximately the same probability of showing a positive ($P_{\text{sup}} = 49.76$) or a negative correlation ($P_{\text{inf}} = 50.24$). Moreover, 13 samples are above IPO, displaying a higher probability of having a positive slope, whereas under IPO, 16 samples have a higher probability of having a negative slope. This quasi-perfect symmetry confirms the absence of correlation between IA and AP for intra-relation study. As a consequence, we conclude that for a given sample no relation exists between its STA and its LTA.

3.1.2. Second bootstrap analysis: inter-relation between STA and LTA for all samples

We shall now analyse if there is a relation between STA and LTA for all samples taken together (Fig. 3). For these samples, the mean is calculated on both I and A of the bootstrap (Fig. 4). Then the same bootstrap protocol as described in the previous section is applied. 10^6 simulations are processed and the histogram of the slope values between STA and LTA are plotted

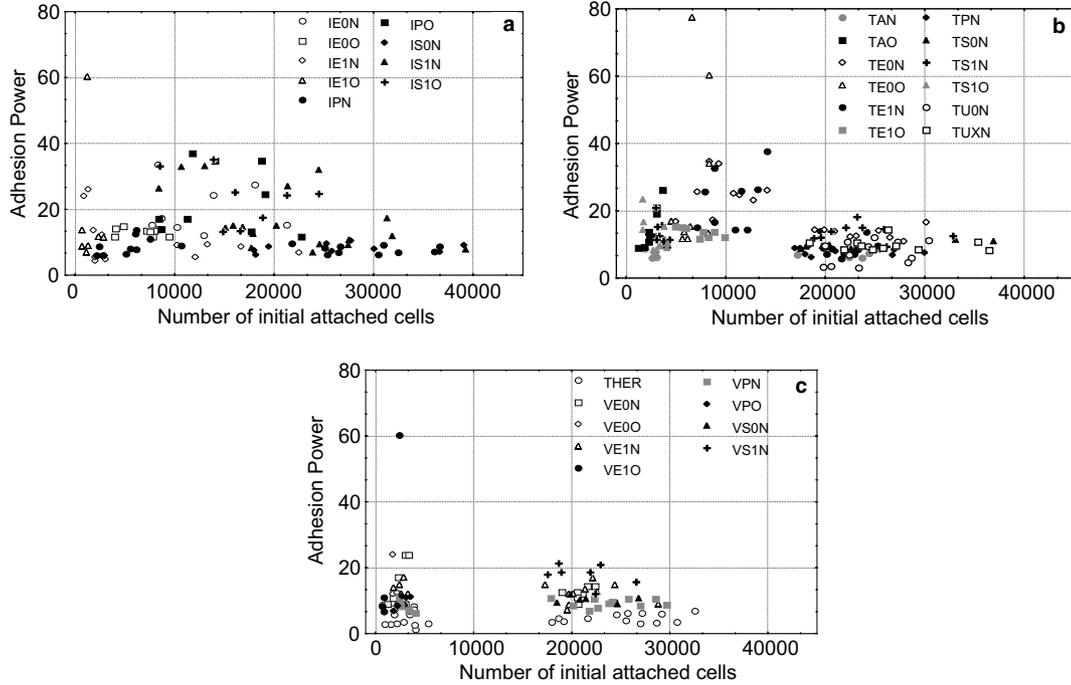


Fig. 3. (a) Plot of the correlation between number of initial attached cells and adhesion power for stainless steel samples. (b) Plot of the correlation between number of initial attached cells and adhesion power for pure titanium samples. (c) Plot of the correlation between number of initial attached cells and adhesion power for titanium alloy samples.

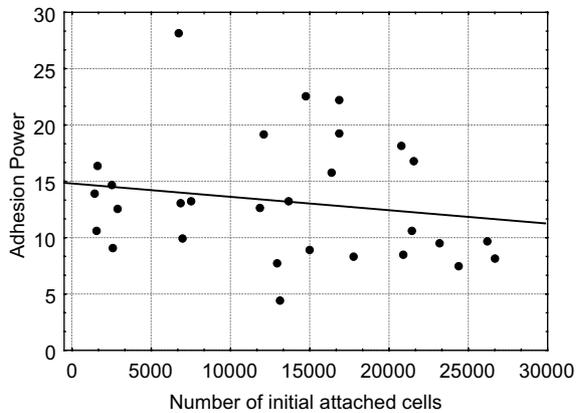


Fig. 4. Mean plot of the correlation between number of initial attached cells and adhesion power for all samples.

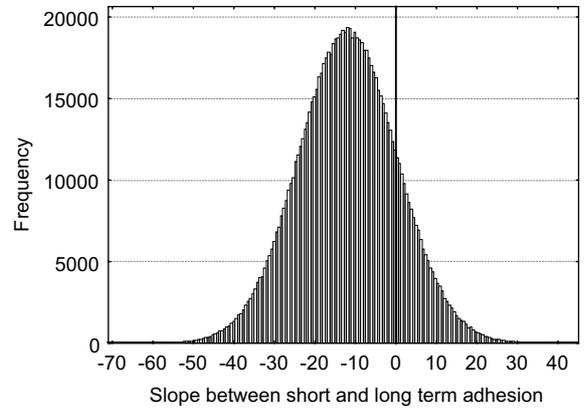


Fig. 5. Histogram of the slope values between short-term and long-term adhesion.

(Fig. 5). According to the descriptive statistics based on the bootstrapped slope, we obtain a probability of 16.59% to have a positive correlation and 83.41% to obtain a negative one. As a consequence, at the usual critical $p = 0.05$, we must reject the presence of a correlation between STA and LTA for samples with different roughness amplitude, coating, processes and materials' composition.

Although no correlation emerges from STA and LTA, it seems logical to analyse if STA and LTA are different for each class of samples. To carry out this analysis, we shall use the bootstrap again. For each class

of sample, the mean is calculated on both A and I of the bootstrap samples $(A, I)_1^B$ that will be denoted $(\bar{A}, \bar{I})_1^B$. Then the procedure is repeated a high number of times (n) to obtain a data bank of the mean values $\{(\bar{A}, \bar{I})_1^B, (\bar{A}, \bar{I})_2^B, \dots, (\bar{A}, \bar{I})_n^B\}$. This databank represents the possible values of the means of (A_i, I_i) and by plotting bivariate histograms of these bootstrap samples, the bivariate mean density function is obtained. Fig. 6 represents the bivariate histogram of the mean obtained with 100,000 data of mean value of both STA (I) and LTA (A) for the TU0N samples. From this histogram, we want to determine the curves that represent the same frequency i.e. curves of iso-probability to find a bivariate mean.

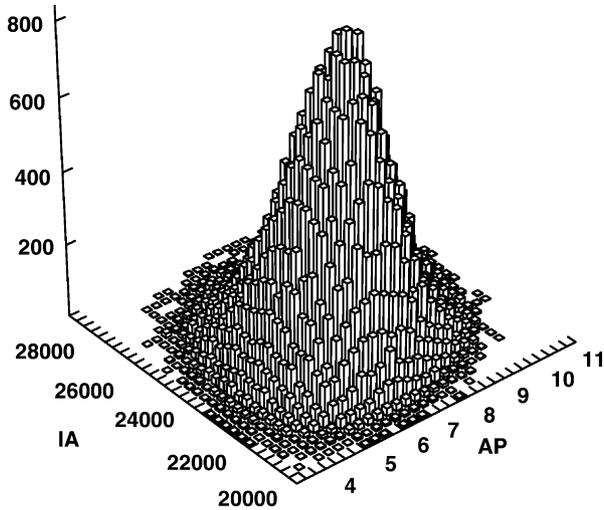


Fig. 6. Bivariate histogram obtained with 100,000 data of mean value of initial attachment and adhesion power.

Then we keep the curve such that 95% of the value are included inside this curve. This curve represents the confidence region at 95% of the mean. However, to obtain a low noise curve, we have to do a high number of simulations to minimize the noise due to the sampling in each bar of the histogram. A Visual Basic[®] software was designed to simulate a high number of loops, i.e. $n = 10^8$

bootstrap samples. Fig. 7a–c represent the 95% iso-confidence intervals of the means for the three different materials (pure titanium, titanium alloy and stainless steel). As observed in these figures, no correlation exists between the STA and the LTA of the cells for all the substrates under study whatever their roughness amplitude, composition or their recovering by coating. However, thanks to the low recovering of intersection of our confidence “circles”, this bootstrap analysis demonstrates that the values of STA and LTA independently depend on the substrates’ characteristics: composition, roughness or presence of coating.

3.2. Relation between the short-term adhesion and the final number of cells

The problem we shall now propose to solve is to see if there is a relation between the initial number of attached cells (I) and the final number of cells (P) representative of the cell’s growth potential.

3.2.1. Third bootstrap analysis: intra-relation between the number of initial attached cells and the final number of cells

Thus, we shall do a new bootstrap with a procedure that is similar to that in the previous paragraph, and then we shall create a bootstrap sample denoted

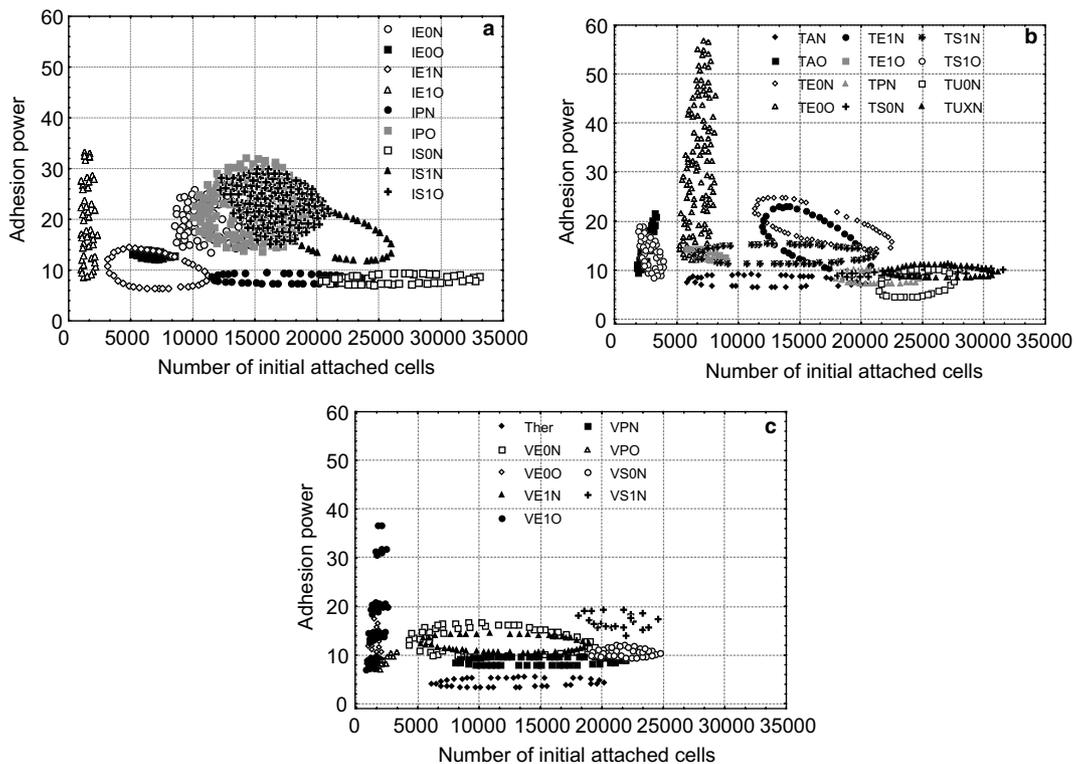


Fig. 7. (a) Plots of iso-confidence intervals of the means of initial attachment and adhesion power for stainless steel substrates. (b) Plots of iso-confidence intervals of the means of initial attachment and adhesion power for pure titanium substrates. (c) Plots of iso-confidence intervals of the means of initial attachment and adhesion power for titanium alloy substrates. Ther: Thermanox[®].

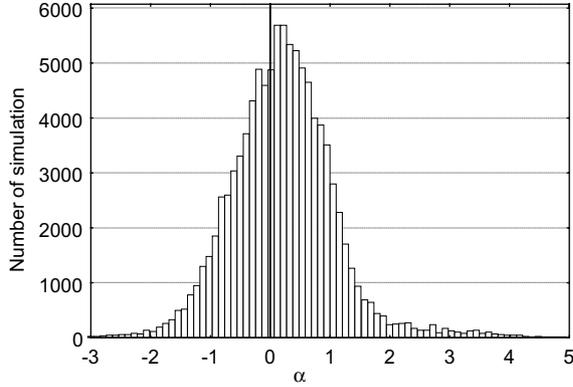


Fig. 8. Probability density functions of the α coefficient (for the 30 classes of samples) from $P = \alpha I + \beta$ with P the number of final cells after 21 days and I the number of initial attached cells.

$\{(\bar{I}, \bar{P})_1^B, (\bar{I}, \bar{P})_2^B, \dots, (\bar{I}, \bar{P})_n^B\}$ where P represents the number of final cells after 21 days in culture and \bar{P} the associated mean for each type of sample. Initially, we shall analyse the relation between the $P = \alpha I + \beta$ as shown in Section 2.5. The results of this analysis prove that all the probability density functions of the α coefficients (for the 30 classes of samples) approximately follow a Student law (Fig. 8). According to the Gauss–Markov hypothesis about the least squares method, this demonstrates that data are Gaussianly distributed. This fact is an important one. Whatever the counting process, the STA and cell growth are stochastic processes that seem to be governed by a Gaussian property, i.e. according to the central limit theorem due to the many small physical influences with the same amplitude. We then proceeded to the analysis of the α coefficients to determine if their correlation was null (Table 3). We processed results as for Table 2.

We observed that for 26 samples out of the 30 tested, the relation between the STA and final number of cells appeared positive. However, this positive relation was statistically asserted at $p > 0.05$ for 16 out of 26. On the other hand, the relation was negative for 4 substrates out of 30 and poorly significant only for the IS1N substrate with a high critical value of 2.5% (Table 3). This means that for a given sample (the same roughness amplitude, the same process, the same materials and coating), the more numerous initial cells are, the more numerous final cells are.

3.2.2. Fourth bootstrap analysis: Inter-relation between the number of initial attached cells and the final number of cells

We carried out the process with the same protocol as defined in Section 3.1.2. Fig. 9 represents the mean values of the initial number of cells versus the final ones for all the 27 classes of samples. By the least squares method, the following linear relation is found $P_f = 3.22P_i - 8000$. Then thanks to the bootstrap, 10^6 simulations are pro-

Table 3

Values of bootstrap probabilities to have a positive slope (P_{sup}) or a negative one (P_{inf}) between the number of initial attached cell and the final number of cells on each substrate. The correlation is statistically relevant (positive or negative) at the usual 5% critical value

Material	P_{inf}	P_{sup}	Correlation
IPN	0	100	Positive
TE0N	0	100	Positive
Thermahox	0	100	Positive
TS1N	0	100	Positive
VE0N	0	100	Positive
VPN	0	100	Positive
TAN	0.001	99.999	Positive
VE1N	0.003	99.997	Positive
VE1O	0.097	99.903	Positive
TE0O	0.197	99.803	Positive
VPO	0.524	99.476	Positive
IE0O	0.882	99.118	Positive
TAO	1.362	98.638	Positive
VE0O	1.575	98.425	Positive
TUXN	2.74	97.26	Positive
VS1N	4.304	95.696	Positive
IE1N	6.197	93.803	Null
TS0N	6.988	93.012	Null
TE1O	7.139	92.861	Null
IE1O	7.28	92.72	Null
TU0N	10.399	89.601	Null
TPN	10.847	89.153	Null
IPO	18.439	81.561	Null
VS0N	29.852	70.148	Null
TE1N	36.568	63.432	Null
IS0N	39.782	60.218	Null
IE0N	56.709	43.291	Null
IS1O	74.263	25.737	Null
TS1O	89.653	10.347	Null
IS1N	97.427	2.573	Negative

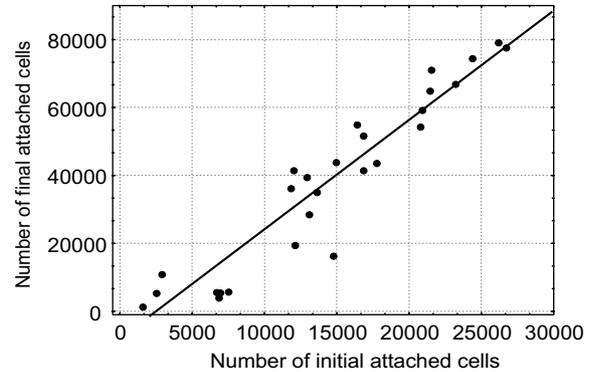


Fig. 9. Mean values of the initial number of cells and the final number of cells.

cessed and the histogram of the slope and intercept values between the initial number of cells and the final ones are plotted (Fig. 10a and b). According to the descriptive statistics based on the bootstrapped slope, we respectively get the 95% confidence intervals for the slope of $[2.85, 3.59]$ and $[-13,800, -2600]$.

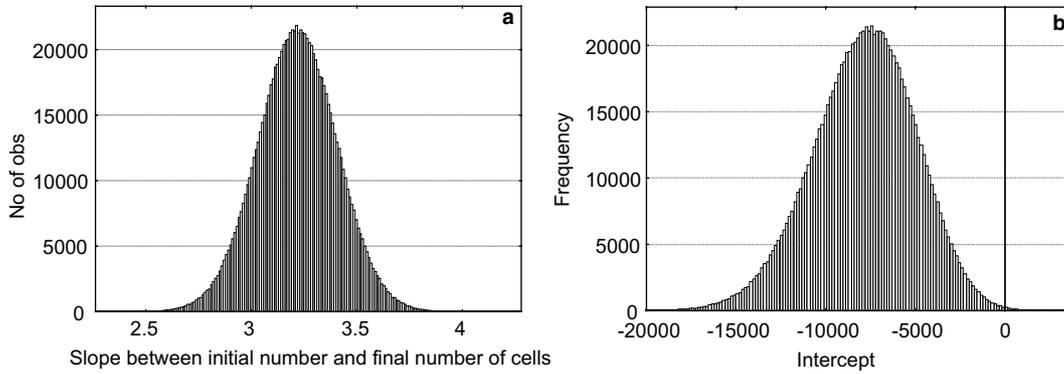


Fig. 10. (a) Histogram of the slope between the initial number of cells and the final number of cells. (b) Histogram of the intercept values between the initial number of cells and the final number of cells.

Two important remarks can be made based on these statistical results:

- The histogram of the slope follows a Gaussian law meaning that the linear relation between the initial number and final number of cells is well adapted. This seems to mean that if cell growth occurs (a bio-compatible substrate), then the number of final cells depends only on the number of initial attached cells

(in our case, the multiplicative factor is around three after 21 days). If we admit this relation, we can infer that after one day in culture the kinetics of cells' reproduction is obtained and is the same whatever the substrate, coating, roughness and process. In addition, the finally attached cells will be three times more numerous than the initial attached cells. These fundamental results, which will be tempered in the fourth bootstrap study for a small number of initial

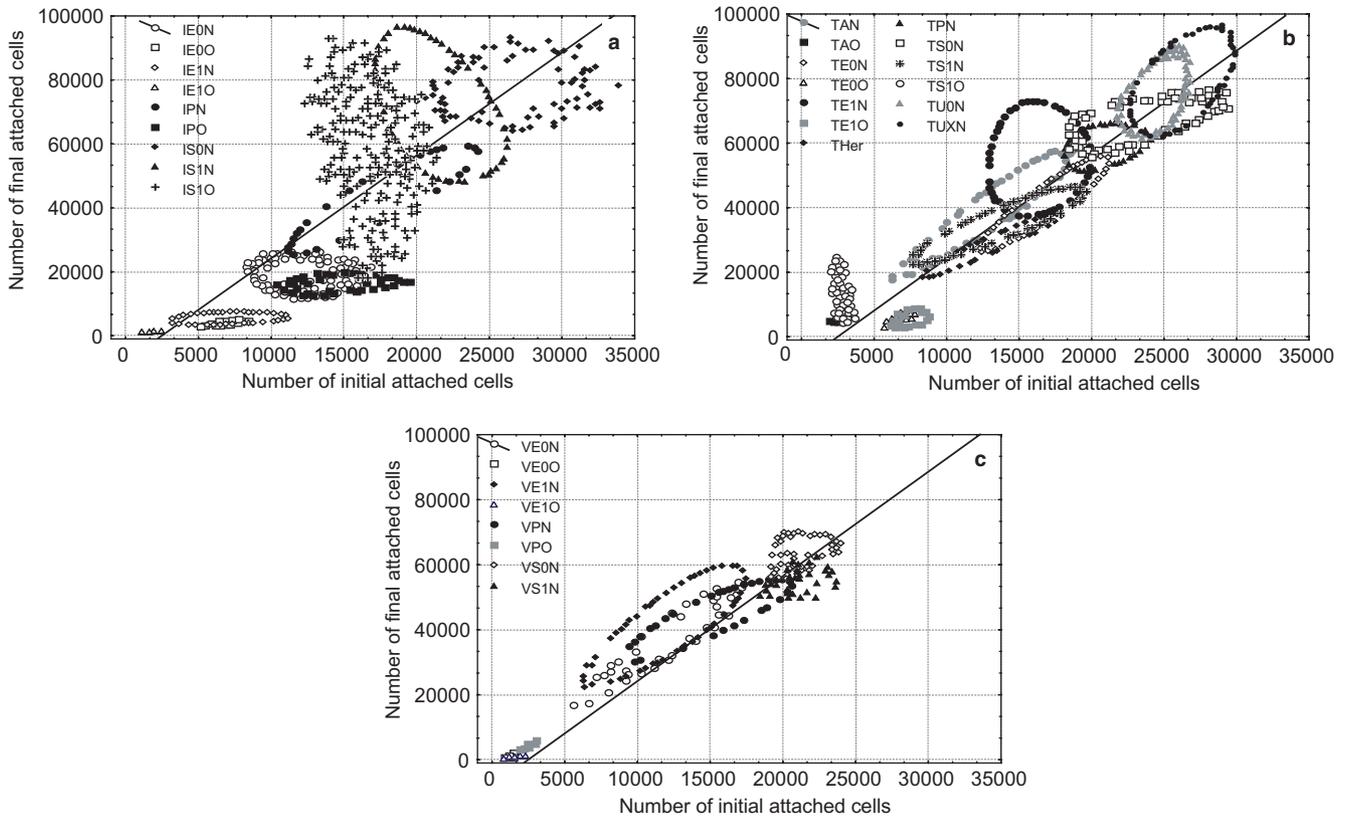


Fig. 11. (a) Plots of iso-confidence intervals of the means of initial number of cells and final number of cells for stainless steel substrates. (b) Plots of iso-confidence intervals of the means of initial number of cells and final number of cells for pure titanium substrates. (c) Plots of iso-confidence intervals of the means of initial number of cells and final number of cells for titanium alloy substrates.

attached cells, shows that the cell growth rates, when cells are attached and when surfaces are biocompatible depend neither on the substrate's chemistry, nor on the substrate's morphology.

- The intercept is statistically negative ($p < 0.002$). This means that when the number of initial attached cells is lower than 2500, the number of final attached cells is null. Under these values, this number will become negative which is a physical nonsense. What does this negative intercept physically mean? We could interpret these values as a critical number of initial attached cells. The number of cells introduced in the culture is always the same but if fewer than 2500 cells attach after one day, then no cell will proliferate over 21 days.

Thanks to a new bootstrap, we then reproduced similar figures as Fig. 7a–c (Fig. 11a–c) but by analysing the number of initial attached cells versus the final ones. The aims of this bootstrap are to introduce the intra-sample variation, to reproduce the same analysis and to specify the cut-off of the linear relation found. The following statistical results can be stated: when the initial number of attached cells is higher than 10,000, then all 95 isoprobability confidence intervals of the mean are confounded with the equations $P_f = 3.22P_i - 8000$ meaning that the previous remarks always hold under this condition. However, under this critical number of cells, these linear laws do not always hold for all samples. This can be interpreted by the fact that for these samples, the STA is not achieved after one day and then the linear relation that represents a kind of equilibrium of the cells' rates is not valid yet. If the number of attached cells after 24 h is less than 10,000 it is not possible to predict the number of cells after 21 days as a function of this linear relation.

4. Discussion

We statistically demonstrated by our correlation study that different mechanisms are involved during short-term adhesion and long-term adhesion. Indeed, the short-term adhesion comprises an attachment phase, which is mainly related to passive events not requiring active cell participation (like physical linkages involving ionic forces, van der Waals forces, and passive formation of ligand–receptor bonds) and an adhesion phase which is an active phase involving various biological molecules like membrane and cytoskeleton proteins. The cell membrane proteins and especially integrins are involved at the same time not only in the adhesion of cells but also in the signal transduction modulating gene expression and further cell proliferation and differentiation. The cytoskeleton proteins by the reorganisation occurring during the adhesion phase, are involved

in the spreading of cells and also play a role in signal transduction. The long-term adhesion involves in addition the extracellular matrix proteins which behave like ligands for integrins and have a structural role at the interface. They also play a role in the transduction of mechanical forces and contribute to the adhesion strength of cells [7]. Actually, the evaluation of long-term adhesion over a 21 day period gives us information on the maturation of the cell/substrate interface, on the densification of integrins/ligand binding and on the thickening of extracellular matrix layer, giving a more and more complex interface closer to the tissue/implant situation.

This correlation result also demonstrated that the mechanism responsible for the STA has no influence on the future LTA of cells. Thus, it appears that the evaluation of the number of attached cells after 24 h cannot let us presume that cells will survive or that they will form an evolving interface by synthesis of extracellular matrix proteins.

Additionally, we demonstrated that STA was positively correlated with cell growth. The higher the number of attached cells after 24 h, the higher the number of final cells after 21 days. This relation appears wholly logical and trivial since adherent cells like osteoblasts need to attach to be able to proliferate and since the final cell number will unsurprisingly depend on the initial number of attached cells. However, it was also demonstrated that this correlation was not influenced either by substrate's chemistry or the substrate's morphology. Thus, knowing the cell growth capacity of the cellular model used, it is possible to predict the final number of cells present on a surface after a particular time in culture.

Interestingly, we found a linear relation between STA and cell growth with a growth rate of about three, meaning that the final number of cells after 21 days will be always three times the initial cell number if the initial attached cell number is above 10,000 cells/cm². Under this threshold, the relation between STA and cell growth is not more linear. If the initial cell number is under 2500 cells/cm², no growth of cells will be possible. This critical initial cell concentration below which no growth will occur is a well known phenomenon for investigators using primary cell cultures. In the 1970s, it was found that cell growth was determined almost entirely by the density of attached cells per unit area of the substratum rather than by the total number of cells per culture vessel or per unit volume of medium [16,17]. The influence of the cell density on cell growth is related to the synthesis by the cells of a diffusible growth factor essential for their own growth. The low rate of diffusion of this growth factor explains the need of a critical number of cells per unit area to reach in the immediate vicinity of the cells a critical concentration of this growth factor. If the population of cells is plated at a too low concen-

tration or if the attachment potential of these cells is decreased by a weakly biocompatible substrate, then the local critical concentration of this growth factor is not reached and the cell growth become impossible [16,17]. Moreover, as the growth potential of cells is lost within the first 12 h if they are not cultured in adequate conditions, this explains why the first hours of attachment are so critical for the further development of cells [16,17].

It is interesting to note that the LTA is also positively correlated with cell growth [11]. Thus, it appears that to display a good growth, cells need to initially well attach to the substrate and then need to adhere well by elaborating a complex interface involving all the adhesion proteins.

The inter-relation study we performed allowed us to define more specifically if an effect of the substrate's characteristics was visible on the interactions between STA and LTA. We observed that the circles representing the iso-confidence intervals of the means of IA and AP in Fig. 7a–c did not recover each other. This absence of recovering demonstrates that the values of STA and LTA depend on the substrate's characteristics (composition, roughness, coating). The statistical demonstration of the effect of these surface characteristics has been done by other statistical methods like multivariate analysis of variance and has shown that the major influencing parameter on STA was the surface's chemistry compared to the surface's topography (submitted for publication). This result is confirmed by a recent study of Lim et al. [18]. They have cleverly demonstrated that short-term biological response to polymeric substrates like attachment and proliferation before 3 days correlated with substratum surface energy, although longer term alkaline phosphatase activity was not influenced by surface energy. We obtained a different result with LTA since CMS adhesion was shown to be influenced rather by the surface's topography and notably the surface's morphology rather than by the surface's chemistry [8,10,13,14]. More recently, we also showed that LTA was more influenced by the surface's morphology than by the surface's chemistry [12]. The major differences between STA and LTA are the formation over the 21 days culture period of a more and more elaborate extracellular matrix layer between the cells and the substrate. We have previously shown that the organisation of the extracellular matrix reflected the organisation of the surface topography [10]. Therefore, it appears logical that LTA is more influenced by the surface morphology than by the surface chemistry.

5. Conclusion

For the first time, we demonstrated by a statistical method the logical positive relation existing between STA (the initial number of attached cells after 24 h)

and cell growth (the final number of attached cells after 21 days). Importantly, this relation is independent of surface topography and surface chemistry—it exists whatever the substrate, coating, roughness or process. Thus, knowing the growth rate of the cellular model used, it is possible to predict the final number of cells from the initial attached cell number. More surprisingly, we demonstrated the absence of correlation between STA and the LTA of cells throughout the culture from 24 h to 21 days. This illustrates that different mechanisms underlie STA and LTA. As LTA represents the maturation of the cell/matrix/substrate interface, this study demonstrates that the evaluation of the number of attached cells after 24 h cannot let us presume that cells will survive or that they will adhere by forming a complex interface by synthesis of extracellular matrix proteins. This is an important feature since it seems illusory to search for a technique in order to increase the STA of the cells with the aim of favouring LTA. Thus, no single test evaluating a particular period of time can expect to deliver all the information needed for the characterisation of biocompatibility. Finally, this study is the first one to describe the statistical correlation between adhesion and growth of cells on substrates with controlled surface chemistry and topography. The originality of this work resides in the extensive statistical correlation analysis performed between biological parameters describing the cell behaviour on a substrate thanks to a model established from experimental results. We hope that in the future these approaches will become generally applied in the biomaterials field, in order to demonstrate definitively the intuitions of investigators interested in cell or tissue/material interactions.

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