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Statistical demonstration of the relative effect of surface chemistry and roughness on human osteoblast short-term adhesion

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Abstract The effects of material composition, surface chemistry or surface topography on cell attachment (short-term adhesion) have been largely studied on bone-derived cells. However, no statistical demonstration of these effects has been performed until now. With this objective, we quantified the attachment after 24 hours of human osteoblasts on pure titanium, titanium alloy and stainless steel substrates presenting 6 different surface morphologies and 2 different roughness amplitude obtained by sand-blasting, electro-erosion, acid etching, polishing and machine-tooling. The coating by a gold-palladium layer of these surfaces allowed determining the relative effect of the surface roughness and of the surface chemistry. By multiple analysis of variance, we demonstrated that neither material composition nor surface roughness amplitude influenced cell attachment except on sandblasted pure titanium substrates. On the contrary, a high significant influence of the process used to produce the surface was observed meaning that the main influent factor on cell attachment could be either the surface morphology or the surface chemistry induced by the process. As the coating of surfaces by a gold-palladium layer decreased significantly the attachment of cells on the majority of substrates, we concluded that attachment is rather influenced by surface chemistry than by surface topography.

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1. Introduction

The effects on cell attachment of materials composition [1–3], as well as the effects of surface chemistry [4–6] or surface topography [7–11] have been largely studied on bone-derived cells. The material composition always influences cell attachment [1–3] whereas variations of surface chemistry of titanium-based substrates following surface treatments like anodization have generally little influence on osteoblasts attachment capacity [5, 6]. Likewise, Ahmad *et al*. described a non significant difference of osteoblastic cell attachment between Grade 1 and Grade 4 pure titanium [4]. On the contrary, the surface roughness of titanium substrates is known to have a considerable effect on osteoblastic cell adhesion as well as on cell proliferation and differentiation [1, 7, 10, 12–17]. Attachment is generally increased on rough surfaces (Sa $> 1\mu$m) produced for example by sandblasting compared to smooth ones [7–9, 18–20] but sometimes no effects are described [11, 21]. In order to bring more definitive response on the effect of topography on attachment we will proceed to an attachment study of human osteoblasts after 1 day of culture on 30 different substrates made of pure titanium, titanium alloy or stainless steel, presenting 5 different surface morphologies and 2 different roughness amplitudes obtained by sand-blasting, electro-erosion, acid etching, polishing and machine-tooling, and recovered or not by a sputtered gold-palladium coating. Each of the 30 different substrates will be tested at

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

		Sa (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)	Parallel grooves	0.7		TU0N (12)				
	Cross grooves	0.7		TUXN (12)				
Acid-etching (A)		0.7	TAO (9)	TAN (18)				
Thermanox®		–			Thermanox® (33)			

least 9-fold to obtain a large number of data for statistical analysis.

2. Materials and methods

2.1. Surface preparation

Ti6Al4V titanium alloy (V), pure titanium (T) or stainless steel samples (I) were treated using different techniques to obtain various surface morphologies. The three different materials were electro-eroded or sandblasted under two different conditions to produce surfaces with two different roughness amplitudes (respectively Sa = 0.85 μm and Sa = 2.35 μm). Ti samples were also polished, acid-etched or machine-tooled under different conditions to produce parallel or cross grooves with a Sa of 0.7 μm. The half of the samples were coated by a layer of gold-palladium. The cell culture treated polystyrene Thermanox® was used systematically as a smooth control in each experiment with a Sa being near 0 (Sa = 42 nm). Finally 30 different substrates were studied (Table 1).

2.1.1. Machine-tooling

Pure titanium Ti40 bars (12 mm in diameter) were machine-tooled using a numeric lathe Cazeneuve HB725 (Groupe CaTo, Pont-Eveque, France) to obtain samples measuring 2 mm in thickness. The conditions for machine-tooling were established to obtain either parallel grooves (200 μm in width and 14.5 μm depth) (TU0N) or cross grooves forming kind of valleys (200 μm in width and 5.5 μm in depth) (TUXN) doing respectively one passing or two perpendicular passing.

2.1.2. Electro-erosion process

We used a specific wire cutting machine to process the three materials (AGIECUT, Premier Equipment, Altamonte Springs, FL, USA). Two process conditions were used to obtain two different roughness amplitudes (Sa = 0.85 μm and Sa = 2.35 μm). The first ones were cut at 3 A and then the tooled face was electro-eroded twice at 0.25 A (VE1N, TE1N, IE1N). The second ones were cut at 3 A and then the tooled face was electro-eroded twice more at decreasing powers (1 A and 0.25 A) (VE0N, TE0N, IE0N).

2.1.3. Polishing

Using a Pedemax 2 automatic polishing machine (Struers S.A.S, Champigny sur Marne, France) the three materials were polished using grade 40 silicon carbide paper (VPN, TPN, IPN).

2.1.4. Acid-etching

To eliminate any risk of contamination by machine-tooling residues, pure titanium samples were extensively polished using grade 220, 320, 500, 1000 and 4000 before being treated during 300 seconds with 10% hydrofluoric acid at room temperature (TAN).

2.1.5. Sandblasting

To eliminate any risk of contamination by machine-tooling residues, all the materials were extensively polished using grade 220, 320, 500, 1000 and 4000 before being sandblasted using silicon carbide particles measuring 120 and 400 μm in diameter giving respectively the samples VS0N, TS0N, IS0N and VS1N, TS1N, IS1N.

2.1.6. Coating

In order to isolate the effect of surface roughness from that of surface chemistry, we covered the half of the IE0N, IE1N, IPN, IS1N, TAN, TE0N, TE1N, TS1N, VE0N, VE1N, VPN samples by sputter-coating with gold-palladium using an Emscope SC 500 (Elexience, Paris, France) for scanning electron microscopy preparation. These samples became respectively IE0O, IE1O, IPO, IS1O, TAO, TE0O, TE1O, TS1O, VE0O, VE1O, VPO.

2.2. Roughness measurement

Roughness was measured using a tactile profilometer (Tencor P-10, KLA Tencor, USA) on a surface of 1mm $\times$ 1mm with one measure each two micrometers on horizontal and vertical scanning. Three-dimensional profiles were drawn and the classical roughness amplitude Sa parameter among many others was calculated from these profiles.

2.3. Cell culture

Human osteoblasts were obtained from trabecular bone taken from the iliac crest of young patients as previously described [22]. Cells were initially cultured in Dulbecco Modified Essential Medium (DMEM, Eurobio, France) containing 10% foetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, until confluence and were then preserved in liquid nitrogen in complete DMEM + 10% dimethylsulfoxyde (Sigma, L'Isle d'Abeau, France) in order to test all the substrates with an homogenous cell line. Before experiments, the cells were thawed and cultured in 75 cm^2 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.

2.4. Short-term adhesion measurement

Samples were inoculated with 4×10^4 cells/sample. The experiments were reproduced at least three fold. In each experiment, three samples were analysed. After 24 hours, the cells were enzymatically detached from the samples using trypsin-EDTA (0.25% v/v) and counted using a Coulter Z1 (Beckman Coulter, Roissy, France).

2.5. Statistical analysis

A multiple analysis of variance (ANOVA) was performed using SAS© software (SAS Institute, Cary, NC) in order to specifically analyse the relative effects of 4 parameters on human osteoblast attachment: the material nature (material), the process used to produce the surface topography (process), the roughness amplitude (roughness), and finally the gold-palladium coating (coating). Alternatively, Duncan's Multiple Range Test will be performed. These tests allow comparing the means of attached cells after 24 hours on the 30 different surfaces. One letter (A to H) is attributed to each group: means with the same letter being not significantly different.

3. Results

3.1. Effect of roughness

Experimentally, we did not observe any difference between the number of cells attached after 24 hours on surfaces with low roughness amplitude and with high roughness amplitude. In order to demonstrate that initial attachment did not depend on the roughness amplitude, we performed multivariate analysis of variance. We demonstrated discrepancies between substrates. Contrary to other substrates, the attachment of cells on TS0N and TS1N substrates was significantly influenced by the roughness amplitude (27 experiments, $F = 6$, $p = 0.02$). By taking only the other values (IE0N, IE0O, IE1N, IE1O, TE0N, TE1N, VE0N, VE0O, VE1N, VE1O, VS0N, VS1N) that represent 149 experiments, it was shown that roughness amplitude did not play a role on attachment ($F = 2.34$, $p = 0.1280$).

Morphologically the cells were more spread and displayed very intimate contact with the surface on substrates with lower roughness amplitude (TU0N, TUXN, TPN, TAN, TE0N and TS0N) than on rougher surfaces (TE1N, TS1N). They appeared to produce fewer extensions on anisotropic surfaces (TUON, TUXN, TPN) than on the isotropic ones (TAN, TE0N, TS0N TE1N, TS1N) (Fig. 1).

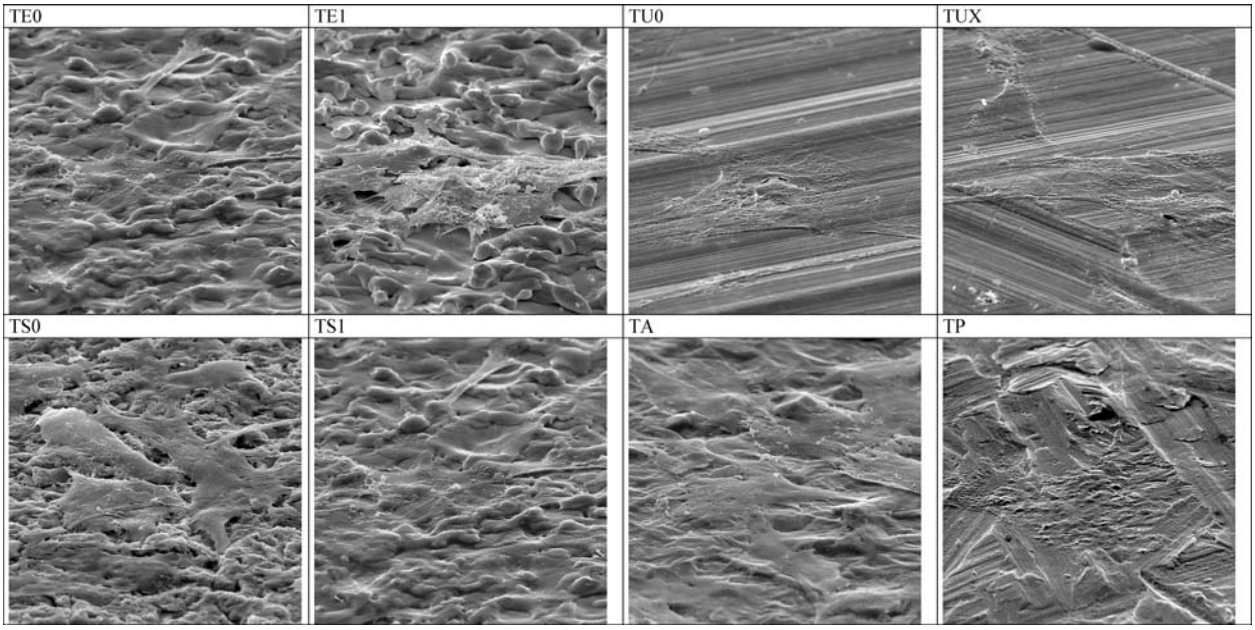

Fig. 1 Scanning electron micrographs of cells after 24 hours of culture on pure titanium substrates with the 8 different surface morphologies tested. TE0: low roughness electro-eroded surface, TE1: high roughness electro-eroded surface, TS0: low roughness sandblasted surface, TS1: high roughness sandblasted surface, TU0: parallel grooved machine-tooled surface, TU0: cross-grooved machine-tooled surface, TP: polished surface, TA: acid-etched surface. The cells were more spread and displayed very intimate contact with substrates with lower roughness amplitude (TU0, TUX, TP, TA, TE0 and TS0) than on rougher ones (TE1, TS1). They appeared to produce less extensions on anisotropic surfaces (TUO, TUX, TP) than on the isotropic ones (TA, TE0, TS0, TE1, TS1). Tilt 90°.

3.2. Effect of material and process

The multivariate analysis of variance in function of process and material demonstrated that materials composition itself did not influence the number of attached cells ($F = 1.07$, $p = 0.34$) (Table 2). On the contrary, the process used to create the surface displayed a high influence on attachment ($F = 14.47$, $p < 0.0001$) (Table 2). Duncan grouping discriminated three classes of process (Table 3): Machine-tooling > Sandblasting = Polishing > Acid-etching = Electro-erosion. Interaction of materials and process was significant with a F value of 5.37 and $p = 0.0004$. This interaction appeared to be linked to the pure titanium sandblasted surfaces which induces a different cellular attachment compared to the 316 L stainless steel or Ti6Al4V titanium alloy sandblasted substrates (Fig. 2). When only Ti6Al4V and 316 L substrates were considered, the interaction of material composition and process did not more exist ($F = 1.59$, $p = 0.2$).

Table 2 Multivariate analysis of variance (ANOVA) of the number of attached cells in function of the material composition (material) and of the process used to produce surface roughness (process). Source: parameter, DF: degree of freedom, Type I SS: Sum of Squares, F value: Fisher Variate, Pr > F: Probability to obtain a F greater than experimental one under the null hypothesis

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Process	4	4335116169	1083779042	14.47	<.0001
Material	2	160626746	80313373	1.07	0.3438
Process*Material	4	1608570669	402142667	5.37	0.0004

Table 3 Duncan grouping in function of process

Duncan Grouping	Mean	N	Process
A	25282	24	Machine-Tooling
B	20322	72	Sandblasting
B	17372	54	Polishing
C	12947	18	Acid-Etching
C	12929	96	Electro-erosion

3.3. Effect of coating

Figure 3 demonstrated that the initial number of attached cells was less important on the gold-palladium recovered surfaces than on the uncoated ones except for IP0 and IS1O where no difference could be statistically detected. To analyse more precisely these results we did proceed to a Duncan analysis of all the 30 substrates that allowed us to distinguish 9 groups. A highly significant result was obtained from this analysis: almost all of the coated surfaces were in the group I (except IS1O and IPO) and thus displayed the lower number of attached cells after 24 hours of culture

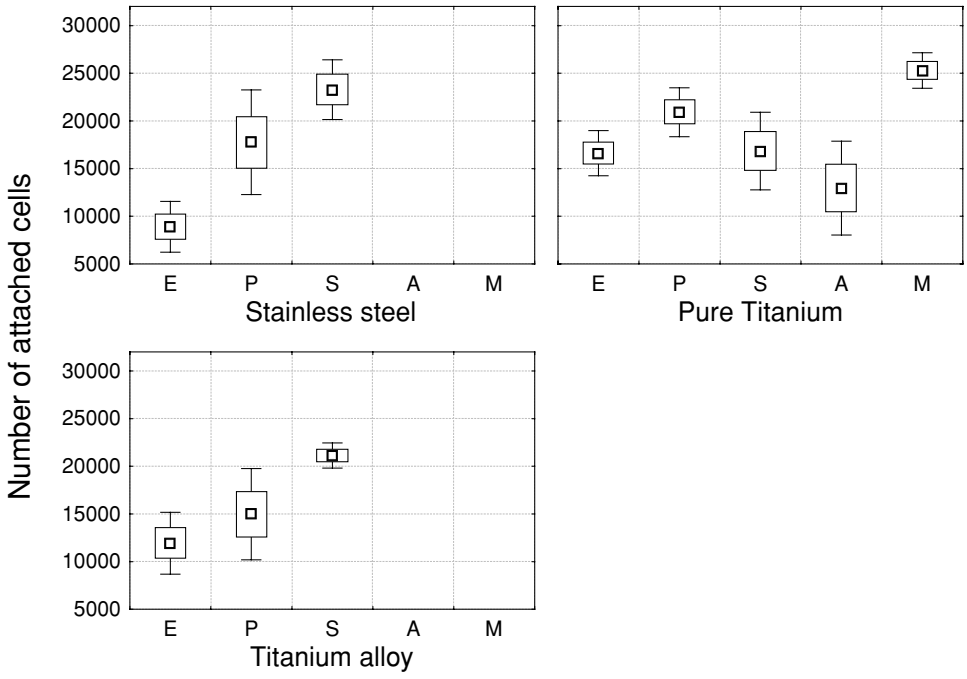

Fig. 2 Mean number (open square) of attached cells on each type of substrates in function of the material and of the process. Open rectangle: Mean value, bars: 95 % confidence interval of the mean.

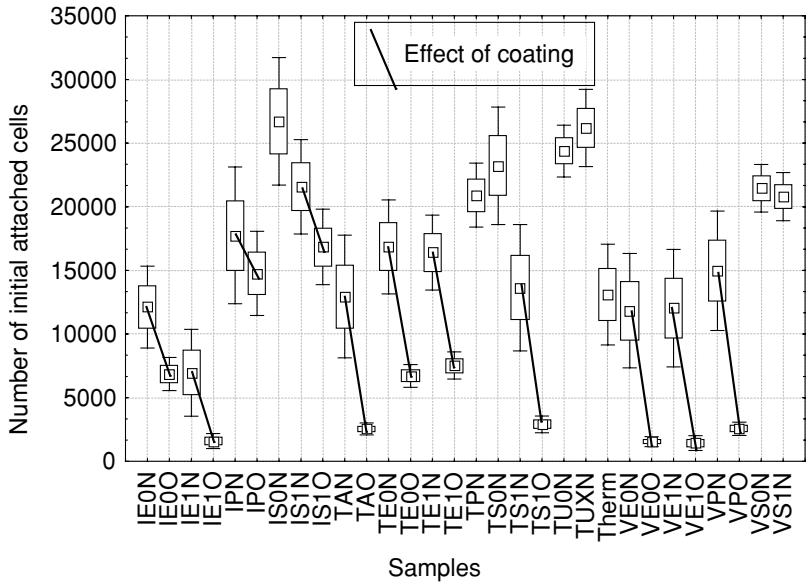

Fig. 3 Mean number (open square) of attached cells on all substrates. Open rectangle: Mean value, bars: 95 % confidence interval of the mean.

(Table 4). The morphological observations of cells on substrates before and after coating confirmed the lower number of cells after coating. Moreover, the cells appeared round and non spread on electro-eroded titanium-based substrates with rough amplitude after coating (VE1O, TE1O) and on all the stainless steel electro-eroded surfaces after coating (IE0O, IE1O). On the non electro-eroded substrates, the cells were less numerous (TS1O, IS1O) or less spread (TAO) after coating except on polished stainless steel substrates (IPO) where the number of cells was higher after coating (Fig. 4).

4. Discussion

Our study asserts with an important number of experiments (374) that the short-term adhesion depends on the surface chemistry of the substrate since the covering of the substrates by a gold-palladium layer impede in the most of cases the cell attachment and more specifically the cell spreading. It is known that attachment is principally governed during its first flattening phase by non specific electrostatic forces like Van Der Waals ones and by passive formation of ligand-receptor bonds [23]. This phase achieved in a fraction of a second is

Table 4 Duncan grouping of all 30 samples. Italic characters: gold-palladium coated samples

Duncan grouping					Mean	N	MAT
			A		26714	9	ISON
			A		26189	12	TUXN
	B		A		24374	12	TUON
	B		A	C	23215	9	TSON
	B	D	A	C	21562	18	IS1N
	B	D	A	C	21452	9	VSON
E	B	D	A	C	20914	12	TPN
E	B	D	A	C	20792	9	VS1N
E	B	D	F	C	17757	21	IPN
E	*B*	*D*	*F*	*C*	*16841*	*9*	*IS1O*
E	B	D	F	C	16839	18	TE0N
E		D	F	C	16394	18	TE1N
E	G	D	F	C	14963	21	VPN
E	*G*	*D*	*F*		*14763*	*9*	*IPO*
E	G		F	H	13640	18	TS1N
	G		F	H	13097	33	Thermanox
	G		F	H	12947	18	TAN
	G		F	H	12116	9	IE0N
	G		F	H	12031	18	VE1N
	G		F	H	11837	18	VE0N
	G		*I*	*H*	*7525*	*9*	*TE1O*
			I	H	6952	15	IE1N
			I	*H*	*6859*	*9*	*IE0O*
			I	*H*	*6706*	*9*	*TE0O*
			I		*2741*	*8*	*TS1O*
			I		*2552*	*9*	*VPO*
			I		*2543*	*9*	*TAO*
			I		*1586*	*9*	*IE1O*
			I		*1542*	*9*	*VE0O*
			I		*1424*	*9*	*VE1O*

followed during the next ten minutes by an alignment phenomenon resulting in the widening of contact area which may take place without active cell participation. Later, the spreading phase going on during the further hours involves active cell reorganization and is much more dependent on cell metabolism [23]. It is during this phase that 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 Pierres et al. as forming the fitting phase, the prerequisite to firm attachment and subsequent events [23]. Finally, it appears that during the first minutes of contact, the surface chemistry could modify considerably the further spreading of cells likely by influencing the first passive phases: the flattening and the alignment. Hence, the gold-palladium layer should be considered as not favourable for cell fitting. Bagno et al. observed also a lower osteoblast attachment on titanium substrates coated with PLLA or PDLA polymers. They considered that it was because their coating did not let the original disk morphology intact [24]. In our case, the gold-palladium layer measuring about hundred nanometers, it statistically did not affect the numerical values of the surface roughness amplitude. Moreover, gold or palladium themselves are not known to induce a specific toxicity versus cells even if they are generally slightly less favourable for cell proliferation and differentiation than titanium [25, 26]. Thus, the effect of the gold-palladium layer on human bone cell attachment that we observed may be rather related to the physico-chemical state of the gold-palladium layer after sputtering and after immersion in culture medium. The difference of human bone cell attachment on coated material compared to bulk material has been also observed by Howlett et al. but they did not propose convincing explanation for this phenomenon except the presence of some underlying elements on the surface after coating [2]. In our case, since the effect of coating appears more important on stainless steel substrates than on titanium-based ones, it seems that physical or chemical interactions occurring between the gold-palladium layer and the metallic substrate could influence the cell attachment. Further investigations are currently done in our laboratory on the coated substrates to test this hypothesis.

We failed to demonstrate any effect of the roughness amplitude on the cell attachment. This result could appear a little surprising since a lot of previous works have demonstrated the contrary [7–11]. How to interpretate this discrepancy? We could think that the higher the roughness, the larger the surface. By surface, we do not mean the projected surface of the samples that stay unchanged between substrates but the developed area. To check statistically this assertion, we have calculated the developed area for the two levels of roughness (low level $= 0.85\mu$m, high level $= 2.35\ \mu$m). The mean developed area for both electro-eroded and sandblasted surfaces with low level of roughness is around 102% and respectively 105% and 109% for the sandblasted and electro-eroded surfaces with high level of roughness. This means that the real area have been increased by a factor 2.5 and 4.5 respectively for low and high roughness. However, in spite of this considerable increase of the available area for cell attachment, there were no more cells attached on the substrates with a low level of roughness than on substrates with a high level of roughness. This means that the cell attachment is not linked to the underlying area. Let us try now to find the stochastic process that could explain this. We propose that the initial attachment could be described by the well known ballistic process [27]. An individual cell would drop on the surface with a given probability of attachment which likely would depend essentially on the surface chemistry since, as we have shown, the probability of attachment is lower for gold-palladium coated surfaces than for un-coated ones. Considering this model, the developed area has not any influence on attachment since the ballistic deposition hypothesis is only

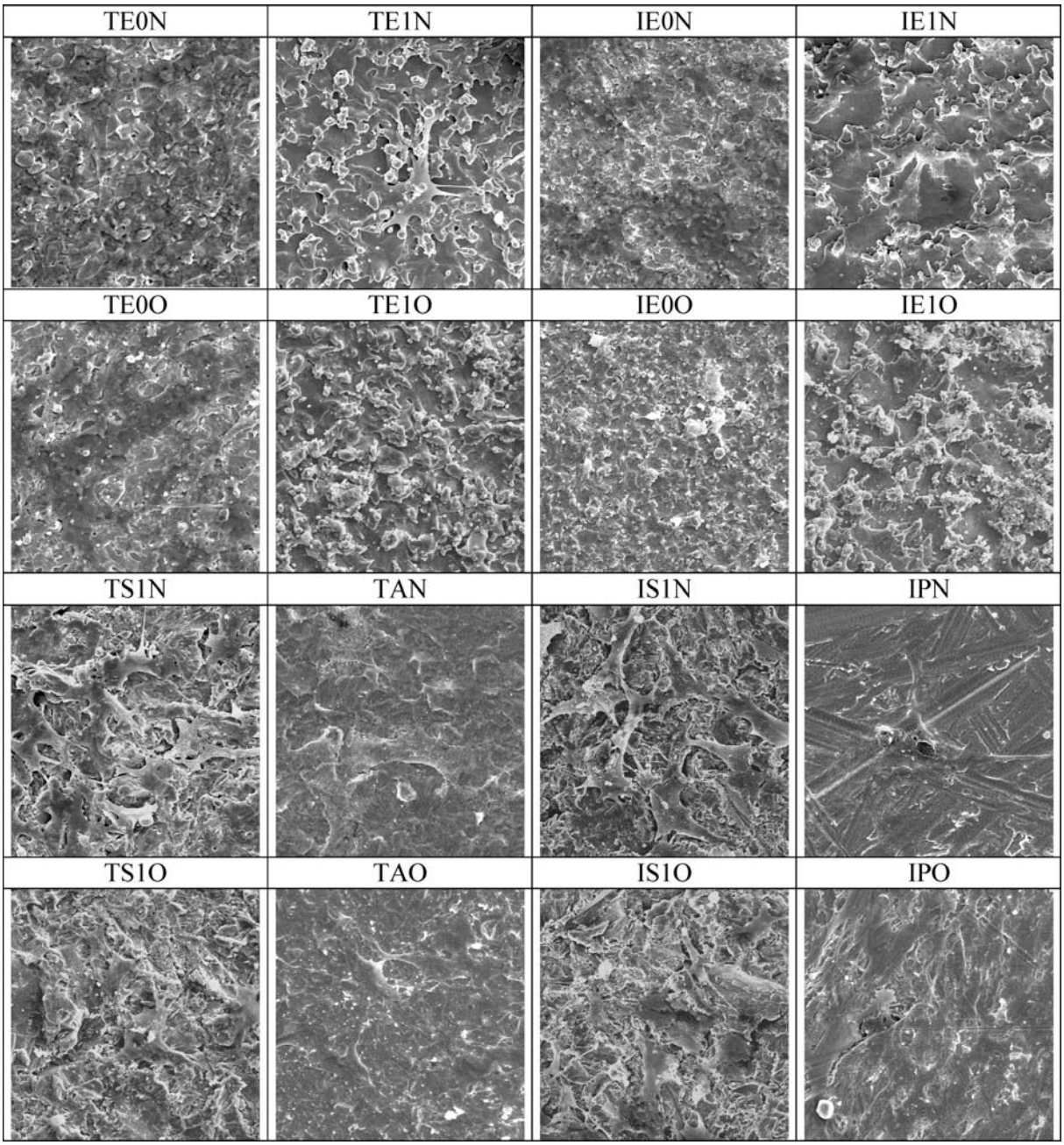

Fig. 4 Scanning electron micrographs of cells after 24 hours of culture on 8 substrates before (N) and after coating (O) with gold-palladium. TE0: low roughness electro-eroded titanium surface, TE1: high roughness electro-eroded titanium surface, IE0: low roughness electro-eroded stainless steel surface, IE1: high roughness electro-eroded stainless steel surface, TS1: high roughness sandblasted titanium surface, TA: acid-etched titanium surface, IS1: high roughness sandblasted stainless steel surface, IP: polished stainless steel surface. The microscopic observations of cells on substrates before and after coating confirmed the lower number of cells after coating. The cells were round and did not spread on electro-eroded titanium-based substrates with rough amplitude after coating (VE1O, TE1O) and on all the stainless steel electro-eroded surfaces after coating (IE0O, IE1O). On the non electro-eroded substrates after coating, the cells were less numerous (TS1O, IS1O) or less spread (TAO) except on polished stainless steel substrates (IPO) where the number of cells was higher after coating.

based on the apparent surface i.e. the projected area of the sample.

Moreover, we observed that the cell attachment was significantly influenced by the process used to prepare the surface. On a physical point of view, this means that the cells react rather to the morphology of the topography than to its roughness amplitude. The cells would attach more on surfaces with an anisotropic roughness (machine-tooled and polished surfaces) than on the isotropic ones (acid-etched and electro-eroded surfaces). This would be consistent with

our previous observations of a different cell behaviour in function of the roughness morphology or organization of surfaces [15]. However, in this new experiment, the sand-blasted surfaces contradict this hypothesis since they induce a high short-term attachment whereas their topography is totally isotropic. Hence, the process would rather influence attachment through a modification of the surface chemistry. The quality and the thickness of the superficial oxide layer could be involved since acid-etched and electro-eroded surfaces which are known to display a relatively thick oxide layer [28] induced the lowest attachment. Again, the surface chemistry analysis currently under investigation will bring some answers on this aspect.

Finally, another important remark has to be stated. It has been proved without ambiguities that long-term adhesion (adhesion after more than 24 hours of culture), cell proliferation and cell differentiation did depend on the roughness of the substrate [1, 7, 10, 12–17]. Thus it appears that the initial attachment or short-term adhesion does not reflect the further behaviour of cells on the substrates. This illustrates again the need to test the in vitro behaviour of cells on substrates not only after some hours to check attachment but also after later delays (days or weeks) to check adhesion, proliferation or differentiation.

5. Conclusion

Our study asserts with an important numbers of experiments (374) that the initial cell attachment is statistically influenced by the process used to produce the surface topographies but not by the roughness amplitude. This demonstrates that short-term adhesion is rather influenced by the surface morphology. However, the process could also influence the cell attachment by modifying the surface chemistry. Since the coating by a gold-palladium layer of surfaces has also a significant negative influence on cell attachment, it appears that human osteoblast short-term attachment would be rather influenced by surface chemistry than by surface roughness of metallic substrates. This could be linked to the electrostatic forces involved in the initial adhesion phase which logically would be more influenced by surface chemistry. This major influence of surface chemistry on attachment could appear contradictory with previous published works designed to study the cellular short-term adhesion on substrates with various topographies. However, most of these works based their assertions on morphological description of cells and on slight quantitative comparison of number of attached cells. Never these works processed to a solid statistical analysis as we did with a high number of experiments allowing to analyse cross effects. Further experiments concerning the characterization of the surface chemistry of the tested surfaces before and after coating will now be performed in order to go deeper in the knowledge of the relation between short-term adhesion and surface chemistry.

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