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A kinetic approach to osteoblast adhesion on biomaterial surface

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Abstract: An incompletely understood question in the field of biomaterials is how eucaryotic cells adhere on material surfaces. The adhesion of cells on materials is generally studied after some hours. Because this evaluation after some hours cannot let us presume about the future of the cells on the material, we have developed a culture model that does allow study in the long term of an elaborate cell/material interface closer to the *in vivo* situation. For that, we used a progressive trypsin-based detachment method. Here we report on the mathematical modeling of long-term human primary osteoblastic cell adhesion on metallic substrates, which allows us to quantify the real adhesion simultaneously by taking into account the effect of cell proliferation. A time-dependent adhesion index t_d is proposed, which varies with culture time t according to the power law: $t_d(t) =$

at^b , a being independent of b . The exponent b is equal to 0.5 ± 0.03 and is independent of the substrate's characteristics, meaning that the long-term adhesion increases proportionally to the square root of culture time. On the contrary, the parameter a significantly depends on the material's nature, the surface's topography, and the surface chemistry of the substrate and is sufficient to characterize cell adhesion. From this relationship, we suggest that a diffusion-based process related to the kinetic of formation of extracellular matrix should be involved in long-term adhesion on materials.

Key words: cell proliferation; modeling; cell adhesion

INTRODUCTION

The clarification of the interactions between cells and materials is recognized as an essential factor in developing biomaterials that are used directly or indirectly in contact with biological entities such as cells. The first phase of these interactions is defined as adhesion. Adhesion is known to be critical for the further proliferation, differentiation, migration, and apoptosis phases.¹ However, the definition of the adhesion process and its division in stages are defined in different ways according to the authors. Generally, the authors consider that adhesion is achieved in the first hours of

contact of a cell with a substrate. Adhesion is principally governed during its first flattening phase by nonspecific electrostatic forces, such as the Van der Waals, and by passive formation of ligand-receptor bonds.² This phase achieved in a fraction of a second is followed over the next 10 or so minutes by an alignment phenomenon resulting in the widening of the contact area, which may take place without active cell participation. Later, the spreading phase going on over the next hours involves active cell reorganization and is much more dependent on cell metabolism. It is during this phase that receptor recruitment and clustering to anchoring sites occur and interactions with cytoskeletal elements are formed. The flattening, alignment, and spreading stages have been defined by Pierres et al.² as forming the fitting phase, the prerequisite to firm attachment and subsequent events. Garcia and Gallant separate the adhesion into two major stages: the stick and the grip stages. The stick stage involves the contribution of integrin-ligand binding parameter to initial adhesion strength although the grip stage represents the contribution of cell spread-

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ing, receptor clustering, and focal adhesion assembly to strengthening responses.³ Murphy-Ulrich distinguished three adhesion stages: the weak adhesion involving attachment of cells because of the integrin-ligand bindings, the intermediate adhesion when cell spreading occurs, and the strong adhesion when the formation of focal adhesions and stress fibres are achieved. For all authors, adhesion is considered as achieved when the focal contacts are established and when the cytoskeleton is stabilized.⁴ In the 1980s, biophysical studies of cell adhesion were attempted either by way of mechanical approaches based on detachment experiments or by thermodynamic approach of receptor-ligand binding.^{5,6} More recently, kinetic and dynamic models of cell adhesion have been established on the basis of these previous experimental results.^{7,8}

For several years, our objective has been to develop a human bone cell culture model allowing the *in vitro* formation of cell/material interface that would be as close as possible to the “implant/tissue-like” interface existing *in vivo* during the first days of contact. Intuitively, it seems likely that the evaluation of the strength of the cell/material interface after only some hours cannot let us presume what the future of the cells on the material will be like. Thus, we developed the culture of primary human-derived osteoblasts on model substrates from some hours to several weeks, and we established an original experimental method to detach cells and to evaluate the cell/material interface strength. Indeed, we considered as essential to take into account not only the short-term adhesion of cells in direct contact with the native substrate surface or with the surface after adsorption of proteins from biological fluids but also the adhesion of cells in the longer term. By this long-term adhesion, we understand the cell/material interface strength at times when the cells themselves have elaborated an interfacial extracellular matrix (ECM), which is an intricate arrangement of glycoproteins, collagens, proteoglycans, and growth factors that act not only as physical scaffold for cell attachment and organization of cellular structures but also as a mediator of intracellular signaling through cell surface receptors that recognize these ECM molecules. Hence, in this article, we call short-term adhesion the classically named adhesion phase as opposed to the long-term adhesion we evaluate by using our original model and which represents the strength of the cell-material interface formed during the 3 weeks of culture. It is important to note that to date and to our knowledge, few studies have focused on the study of the long-term evolution of adhesion, although some authors interested in interactions between osteoblasts and bone-replacing materials have recently emphasized that the differences found in the initial responses of cells cannot predict the cell's behavior after longer periods.⁹

Our method to evaluate the long-term adhesion of human osteoblasts on biomaterials is based on a progressive enzymatic detachment protocol using diluted trypsin-EDTA.¹⁰⁻¹³ The curves of cell detachment were established by summing the number of detached cells after increasing times of treatment with diluted trypsin-EDTA. To quantify this long-term adhesion, we defined some years ago two adhesion parameters from the curves of cell detachment: the Adhesion Index (AI) corresponding to the area under the curve and the Detachment Index Percentage (DIP) corresponding to the ratio AI on substrates/AI on control material (cell culture-treated plastic coverslips: ThermanoxTM).¹⁰⁻¹³

In the present article, we study the long-term adhesion of human osteoblasts on metallic substrates by presenting 26 different surface topographies and chemistries. This high number of surfaces was tested at least eightfold at four times in culture, giving a high number of data essential for the extensive statistical analysis needed in this study. Because the cells growing on the substrates in our *in vitro* model alternate different phases (adhesion, detachment for proliferating or migrating, re-adhesion, and synthesis of extracellular matrix), we have to bring a mathematical correction to our long-term adhesion parameter. This correction is needed to take into account the time difference between inoculation and the beginning of proliferation for cells having proliferated during the culture time and to consider this delay in the calculation of adhesion. Thus, we first present in this article the definition of a new quantitative parameter truly representative of adhesion. The relevance of this parameter to discriminate the response of cells on the metallic substrates with various surface topographies is further stated. Second, we determine the law governing the evolution of long-term adhesion with the culture time, and this is discussed in function of physical and biological processes involved in the elaboration of the cell/material interface during the culture time.

MATERIALS AND METHODS

Surface preparation

One of our objectives in this work is to test the influence on human osteoblast adhesion of three major parameters:

- The material nature: Pure titanium (Ti), titanium alloy (TiAl6V), or stainless steel 316L substrates were compared.
- The surface topography: Five various surface topographies obtained by five different surface treatments (sandblasting, electro-erosion, grinding, tooling, and etching) were compared with a control surface (cell culture-

TABLE I
Experiment Designs of 27 Different Substrates Used in Culture Model

	Pure Titanium (T)		Titanium Alloy (V)		Stainless Steel (I)	
	C	NC	C	NC	C	NC
Sand-blasting (S)	TS1O (9)	TS1N (18)		VS1N (9)	IS1O (9)	IS1N (18)
Electro-erosion (E)	TE0O (9)	TE0N (18)	VE0O (9)	VE0N (18)	IE0O (9)	IE0N (9)
	TE1O (9)	TE1N (18)	VE1O (8)	VE1N (18)	IE1O (8)	IE1N (15)
Polishing (P)		TPN (12)	VPO (9)	VPN (21)	IPO (9)	IPN (21)
Machine-tooling (U)	TU0N (12)					
	TX0N (12)					
Acid-etching (A)	TAO (9)	TAN (18)				
Thermanox TM			THER (33)			

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

treated plastic coverslips, ThermanoxTM, NUNCTM, NY, USA). The electro-eroded surfaces presented two different roughness amplitudes: $S_a = 0.8 \mu\text{m}$ or $2.5 \mu\text{m}$. The machined surfaces presented alternatively parallel grooves (TU0N) or cross grooves (TUXN).

- The surface chemistry: One half of each type of samples was coated by electro-sputtering by a nanometric layer of gold-palladium to test either the effect of the surface topography and chemistry on the noncoated surfaces (NC) or the effect of the surface topography alone on the coated ones (C).

Each surface was tested at least eight times. Finally, 27 different substrates were compared with our culture model, and the long-term adhesion of human primary bone-derived cells was quantified on these substrates (Table I).

Roughness measurement

Roughness was measured by using a tactile profilometer (Tancor-P3, Optilas, France) on a surface of $1 \text{ mm} \times 1 \text{ mm}$ with one measure every $2 \mu\text{m}$ on horizontal and vertical scanning. Three-dimensional (3D) profiles were drawn and analyzed on a computer using personal software. Classical roughness amplitude parameters were used: S_a (mean roughness amplitude), S_k (skewness of the roughness amplitude), and S_t (range of the roughness amplitude). Frequency roughness parameters were also analysed: the auto-correlation length and the order parameter (which describes the periodicity of a profile and lies between 0 for a white noise profile and 100 for a perfect periodic profile without noise).¹²

Adhesion measurement

Samples of each surface were inoculated with 4×10^4 cells/sample. We used human primary osteoblasts prepared as previously described.¹⁴ In each experiment, three samples were analyzed after each incubation period t : 24 h, 7, 14, and 21 days. The cells were progressively enzymatically detached from the samples by a diluted trypsin-EDTA (0.025%

v/v) treatment (Eurobio, Les Ulis, France) as previously described.^{10,14,15} Cells detached after a time d of 5, 10, 20, 30, and 60 min of contact with the diluted enzyme were counted with a Coulter Z1 (Beckman Coulter, Roissy, France). In the end, the remaining cells were detached by two successive final 15-min treatments with nondiluted trypsin-EDTA (0.25% v/v). The complete detachment of cells was checked by scanning electron microscopy. The curves of the number of cells released versus the trypsination time were established. The experiments were repeated three or four times.

STATISTICAL ANALYSIS

Adhesion modeling

By suppressing the bias on the characterization of adhesion, authors have shown^{14,15} that the ratio of cells $p(t,d) \in [0..1]$ detached by the action of trypsin during d minutes and which were formed by division over a time t (the beginning being the origin $t = 0$) is given by the following integro-differential equation:

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

with t the culture time, $P(t)$ the number of cells at time t (in cells per cm^2) where H is the Heaviside step distribution

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

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

and time t_d is the adhesion law for an individual cell (i.e., time taken by the trypsin to detach the cell). We have proposed the following analytical expression for $t_d(t)$, which finally describes the adhesion of cells on biomaterials after a time t in culture (without proliferation):

$$t_d(t) = at^b \text{ with } a \geq 0 \quad (2)$$

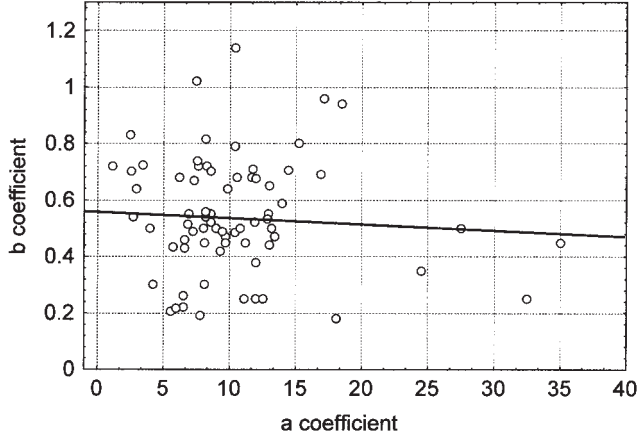


Figure 1. Relation between a and b coefficients of the relation $t_d(t) = at^b$ calculated from Eq. (3) for all experiments given in Table I.

where the two parameters a and b are, respectively, a scale coefficient (a) and a kinetic coefficient (b) and are determined by minimizing from 20 experimental data the following functional:

$$\min_{a,b} \sum_{i=1}^4 \sum_j^5 \|p(t_i, d_j) - \frac{1}{P(t_i) - P(0)} \times \int_0^{t_i} (\partial P(\tau)/\partial \tau) H(d_j - t_d(t_i - \tau)) d\tau\|_{L^2} \quad (3)$$

where $d_1 = 5, d_2 = 10, d_3 = 20, d_4 = 30, d_5 = 60$ are the five trypsination times and $t_1 = 1$ day, $t_2 = 7$ days, $t_3 = 14$ days, and $t_4 = 21$ days the four different times in culture.

Analysis of results

The resolution of Eq. (3) is carried out by the simplex method for all the 396 curves, which gives 396 pairs of (a, b) coefficients. We now want to analyze the values of these coefficients for the different parameters (material/topography/chemistry). We have to answer the following three questions:

1. Is there a correlation between a and b ? If the answer was positive, that would mean that our model would be badly formulated because the coefficient of

the adhesion rate b would depend on the scale factor a and then they would be not intrinsic parameters. We shall then analyze if the relation $a = \alpha b + \beta$ is significant (Fig. 1). With use of the least-square method, we obtain $\alpha = 1.0 \pm 6.9$, meaning that the slope α is not statistically different from zero at the 5% level (critical probability: $p = 0.57$). As a consequence, our model given by Eq. (8) is not statistically ill conditioned (in robustness sense) to describe the adhesion process.

2. Does the coefficient b depend on the different substrate parameters (material/processing/coating)? To test this hypothesis, we carry out a multivariate analysis of variance on b values for the three variables (i.e., material, topography, and chemistry).

At the usual 95% confidence levels, there is no effect resulting from the material because we obtained a Fisher values of $F = 1.8$, which gave a critical probability of $p = 0.14$ (Table II). Then at the usual critical level of 5%, it is impossible to accept the hypothesis that the value of b depends on the materials. In the same way, it is impossible to claim that the surface topography ($p = 0.90$) or the surface chemistry ($p = 0.26$) has any influence. This is an important feature. Coefficient b does not depend on the material's composition, the surface topography, and the surface chemistry, meaning that $t_d(t) \propto at^{\text{constant}}$. On the contrary, the analysis of variance performed on a demonstrates that a depends on the three different variables (material, topography, and chemistry) with a high confidence level ($F = 3.04; p = 0.0004$).

3. What is the value of the invariant parameter b ? We analyze the values of b because the previous analysis of variance showed that b does not depend on the physical and chemical aspect of the substrate. It is possible to plot the frequency histogram of b (Fig. 2). The shape of this histogram looks like a Gaussian curve (plotted in thin line). To test this hypothesis, we performed a chi-squared test and obtained $\chi^2 = 4.3$ with $p = 0.1$. Then the Gaussian hypothesis was not rejected at the usual confidence level of 5%. As a consequence, we can calculate the 95% confidence interval of the mean for b , and we obtain $b = 0.52 \in [0.47; 0.56]$. The value of $b = 0.5$ is not rejected, meaning that the relation $t_d(t) \propto \sqrt{t}$ holds.

From these results, adhesion can be characterized only by the " a " coefficient we call the "Adhesion Power (AP)." To statistically minimize noise in this determination and admitting that $b = 0.50$, the " a " coefficient is recalculated by:

TABLE II
Results of Multivariate Analysis of Variance on b Values for the Three Variables Material, Topography, and Chemistry

Source	DF	Type III SS	Mean Square	F Value	P > F
Material	3	0.51828	0.27276	1.8	0.14
Topography	1	0.00091	0.00091	0.02	0.90
Chemistry	1	0.07601	0.07601	1.28	0.26

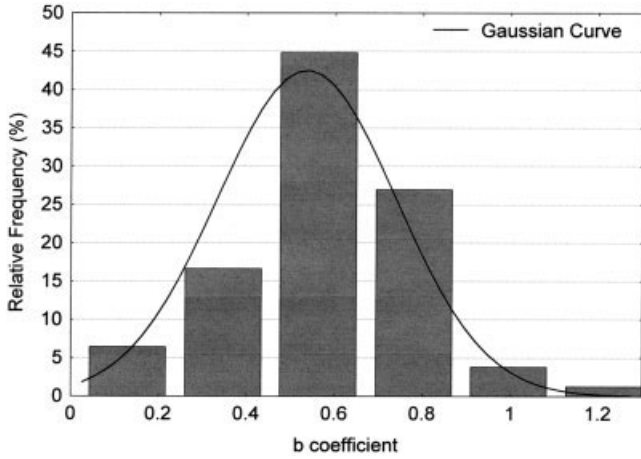


Figure 2. Histogram of b coefficient of the relation $t_d(t) = at^b$ calculated from Eq. (3) for all experiments given in Table I.

$$\min_a \sum_{i=1}^4 \sum_{j=1}^5 \|p(t_i, d_j) - \frac{1}{P(t_i) - P(0)} \times \int_0^{t_i} (\partial P(\tau) / \partial \tau) H(d_j - a\sqrt{(t_i - \tau)}) d\tau\|_{L^2} \quad (4)$$

A new analysis of variance is performed and we obtain $F = 10$, which leads to a highly discriminating value of the “ a ” parameter to characterize the cell adhesion. To illustrate the accuracy of our model, we plot the ratio of experimental detached cells $p(t_i, d_j)$ and the modeled one

$\frac{1}{P(t_i) - P(0)} \int_0^{t_i} (\partial P(\tau) / \partial \tau) H(d_j - a\sqrt{(t_i - \tau)}) d\tau$ for the two biomaterials, Thermanox™ (Fig. 3, low adhesion, $a = 3.6$) and TE1N (Fig. 4, high adhesion, $a = 37.5$). As observed, modeled data and simulated data are quite similar, meaning that our model is well adapted to describe adhesion.

Modeling validation

The aim of this part is to validate Eq. (4) (i.e., to statistically analyze if our model is well accurate to describe adhesion and proliferation. We now analyze the possible origins of error in modeling.

Type 1: Because our model needs a modeling of proliferation, an error could occur by a wrong choice among the models proposed for proliferation.

Type 2: To calculate coefficients of the proposed proliferation models, we used regression tools, and coefficients were calculated with use of the least-square method. Consequently, coefficients possess an intrinsic error that involves uncertainty on proliferation equation.

Type 3: We claim that t_d follows the power law $t_d(t) = at^b$ and prove that it could be simplified in $t_d(t) = at^{0.5}$. This assumption could be seen as a simplification of the reality and could involve error.

Type 4: More simply, experimental errors occur during the measure of detachment. This error can be

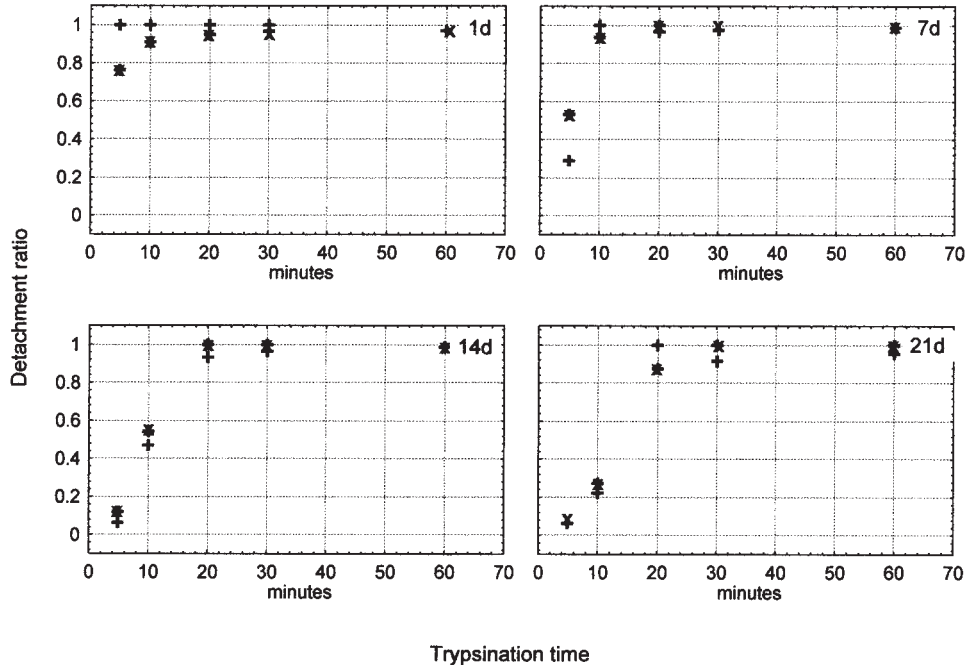


Figure 3. Plot of the experimental ratio (+) and the modeled ratio (*) of detached cells $p(t_i, d_j)$ with $t_d(t) = 3.6t^{0.5}$ for cells cultured on Thermanox™.

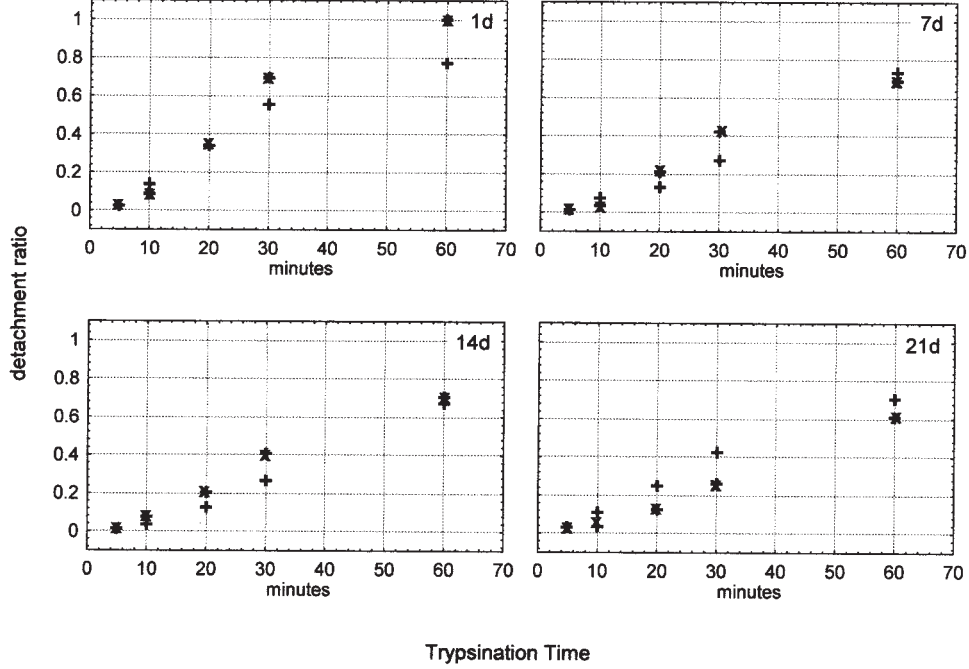


Figure 4. Plot of the experimental ratio (+) and the modeled ratio (*) of detached cells $p(t_i, d_j)$ with $t_d(t) = 37.5t^{0.5}$ for cells cultured on uncoated electro-eroded titanium alloy with high roughness amplitude (TE1N).

seen in the repetition error, meaning that for a given sample, a same time in culture and a same trypsination time, the ratio of detached cells possesses an intrinsic error that corresponds to the real experimental error.

Before analyzing error modeling, we emphasize an important fact. Our adhesion model given by Eq. (4) is only described by one coefficient called the adhesion power. To calculate it, we used 20 experimental data. As a consequence, our model is highly robust in a statistical sense. If we would have retained a model with more than one coefficient, it is sure that the error modeling would have decreased [e.g., the minimal value given by Eq. (3) with two coefficients would have been lower than the minimal value given by Eq. (4) with one coefficient]. However, the coefficients would have lost drastically their physical meaning when they would have been estimated from experimental data.

We now estimate modeling error. In a first time, we have to estimate the intrinsic experimental error (error of type 5). For a given sample, all experiments are repeated three times to obtain three experimental values of $p_n(t_i, d_j)$ with $n = \{1, 2, 3\}$ for a given trypsination time d_j and time in culture t_i . To estimate the error in the detachment of the k^{th} experiments, we use the unbiased standard deviation in the $p_n(t_i, d_j)$ measurements noted $\sigma_{k,i,j}^e$ with

$$\sigma_{k,i,j}^e = \sqrt{\frac{1}{2} \sum_{n=1}^3 (p_n(t_i, d_j) - \bar{p}(t_i, d_j))^2} \quad (5)$$

The empirical distribution of 2087 measures of $\sigma_{k,i,j}^e$ is represented in Figure 5. We obtain the mean error value of $\bar{\sigma}^e = 0.051$ which represents our experimental error. Then, we calculate the error modeling noted $\sigma_{k,i,j}^m$ with:

$$\sigma_{k,i,j}^m = \sqrt{\frac{1}{2} \sum_{n=1}^3 \left(p_n(t_i, d_j) - \frac{1}{P_n(t_i) - P_n(0)} \tau \int_0^{t_i} (\partial P_n(\tau) / \partial \tau) H(d_j - a_n \sqrt{(t_i - \tau)}) d\tau \right)^2} \quad (6)$$

where a_n is calculated from Eq. (4) with the 20 detachment measures of the n^{th} study.

Note that statistically, we must always have $\sigma_{k,i,j}^e \leq \sigma_{k,i,j}^m$. The empirical distribution of 2087 measures of $\sigma_{k,i,j}^m$ is also represented in Figure 5, and we obtain $\bar{\sigma}^m = 0.065$. Figure 5 shows that intrinsic experimental

error and modeling error distribution are quite similar, indicating that our monocoefficient model is well adapted to describe adhesion (whatever the substrate nature) and that proliferation is also well modeled. All coefficients a shown in Table III are statistically non null and characterize well the dependence between

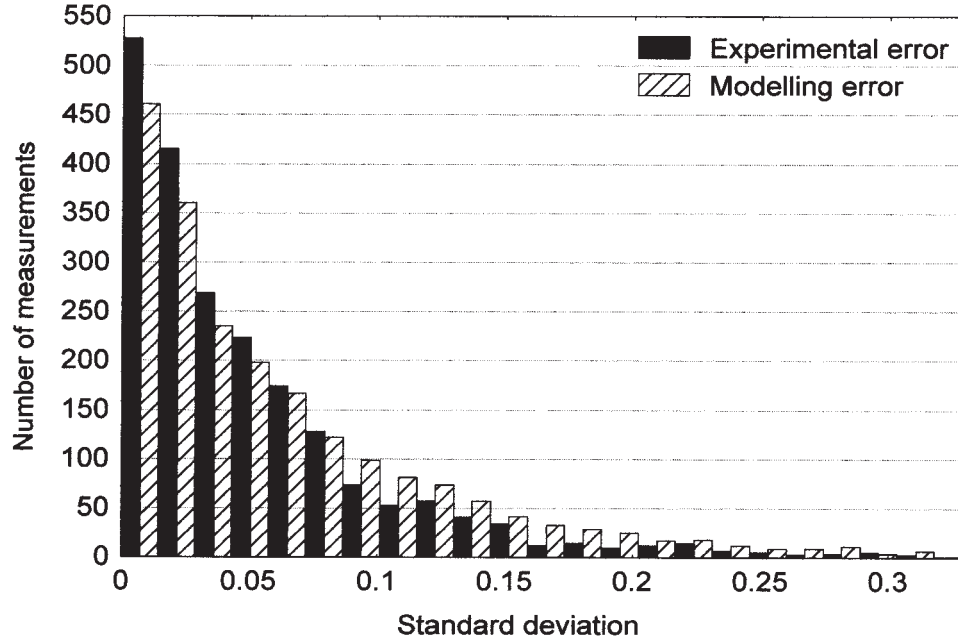


Figure 5. Empirical probability density function of error in adhesion measurement [$\sigma_{k,i,j}^e$ in Eq. (5)] and modeling error [$\sigma_{k,i,j}^m$ in Eq. (6)] of adhesion model [Eq. (1)] where solution is determined by solving Eq. (3).

time in culture and adhesion, proving that $t_d(t) = at^{0.5}$ is statistically validated, meaning that the adhesion power a is an appropriate parameter to characterize adhesion.

The adhesion power as a new adhesion index

With use of a bootstrap technique,¹⁶ the mean histograms of the adhesion power of all samples were established. Three sampled histograms are plotted in Figure 6. As shown in this figure, our original adhesion parameter helps to discriminate the adhesion on these three surfaces well. Detachment slowed down with culture time and was slower on etched (TAN), electro-eroded (TE0N, TE1N), and sand-blasted surfaces (TS1N) than on ma-

chine-tooled surfaces (TU0N, TUXN) or on polished surfaces (TPN) (Table III). Table III shows a relation between adhesion and the order of the surface: adhesion on disordered surfaces (TE0N, adhesion power = 28.1; order = 13.9) is fourth higher than on an ordered one (TU0N, adhesion power = 7.4; order = 49). The highest adhesion was obtained on isotropic rough surfaces (electro-eroded, acid-etched, and sand-blasted surfaces) but with a much higher value of adhesion power for TE0N surfaces (Table III). A more complete study of the pertinence of the adhesion power for discriminating the long-term adhesion of human osteoblasts in function of sur-

TABLE III
Values of Roughness Parameter and Adhesion Power for Titanium Substrate

Sample	Sa (μm)	Sk (μm)	St (μm)	Lac (μm)	Order (%)	Adhesion Power a
TU0N	0.79	0.676	5.28	17.9	49.0	7.4
TPN	0.53	0.362	4.56	21.1	27.4	8.5
TUXN	0.75	0.437	4.00	27.2	43.7	9.6
TS1N	2.29	0.022	16.53	18.4	21.2	12.5
TE1N	2.52	0.392	16.06	11.3	15.9	13.2
TAN	0.71	0.710	5.33	10.9	15.7	14.6
TE0N	0.76	-0.001	6.25	9.2	13.9	28.1

Sa = mean roughness amplitude; Sk = skewness of the roughness amplitude; St = range of the roughness amplitude; Lac = autocorrelation length; and Order = order parameter.

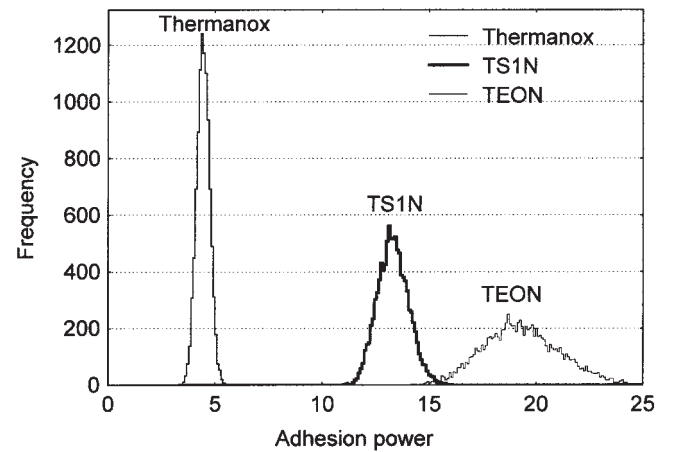


Figure 6. Bootstrapped probability density function of the Adhesion power coefficient characterising the adhesion on three substrates: ThermanoxTM, sand-blasted titanium substrate (TS1N), and titanium electro-eroded substrate (TE0N).

face characteristics of substrates has been published elsewhere.¹⁴

DISCUSSION

The term “adhesion” in biomaterials covers different phenomena, such as the short-term attachment phase, which occurs in the first seconds or minutes of contact, and the adhesion phase occurring in the longer term from minutes to hours. The attachment phase involves physicochemical linkages formed by ionic forces, Van der Waals forces, and so forth between cells and materials. The adhesion phase involves various biological molecules such as cell membrane proteins and cytoskeleton proteins, which interact together to induce signal transduction, promoting the action of transcription factors and, consequently, regulating gene expression; the primary family of cell membrane proteins, mediating the adhesion of cells to substrate being the integrins.^{1,9} In the very long term (several days), the cell-material interactions involve in addition the extracellular matrix proteins synthesized by the cells at the interface with the material.¹ We have proposed the term “long-term adhesion” to describe the strength of the cell-material interface after some hours to several weeks of culture, our aim being to consider an interface involving all the adhesion proteins including extracellular matrix proteins. Consequently, for several years, we have developed an *in vitro* long-term culture model allowing the expression by human osteoblasts all along the culture time of adhesion proteins associated with the focal contacts, such as vinculin and integrin ($\alpha 3$ and $\beta 1$ subunits), with cytoskeleton-like actin fibers and with extracellular matrix proteins, such as fibronectin, osteopontin, and type I collagen. All these proteins have been shown to display an organization reflecting the underlying surface topography.¹⁰

In the present work, we studied the long-term adhesion of human osteoblasts on metallic substrates presenting 26 different surface topographies and chemistries by using our original method of cell detachment based on an enzymatic digestion of the cell-matrix-material interface. Our aim was to determine the kinetic law governing the long-term adhesion on biomaterials in function of culture time t from the first hours of interactions to several weeks of contact.

We demonstrated that $t_d(t) \propto \sqrt{t}$ with t_d , the time of digestion with trypsin. That involves an important feature: the long-term cellular adhesion on biomaterials varies with the square root of culture time, meaning that whatever the nature of the material, the surface topography or the surface chemistry, four times are necessary to double adhesion.

As was proved by the diffusion theory, it is well

known that the front of the diffusion process varies with \sqrt{t} if no external forces are present during the migration of the particles (no convection, no electrostatic potential, etc.).¹⁷ Moreover, it is proved that in the stochastic process of diffusion, the density probability function of the $b = 0.5$ coefficient follows a Gaussian distribution as we also have observed. As a consequence, we could assume that the process involved in cells’ long-term adhesion is assimilated to a pure diffusion process. Hence, a diffusive physical or biological phenomenon, not influenced by the substrates’ features, should occur in the long-term adhesion of cells. A crucial point is that the temporal evolution of this diffusive phenomenon is at the scale of days and not of hours or minutes like the diffusive phenomenon previously described in cell adhesion.⁷

Is there a diffusive biological phenomenon underlying the long-term adhesion?

Because the long-term cellular adhesion involves several cell compartments and different proteins, we consider each of them with the aim to determine if a diffusion-based phenomenon would be involved in their function. Different biological phenomena involve the diffusion of molecules in cellular compartments such as the intracellular, the intra-membranous, or the extracellular one. Newly synthesized molecules diffuse from the internal cytoplasmic organites, such as Golgi complex, to fuse with the cell membrane.^{18,19} Membranous receptors, such as integrins inside the membrane, are able to diffuse inside the membrane to crowd in a focal contact during the adhesion phase.^{7,3,20,21} The extracellular matrix proteins are excreted and diffuse across the membrane before being organized in a three-dimensional matrix at the cell-material interface.

Concerning the intracellular phenomenon, the time-scale of the secretory protein traffic is of another size order than the culture time concerned in the present work because the constitutive secretion, the process that delivers newly synthesized proteins to the plasma membrane and external media, requires less than some hours, and this transport is governed by a first-order rate law.¹⁸ Moreover, the exocytosis of these newly synthesized proteins occurs in only a few seconds.¹⁹

The cell membrane is considered as a bilayered mosaic of lipidic domains and membrane proteins displaying a dynamic structure with permanent rearrangement due to the fluidity of the membrane and the translational diffusion of proteins among the lipidic domains.²² The membranous proteins interact with cytoskeletal or other cytosolic components or with ligands. These interactions are at the origin of the

clustering of receptors in the membrane observed, for example, during adhesion.^{7,3,20,21} This aggregation process has been shown to follow a \sqrt{t} regimen at small ligand concentration, whereas at high concentration a linear law was found.²¹ Brochard-Wyart and De Gennes in their model of cell adhesion have demonstrated that the diffusion of integrin receptors inside the cell membrane to join the focal contacts followed a square root law, whereas the growth of the patch and the accumulation of binders followed a linear law.⁷ However, again, these aggregation processes and clustering of integrin receptors occur in a short term (some hours). As a consequence, it appears unlikely that these processes could explain the evolution with the square root of culture time of the long-term adhesion that we observed.

More probably, these evolutions of adhesion along days have to be related to the evolution of the quality of the cell-material interface and to the synthesis and maturation of the 3D-matrix in contact with the material surface. Geiger stresses the differences in the adhesion matrix formed *in vivo* or in culture, which have a unique combination of topography, molecular composition, and mechanical properties. Therefore, focal adhesions and fibrillar adhesions, which are met *in vitro*, are associated, respectively, with a rigid substrate of limited molecular heterogeneity or with a matrix of soft, partly 3D, fibronectin fibrils. In contrast, 3D-matrix adhesions, which are met *in vivo*, form with a matrix that is 3D, moderately pliable, and complex in its molecular composition.²³ We observed in our culture model the formation of an important extracellular matrix mainly composed by fibronectin fibrils and the presence of $\beta 1$ integrin subunit-positive thin filaments,¹⁰ which could be fibrillar adhesions because Cukierman et al.²⁴ describe fibrillar adhesions as composed prominently of $\alpha 5 \beta 1$ integrin and tensin. Hence, we could hypothesize that the evolution with the square root of time of the adhesion strength we observed would be linked to a progressive change over the culture time from focal adhesions to fibrillar adhesions; this hypothesis involving the fibrillar adhesion would be stronger than focal adhesion and less sensitive to the trypsin action. The trypsin has been shown by Rees et al. to detach cells dissociating “focal grip,” which is the consequence of the holding of macromolecules (glycoproteins, cytoskeleton proteins) in the appropriate organized configuration at areas of contact with substrate. The dissociation by trypsin of glycoproteins would lead to the progressive fragmentation and the eventual disappearance of microtubules and microfilaments close to the underside of the cell membrane prior to cell detachment.²⁵

However, because the relative adhesion strengths of focal and fibrillar adhesions are not known yet, it is impossible to conclude now. Eventually, we need to be careful with these interpretations because the ex-

periments done by Cukierman et al. concern fibroblasts, whereas we used osteoblasts. Indeed, these two types of cells are known to synthesize different extracellular matrix proteins and to express different integrin subunits.¹ Further studies will be needed to check in our culture model the formation kinetics of focal and fibrillar adhesions.

Few articles have tackled the kinetics of the *in vitro* formation of the extracellular matrix by bone-derived cells in a culture model. Gerstenfeld et al.²⁶ studied extensively the synthesis of collagen in cultures of chicken embryo osteoblasts during periods of time consistent with our study (i.e., over a 30-day period). By biochemical and ultrastructural analysis they showed the formation in 30 days of about 15- μm -thick extracellular matrix under the cell layer. Human osteoblast-like cells have also been shown to be able to synthesize a 30- μm -thick extracellular matrix after a 20-day culture, which could reach 80 μm in thickness after treatment with 10^{-11} M estradiol.²⁷ In our experiment, the morphological analysis by scanning electron microscopy of human osteoblasts after 14 or 21 days of culture on metallic substrates has always shown the presence of a layer of fibrils under the cell layer.^{10,12} Consequently, it appears that the evolution of adhesion following the square root law could be in fact due to an evolution of the extracellular matrix following a square root law, which could concern either the kinetics of synthesis or the kinetics of maturation of the ECM. However, to date, we have failed so far to find experimental and model studies concerning the kinetics of the *in vitro* formation of an extracellular matrix by cultured cells.

Is there a diffusive physical phenomenon underlying the long-term adhesion?

Our detachment method is based on an enzymatic detachment based on trypsin. Trypsin is a proteolytic enzyme member of the serine protease family that cleaves peptides on the C-terminal side of lysine and arginine amino acid residues. Trypsin is classically used to detach cells from culture vessels but can also be used for dissociating cells from primary tissues such as skin or for decellularization of heart valves.²⁸ Two hypotheses can be proposed to link the action of trypsin with the kinetics of detachment we obtained. First, the detachment we obtain would be linked to the kinetics of diffusion of the trypsin at the cell-material interface, which can be more and more difficult with culture time from a mechanical point of view. In fact, the presence of a more and more dense interface composed of ECM proteins could make it even harder. However, this is unlikely because the trypsin is able to penetrate in the heart of cardiac valves and to com-

pletely digest all the cells.²⁸ Second, the action of trypsin could be more and more inhibited with culture time from a biochemical point of view again because of the presence of the ECM proteins. Indeed, it is well known that the action of the trypsin is inhibited by the presence of proteins because the classical method to stop its action consists in adding a volume of serum or medium containing serum to the cell suspension detached by trypsin. Thus, the action of the diluted trypsin used to detach cells in our model could be inhibited by the increasing quantity of ECM proteins present at the cell-material interface. Again, in this case, the square root law we obtained could be linked to the increase in ECM quantity at the cell-material interface with kinetics following a square root law.

Moreover, we recently developed a new method of detachment based on ultrasounds. The curves corresponding to the number of cells detached by ultrasounds with culture time were very close to those obtained with enzymatic detachment (unpublished results). This result would confirm that the diffusion phenomenon we are searching would not be a physical phenomenon linked to trypsin because the same results are obtained with another detachment method. Hence, the evolution with the square root of time of the long-term adhesion would rather be linked to the kinetics of formation of the extracellular matrix. Further kinetic studies of synthesis and maturation of the ECM in our culture model will thus be needed especially to determine which law it follows.

CONCLUSIONS

For the first time, we have reported here on the mathematical modeling of long-term cell adhesion on biomaterials. An adhesion index t_d is proposed, which varies with culture time according to a power law: $t_d(t) = at^b$; a is independent of b . The exponent b is equal to 0.5 ± 0.03 and is independent of the substrate's characteristics. On the contrary, the parameter a significantly depends on the material's nature, the surface topography, and the surface chemistry of the substrate. From this relationship, we suggest that a diffusion-based process should be involved in long-term adhesion on materials. More probably, the kinetics of adhesion following a square root law and evolving with days in culture could be related to the presence of the ECM. 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.

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References

1. Anselme K. Osteoblast adhesion on biomaterials. *Biomaterials* 2000;21:667–681.
2. Pierres A, Benoliel AM, Bongrand P. Cell fitting to adhesive surfaces: a prerequisite to firm attachment and subsequent events. *Eur Cells Mater* 2002;3:31–45.
3. Garcia AJ, Gallant ND. Stick and Grip: measurement systems and quantitative analyses of integrin-mediated cell adhesion strength. *Cell Biochem Biophys* 2003;39:61–73.
4. Murphy-Ullrich JE. The de-adhesive activity of matricellular proteins: is intermediate cell adhesion an adaptative state? *J Clin Invest* 2001;107:785–790.
5. Bell GI, Dembo M, Bongrand P. Cell adhesion: competition between nonspecific repulsion and specific bonding. *Biophys J* 1984;45:1051–1064.
6. Evans EA. Detailed mechanics of membrane-membrane adhesion and separation. I. Continuum of molecular cross-bridges. *Biophys J* 1985;48:175–183.
7. Brochard-Wyart F, De Gennes PG. Adhesion induced by mobile binders: Dynamics. *Proc Natl Acad Sci USA* 2002;99:7854–7859.
8. Zhu C. Kinetics and mechanics of cell adhesion. *J Biomech* 2000;33:23–33.
9. Siebers MC, Ter Brugge PJ, Walboomers XF, Jansen JA. Integrins as linker proteins between osteoblasts and bone replacing materials. A critical review. *Biomaterials* 2005;26:137–146.
10. Anselme K, Bigerelle M, Noel B, Dufresne E, Judas D, Iost A, Hardouin P. Qualitative and quantitative study of human osteoblast adhesion on materials with various surface roughness. *J Biomed Mater Res* 2000;49:155–166.
11. Anselme K, Linez P, Bigerelle M, Le Maguer D, Le Maguer A, Hardouin P, Hildebrand HF, Iost A, Leroy J-M. The relative influence of the topography and chemistry of Ti6Al4V surfaces on osteoblastic cell behaviour. *Biomaterials* 2000;21:1567–1577.
12. Bigerelle M, Anselme K, Noël B, Ruderman I, Hardouin P, Iost A. Improvement in the morphology of surfaces for cell adhesion: a new process to double human osteoblast adhesion on Ti-based substrates. *Biomaterials* 2002;23:1563–1577.
13. Anselme K, Bigerelle M, Noël B, Iost A, Hardouin P. Effect of grooved titanium substratum on human osteoblastic cell growth. *J Biomed Mater Res* 2002;60:529–540.
14. Anselme K, Bigerelle M. Topography effects of pure titanium substrates on human osteoblast long-term adhesion. *Acta Biomaterialia* 2005;1:211–222.
15. Bigerelle M, Anselme K. Statistical correlation between cell adhesion and proliferation on biocompatible materials. *J Biomed Mater Res* 2005;72:36–46.
16. Bigerelle M, Najjar D, Iost A. Relevance of roughness parameters for description and modelling of machined surfaces. *J Mater Sci* 2003;38:2525–2536.
17. Shull KR, Kellock AJ. Metal particle adsorption and diffusion in a model polymer/metal composite system. *J Polym Sci Part B Polym Phys* 1995;33:1417–1422.
18. Hirschberg K, Miller CM, Ellenberg J, Presley JF, Siggia ED, Phair RD, Lippincott-Schwartz J. Kinetic analysis of secretory protein traffic and characterization of golgi to plasma membrane transport intermediates in living cells. *J Cell Biol* 1998; 143:1485–1503.
19. Schmoranzler J, Goulian M, Axelrod D, Simon SM. Imaging constitutive exocytosis with total internal reflection fluorescence microscopy. *J Cell Biol* 2000;149:23–31.

20. Miyamoto S, Akiyama SK, Yamada KM. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science* 1995;267:883–885.
21. Boulbitch AA, Guttenberg Z, Sackmann E. Kinetics of membrane adhesion is mediated by ligand-receptor interaction studied with a biomimetic system. *Biophys J* 2001;81:2743–2751.
22. Vereb G, Szöllösi J, Matko J, Nagy P, Farkas T, Vigh L, Matyus L, Waldmann TA, Damjanovich S. Dynamics, yet structured: the cell membrane three decades after the Singer-Nicolson model. *Proc Natl Acad Sci USA* 2003;100:8053–8058.
23. Geiger B. Encounters in space. *Science* 2001;294:1661–1663.
24. Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. *Science* 2001;294:1708–1712.
25. Rees DA, Lloyd CW, Thom D. Control of grip and stick in cell adhesion through lateral relationships of membrane glycoproteins. *Nature* 1977;267:124–128.
26. Gerstenfeld LC, Riva A, Hodgens K, Eyre DR, Landis WJ. Post-translational control of collagen fibrillogenesis in mineralizing cultures of chick osteoblasts. *J Bone Miner Res* 1993;8:1031–1043.
27. Slater M, Patava J, Mason RS. Thrombospondin co-localises with TGF β and IGF-I in the extracellular matrix of human osteoblast-like cells and is modulated by 17 β estradiol. *Experientia* 1995;51:235–244.
28. Schenke-Layland K, Vasilevski O, Opitz F, König K, Riemann I, Halhuber KJ, Wahlers T, Stock UA. Impact of decellularization of xenogeneic tissue on extracellular matrix integrity for tissue engineering of heart valves. *J Struct Biol* 2003;143:201–208.