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Relative influence of surface topography and surface chemistry on cell response to bone implant materials.

Part 2: biological aspects

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Abstract: A current medical challenge is the replacement of tissue which can be thought of in terms of bone tissue engineering approaches. The key problem in bone tissue engineering lies in associating bone stem cells with material supports or scaffolds that can be implanted in a patient. Beside bone tissue engineering approaches, these types of materials are used daily in orthopaedics and dental practice as permanent or transitory implants such as ceramic bone filling materials or metallic prostheses. Consequently, it is essential to better understand how bone cells interact with materials. For several years, the current authors and others have developed *in vitro* studies in order to elucidate the mechanisms underlying the response of human bone cells to implant surfaces. This paper reviews the current state of knowledge and proposes future directions for research in this domain.

Keywords: surface topography, surface chemistry, cell adhesion, cell proliferation, cell differentiation, protein adsorption, bone, implant, roughness parameters

1 INTRODUCTION

Understanding the way in which cells interact with materials is a crucial topic in the biomaterial and tissue engineering areas. Cells respond to nanostructures and microstructures, for example, with the extracellular matrix (ECM) where the nanometre-sized collagen fibrils are organized in three-dimensional (3D) meshes of micrometre-sized fibres. Moreover, because the ECM is composed of a variety of specific proteins, and in the case of bone, also of mineral components, the cellular environment exhibits a chemistry and mechanical behaviour with which the bone cells are used to interacting. A current medical challenge is the replacement of tissue which can be thought of in terms of bone

tissue engineering approaches. The key problem in bone tissue engineering lies in associating bone stem cells with material supports or scaffolds that can be implanted in a patient. This approach to promoting the growth of new bone tissue has the potential to replace bone grafts. The implantation of the cell-containing scaffolds can occur immediately after their synthesis or it can be delayed until after a period of *in vitro* culture. This period allows the acceleration of the interactions of cells with the scaffold thus improving the survival chances of the hybrid material after implantation. Beside bone tissue engineering approaches, these types of materials are used daily in orthopaedics and dental practice as permanent or transitory implants such as ceramic bone filling materials or metallic prostheses. Consequently, it is essential to better understand how bone cells interact with materials. For several years, the current authors and others have developed *in vitro* studies in order to elucidate the mechanisms underlying the response of human bone cells to

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implant surfaces [1–5]. This paper reviews the current state of knowledge and proposes future directions for research in this domain.

2 DEFINITION OF MECHANISMS INVOLVED IN CELL–SURFACE INTERACTIONS

The majority of cells in the body (with the major exception of blood cells) adhere to an ECM. In bone tissue, the cells that form bone, the osteoblasts, and the cells that degrade bone, the osteoclasts, both adhere and grow on ECMs composed of proteins (90 per cent collagen and 10 per cent other proteins) and proteoglycans filled with calcium phosphate minerals. *In vitro*, these cells can survive only if they can adhere to a surface and thus only if this adhesion is achieved can the cells continue to grow and differentiate. The first interactions between the cell and the surface, both *in vitro* and *in vivo*, will define the quality of the cell–implant and tissue–implant interfaces. After only a few seconds of contact between the surface and the fluid (the culture medium *in vitro* or interstitial fluids or blood *in vivo*), the surface becomes coated by water and proteins. Thus, the remaining cells will sense the surface features of the ECM through this adsorbed layer. Cells first adhere through physicochemical interactions such as ionic forces and van der Waals forces and later through

various biomolecules, both those adsorbed on the surface and those involved in cell response to the environment. The key molecules in this process are the cell’s surface receptors, called integrins, that are transmembrane molecules that *in vivo* mediate the interactions with ECM molecules. Integrins are heterodimers that consist of one alpha and one beta subunit that can be combined to form receptors specific for amino-acid sequences such as the arginine–glycine–aspartic acid (RGD) recognition motif present in many ECM proteins. There are eight different alpha sub-units and 11 different beta sub-units, that can be combined to form 24 different integrin receptors. These integrin receptors act as an interface between the intracellular and extracellular compartments. On the extracellular side they interact with the ECM and on the intracellular side they interact with the molecules of the cytoskeleton and with signalling molecules at the adhesion sites, called focal adhesions (Fig. 1). Thus, they can transmit a signal from the ECM to the nucleus through a biochemical signal transduction pathway. This signal transduction involves the accumulation and phosphorylation of several proteins such as FAK, Src, Rho GTPases, ERK, JNK etc. [6]. Alternatively, the signal can be transmitted mechanically since there exists a physical link between the focal adhesion sites and nuclear membrane created by the cytoskeleton [7]. Actually, integrins are physically ‘hardwired’ to the

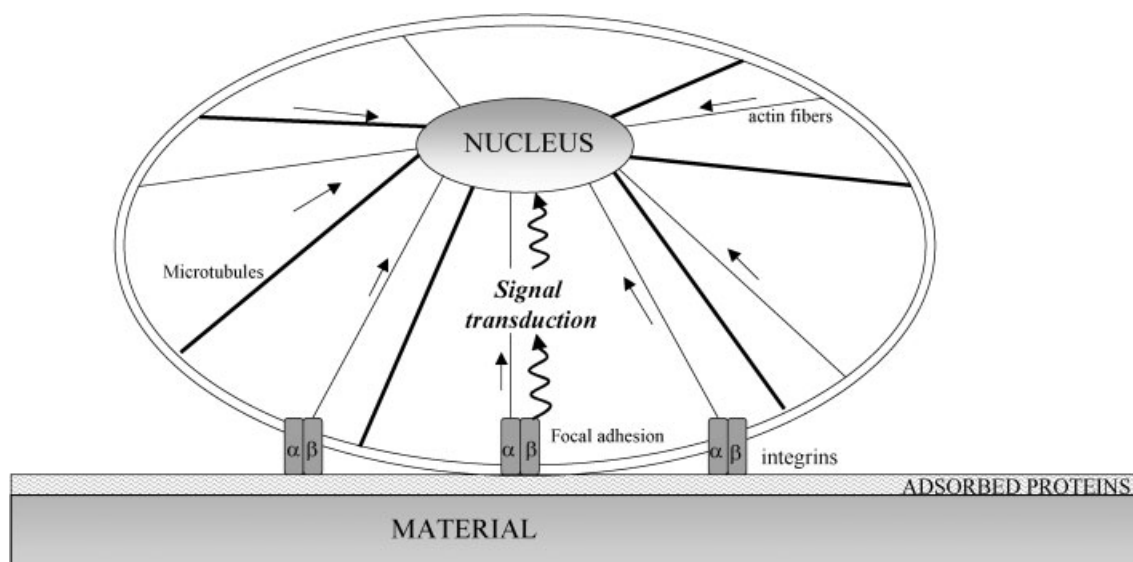


Fig. 1 Schematic diagram of the proteins involved in adhesion of eukaryotic cells on materials. Integrins are transmembrane proteins that form with other intracellular proteins the focal adhesions. On these focal adhesions are connected actin fibres (thin lines) that physically join the cell membrane and nuclear membrane such as microtubules (bold lines). Signal transduction from cell membrane to nucleus can pass through a direct mechanotransduction way (straight arrows) or indirectly by biochemical signal transduction (twisted arrows)

nucleus through the cytoskeleton. This involves the LINC complex (for linker of nucleoskeleton and cytoskeleton) and the coupling between KASH and SUN proteins such as nesprins with intermediate filaments or actin fibres [8]. This 3D configuration may act so as to order the nucleus and then coordinate transcription [9]. The potential use of nanoimprinting to directly imprint the topography onto the cell has been proposed [10].

When the integrin receptors interact with the ECM-adsorbed biomolecules, they cluster to form focal adhesions. At this point the cytoskeleton is reorganized to allow the spreading of cells to the substrate. The cytoskeleton is composed of three different molecules: actin fibers, tubulin microtubules, and vimentin, desmin, or keratin microfilaments. In the focal adhesions, these cytoskeletal molecules interact with signalling molecules such as talin, paxillin, vinculin, tensin, and protein kinases [11]. Focal adhesions are closed junctions in which the distance between the substrate surface and the cell membrane is between 10 and 15 nm. Some authors call them focal complexes when they are shorter than 1 μm , focal adhesions when they measure between 1 and 5 μm , and supermature adhesions when they are over 5 μm [12]. There is kinetic activity in these systems in that nascent close contacts transform into focal contacts before they become focal adhesions. The transition between these different phases is force dependent [5]. Moreover, in tissues or inside the 3D ECM, fibrillar adhesions have also been described [13].

Another group of essential proteins that are involved in a cell's capacity to adhere, spread, divide, and migrate are the myosins [14]. They are actin-based motor proteins that translocate along actin fibres and allow their contraction. After adhesion and spreading, the cells secure their shape stability through a prestress state maintained by myosin motors transmitted by actin fibres throughout the cell body and balanced by microtubules [15, 16]. If needed, cells are able to migrate in order to find a more suitable place for their further development. Also, if the point on the substrate at which they are cultured has an anisotropic topography such as a groove, the cells migrate and orient themselves as a function of the axis of the groove. This phenomenon is called 'contact guidance' and has been only observed *in vitro*.

It has been demonstrated by several authors that filopodia are the cell's tools for exploring its surroundings [17]. Recently, Fujita *et al.* [18] performed a time lapse analysis of cell alignment on nano-grooved patterns and demonstrated that the

filopodia probed the area surrounding the cell protrusion before movement into this area. However, it was also shown that when the cell protrusions extend perpendicularly to a groove axis they retract more rapidly than those parallel to the groove axis. Also, in protrusions parallel to the groove axis, actin filaments form wider focal adhesions at filament termination whereas when the protrusions are perpendicular to the groove axis, the focal adhesions are formed less easily and appear fragmented (Fig. 2). Thus, it appears that the major mechanism in cell alignment on grooves is not the filopodial sensing but rather the retracting phase of the cell protrusion that is related to the quality of the focal adhesions [18].

All these biochemical and mechanical interactions allow signal transduction to the nucleus that either induces cell death through apoptosis if the adhesion is not possible or cell growth and cell differentiation if cell adhesion and spreading can be achieved.

After adhesion and migration, cells divide and proliferate across the surface. They further differentiate which means that they synthesize the molecules they normally synthesize in their tissue of origin. Osteoblasts will synthesize collagen and the other proteins normally found in bone tissue. Also, they are able to induce their own *in vitro* mineralization in certain culture medium conditions and to produce a mineralized bone-like tissue [19].

Generally, the investigation of cell-surface interactions tends to be focussed on the initial cell adhesion phase and little attention is paid to cell-surface interactions after this point. It is the current authors' contention that it is also necessary to consider the influences of surface topography and chemistry at the complex interfaces between the cell and the ECM [20]. Various methods have been developed to quantify the cell-surface adhesion strength. When the ECM is not involved, aspiration, centrifugation, and fluid flow techniques have been used to detach the cell populations. Micromanipulation methods based on micropipettes, microcantilevers, and optical or magnetic tweezers, have been used to detach isolated cells [3]. The cell-surface interactions can also be evaluated using an enzymatic detachment method. First, the adhesion of human osteoblasts after 24 h, called short-term adhesion, is quantified. At the end of this first phase, all the cells are spread on the surface and their cytoskeleton is well organized. However, at this point they have neither expressed $\beta 1$ and $\alpha 3$ integrin molecules nor synthesized matrix proteins [21]. In addition, the long-term adhesion, which represents

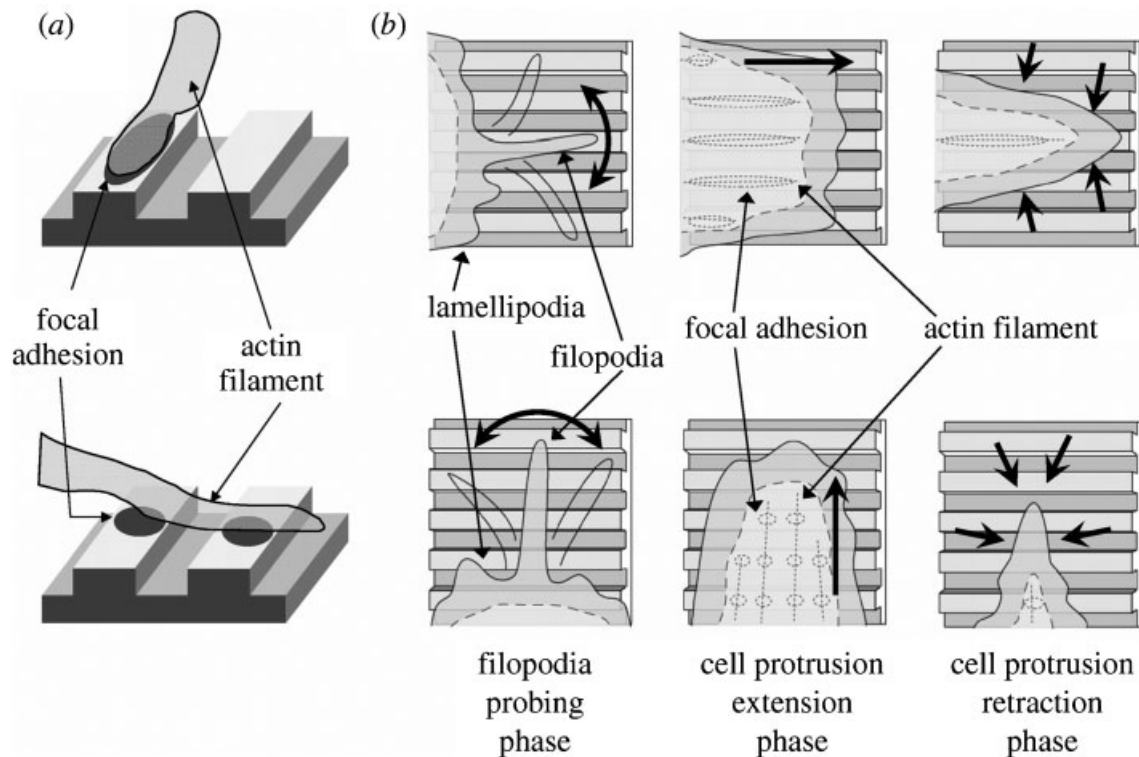


Fig. 2 Model for cell alignment on the nanogrooved substrate. (a) Actin filaments parallel to the grooves form wide focal adhesions at filament terminations. However, termination of perpendicular filaments is fragmented because a focal adhesion is formed only on the ridge. (b) Filopodia movements are isotropic, i.e. no specific direction is observed for their extension and retraction against the nanogrooved structure. This finding suggests that filopodia probing does not play a major role in cell alignment. Cell protrusions extend isotropically, and those that are perpendicular to the nanogrooved pattern retract more rapidly than those parallel to the nanogrooved pattern. These cell protrusion dynamics force a cell to elongate and align along the nanogrooved pattern (Reproduced with permission from [18])

the strength of the cell–material interface formed during 3 weeks of culture involving the ECM proteins synthesized by the cells themselves and the cell–cell contacts, can also be quantified [20]. This long-term adhesion has been shown to be better correlated with topography, especially hybrid roughness parameters that quantify roughness organization, whereas short-term adhesion is more influenced by surface chemistry [22–28].

Another very important point to consider in the study of cell–surface interactions is the phenotype of the cells which is related to their original tissue type. Cell lines produced from normal healthy tissue are called primary cell lines. Their phenotype is very close to the one of cells inside the tissue but they are in general more difficult to culture *in vitro*. Moreover, they cannot be cultured for more than a few weeks and for only a limited number of passages (change from one culture dish to a new one). Other cell lines, called permanent cell lines, are produced

by the immortalization of primary cell lines or are derived from bone tumours. The major advantages of these permanent cell lines are their high proliferation capacity and the ability to culture them for almost indefinite periods. However, their phenotype can be slightly different from their initial or normal phenotype. It has been frequently observed that the cell phenotype has a strong influence on cell response to a material surface [29]. For example, it is clear that the adhesion mechanism of blood cells is different to that of cells originating from connective tissues such as fibroblasts, keratinocytes, or vascular cells since the extracellular environment in their tissues of origin are very different. Primary and permanent cell lines are also very different in term of their adhesiveness. Tumour cells tend to lose part of their ability to adhere to an ECM as the number of proliferations increases. It has been shown that both tumour cells and immortalized cells have a more diffuse cytoskeleton and are more deformable than

normal cells [30]. The current authors recently obtained important results using two osteosarcoma-derived cell lines that are frequently used for analysing the influence of surface topography on bone cells: SaOs-2 and MG63 [31]. Those cells cultured on poly-L-lactic acid (PLLA) micro-pillared substrates displayed a previously unreported extensive deformation of their nucleus. This deformation was not seen using bone-marrow-derived human mesenchymal stem cells (hMSCs). The shape of the nuclei reflected the shape of the micropillars; as if the cells had pushed their nuclei in-between the pillars. More surprising, whilst this deformation should impair future growth and differentiation, the two osteosarcoma cell lines demonstrated a proliferation and differentiation capacity comparable to the one they display on flat PLLA substrates. These results have been reproduced using immortalized cell lines originating from bone and other tissues thus demonstrating that the response of both cancerous and immortalized cell lines to topography can be abnormal [32]. Remarkably, this deformation is not due to any external pressure and is reminiscent of the type of deformation that a cancerous cell can encounter when it undergoes metastatic migration. The mechanical properties of cancerous cells can be related to their metastatic potential; cells that are more likely to form tumors *in vivo* are more deformable [33, 34]. Additionally, this change in shape of the nucleus is surprising given that the nucleus is thought to require a particular architecture for correct functioning. Indeed, it has been found that locating genes close to the nuclear lamina induces a switching off of the genes in question [35, 36]. Deformation of the nucleus increases the surface-to-volume ratio and, hence, the proportion of switched off genes should increase. Thus, it would be of interest to continue to study these deformed cells in order to test if they can act as a surrogate system for the biological behaviour of malignant cells during their metastatic dissemination. From this section of the review it is clear that non-cancerous and non-immortalized cell lines must be preferred for use in the study of cell-surface interactions and in particular for the investigation of the influence of topography on bone cells.

Concerning the mechanisms of the cell response to surface topography, it has been proposed that cells react to the presence of discontinuities on the surface. Discontinuities have been defined as structures

with a radius of curvature less than the average length of a pseudopodium or of the distance part of the sensing elements that control cell movements [37].

Thus, the reaction to discontinuities involves focal adhesions and the actomyosin cytoskeleton and is related to the mechanisms of the mechanical sensitivity of cells. When a cell or a pseudopodium meets a concave or convex structure, the cell receptors and actomyosin cytoskeleton undergo varying degrees of deformation or compression [38] and this induces a reorganization of the attachment and cytoskeletal structures [39]. Dunn and Heath [40] have shown that fibroblasts were affected by a convex ridge in a prism substrate if its angle was greater than 4° . The cells displayed discontinuities in their cytoskeletal microfilament bundle system which coincided with a discontinuity in the substrate [40]. It has also been shown that the cytoskeleton is involved in cell interaction with grooved surfaces thus demonstrating cell sensitivity to concave surfaces [41]. Recently, a very interesting study has demonstrated that the response of cells to equivalent concave and convex structures made in polymethylsiloxane was different. The cells preferentially adhered and migrated on the convex structures rather than on the concave structures [42]. This definitively confirms that the cells are able to identify and discriminate between concave and convex surfaces.

Another proposed mechanism for the cell response to surface topography is that the strains introduced into the cytoskeleton could in turn influence ion channels as observed in the stretch receptors of a variety of organisms [43].

Different hypothesis have been proposed for the mechanisms underlying the identification of discontinuities. One is based on the thermodynamics of the ECM adsorption. The discontinuities may differ in reactivity from nearby planar surfaces due to the existence of unsaturated bonds. This would alter the adsorption of ECM proteins and then modify the response of cells that would reflect the differences in adhesiveness related to surface topography [44]. Another hypothesis has proposed that the discontinuities represent energy barriers, the surface geometry and chemistry influencing the size and position of these energy barriers. Then cells modify their orientation, adhesion, or spreading in order to minimize their contact with high-energy discontinuities [45].

3 RESPONSE OF CELLS TO SURFACE TOPOGRAPHY

Historically, the first *in vitro* studies of cell response to surface topography were performed on surfaces whose topography was controlled and measured at

the micron scale. When the objective was to compare surfaces with different roughness amplitudes, very frequently, the authors changed the process without considering the consequences of this change on the surface morphology or the surface chemistry [46–48]. Another cause for misunderstandings in this field is the practice of defining a surface by its manufacturing process instead of concisely defining it by its topographical parameters [19]. This practice has led to erroneous comparisons of surfaces since it is known that a process can induce different topographies when it is applied to different materials. Indeed, it is extremely rare in the literature that the surface topography is correctly characterized: by associating amplitude, frequency, and hybrid parameters [49]. In general, the amplitude parameters (R_a , R_v , R_z) are commonly considered but the frequency and hybrid parameters are more rarely quantified [50]. It should be noted that the current authors compute more than 100 roughness parameters and then statistically correlate these parameters with biological parameters in order to determine the surface parameter(s) to which the cells respond. Using this approach it has been shown that the parameters that describe the morphology of the topography and particularly its degree of organization are the parameters that better correlate with the adhesion of human primary osteoblasts [21, 51].

The proliferation and differentiation potential of MG-63-osteosarcoma-derived cells has been reported to be positively influenced by surface roughness although the attachment level is reduced [47, 52, 53]. These findings have been independently confirmed in several papers in the literature [27, 54–59], however, there are papers that contradict these findings [60–62]. This disparity highlights the influence of the used cell model and the necessity for detailed characterization of the roughness of substrates to be performed in order to allow valid comparison of results. The topography of substrates is in general either poorly defined or the use of different surface treatments (polishing, turning, blasting, acid etching, plasma spraying etc.) renders comparisons invalid.

There have been extensive attempts to elucidate the mechanisms underlying the MG63 cells response to topography. It has been demonstrated that the cells exhibit a more differentiated phenotype on rough surfaces and that their response to systemic factors such as the vitamin D metabolite 1α , $25(\text{OH})_2\text{D}_3$ or 17β -estradiol is also stimulated by roughness [63–66]. In addition to how cells respond to exogenous factors, surface roughness has been shown to affect regula-

tory factors synthesized by cells such as prostaglandins (PGE1, PGE2), transforming growth factor beta1 (TGF β 1) [52, 53, 63], and osteoprotegerin [67, 68]. It has been shown that this effect can be mediated by protein kinase A and phospholipase A2 [4, 65, 69, 70]. These effects have been reproduced using bone cell lines immortalized from mice or derived from rat calvaria that present a more differentiated maturation state than the MG-63 cells. The sensitivity to roughness appears to be higher for immature cells compared to more mature ones [64].

It has been demonstrated that $\alpha 5\beta 1$ and $\alpha 2\beta 1$ integrins are involved in the MG-63 cells response to titanium substrates with different surface roughness levels. In particular it was highlighted that $\alpha 5\beta 1$ mainly influenced cell proliferation whereas $\alpha 2\beta 1$ signalling was required for MG-63 cell differentiation [71–73]. However, contradictory results that show no expression of $\alpha 5$, $\alpha 3$, and $\alpha 6$ integrin subunits on sandblasted titanium substrates and $\alpha 5$ integrin subunits on polished substrates but with $\alpha 2$, $\alpha 4$, αv , $\beta 1$, and $\beta 3$ integrin subunits being expressed on both substrates have been published in the literature [74]. Human primary osteoblasts express $\alpha 3\beta 1$ integrins but not $\alpha 2\beta 1$ integrins whatever the roughness of the titanium substrates [21]. However, expression of the $\beta 1$ and $\beta 3$ integrin subunits by human primary osteoblasts and MG-63 osteoblastic cells was observed in fibrillar adhesions visualized by immunofluorescence on smooth-structured titanium surfaces but not on coarser surfaces [75, 76]. Flow cytometric analysis was used to confirm a slight increase in the expression of $\beta 1$ and $\beta 3$ integrins on cells detached from coarse surfaces compared to smooth ones [76, 77]. These observations illustrate the difficulty in comparing results obtained by different experimental methods or using different cell lines. The role of integrins in the response of osteoblasts to bone materials has been comprehensively reviewed in [6].

In addition to substrates treated by the classical methods used for bone implant surface preparation, some experiments have been performed on grooved substrates in order to analyse the influence of roughness on anisotropic grooved surfaces such as can be found on screws or dental implants. A systematic orientation of cells in the direction of grooves has been observed [44, 78–82] and it has been elucidated that the cell orientation is not a function of the chemical nature of the substrate although the morphology of cells was influenced both by topography and chemistry [79]. By comparing isotropic rough surfaces obtained by sandblasting and acid etching to grooved surfaces, a higher osteoblastic cell adhesion

and proliferation was isolated on the latter surfaces [78]. The dimension of the grooves is also critical and grooves measuring about 10 μm in width have been shown to significantly increase viability [81] and adhesion of osteoblastic cells [44]. Finally, the presence of a micro-topography inside the grooves has been shown to further increase the orientation of cells [82].

3.1 Scale effect

As seen in part 1 a very important parameter to consider is the scale used to calculate the roughness parameter relative to the cell scale. Surfaces prepared by electro-erosion are particularly favourable for long-term adhesion of human osteoblastic cells. The topography of electro-eroded titanium surfaces has been analysed at two different scales: above the human osteoblastic cell scale ($> 50 \mu\text{m}$) and below the cell scale ($< 50 \mu\text{m}$). Above the cell scale, the surfaces were considered to be rough with a melted relief and smooth edges whereas below the cell scale, these surfaces were relatively smooth and presented a flat area permitting the adhesion of cells in a favourable environment that resembles a basin between reliefs [83]. Thus, it appears that rough surfaces are preferred by human bone cells; particularly when they can provide relatively flat areas at their own scale that help the anchoring of the cells. In order to control the surface chemistry, the electro-eroded samples were coated with a thin (nanometre range) gold layer. The previous results were confirmed demonstrating that the cell response was solely a topography-related effect [83].

An interesting way of systematically exploring the dependence of surface chemistry on surface roughness is to use a single process to produce a gradient of roughness on a single sample. Kunzler *et al.* [84] used sandblasting followed by a chemical smoothing process to create a surface on an aluminium sheet whose R_a values changed between 1 and 6 μm . To ensure a constant surface chemistry, the samples were further replicated in an epoxy resin and coated with titanium. A linear increase of proliferation of rat calvaria osteoblasts was observed although human gingival fibroblasts showed a decreased proliferation. Thus, it appears that for this range of R_a and for the same surface organization, the cell response is linearly dependent on the roughness amplitude but the nature of this response is cell dependent. Again this study illustrates the importance of the cell phenotype. Here, the cell phenotypes influenced the cell response either by their different tissue

origin or by their different size and morphology since rat calvaria osteoblasts are smaller and present a more cubical morphology than human fibroblasts.

Recently, driven by the development of nanopatterning methods, the influence of nanotopography on cell response has started to be studied. This is particularly pertinent since the cells reaction to topography is mediated by proteins that have nanometre scales. Using nanofeatures prepared by colloidal lithography, Dalby *et al.* [85] studied the response of fibroblasts to nano-columns 160 nm high and 100 nm in diameter. They demonstrated a decreased cell adhesion and spreading on the nanocolumns characterized by smaller, fainter focal adhesions and a disorganization of the cytoskeleton. Andersson *et al.* [86] observed that on columns of 58, 91, 111, and 166 nm width, the larger the feature, the more spread the cells became. On surfaces with nano-pits with diameters of 35, 75, and 120 nm, fibroblasts were able to sense topography down to 35 nm using their filopodia [87]. On nano-grooved polystyrene substrates with groove depths from 5 to 350 nm and widths from 20 to 1000 nm, Loesberg *et al.* [88] concluded that depth was the most important parameter influencing cell alignment and that 35 nm was the threshold feature size for fibroblasts since below this value contact guidance was no longer apparent. This dimension can be related to the optimum distance for cell adhesion (58 nm) that was determined in elegant studies on surfaces presenting nanoparticles decorated with the RGD peptides involved in integrin recognition and separated by a controlled distance [89–91].

One area of considerable interest is the determination of the size threshold to produce an effect in cells. Dalby *et al.* [92] demonstrated that cells are able to respond to 13 nm tall islands and Dalby *et al.* [93] reported increased filopodia production and smaller focal contacts on 10 nm tall islands compared to the values obtained for flat surfaces. These observations were confirmed by Lim *et al.* [94] who showed that worm-like structures 14 nm tall increased human fetal osteoblastic cell attachment, spreading, integrin subunit expression, and focal adhesion protein synthesis compared to taller structures (29, 45 nm) and flat control surfaces. In conclusion, these results suggest that the size threshold for cell detection could be less than 10 nm; however, the control of the topography under this threshold remains an open problem.

These results obtained on model surfaces are useful for elucidating mechanisms for specific nano-scale cell features; however, they are not really re-

representative of the implants' actual nanotopography. In general, the materials used for implants have a random nanotopography, meaning that the surface does not present any organization or direction. An anodization process can be used to produce nanotubes or nanopores [95–98] and it has been shown that small nanotubes (~ 30 nm in diameter) promote adhesion of osteoblasts [99] or hMSCs [100] without noticeable differentiation whereas larger nanotubes (70–100 nm in diameter) elicit a dramatic cell elongation which induces cytoskeletal stress and selective differentiation. The same anodization process can be used to create random nano-sized patterns on oxide layers such as titanium dioxide and alumina [101, 102]. An experiment has been performed on titanium substrates micropatterned using electrochemical micromachining and further treated by acid etching and anodization in order to change the nanotopography of the microfeatures. Hemispherical cavities measuring 10, 30, and 100 μm in diameter were produced [101]. After anodization or acid etching of the 30 μm cavities, it was shown that MG63 cells adhered and proliferated better on the treated cavities than on the polished ones, especially after the anodization treatment. These studies confirm that implant surfaces should be controlled at every scale since the cells are able to identify and react to both nano-sized features and micron-sized features.

The concept of nanophase materials or nanomaterials has been proposed to describe materials with structural units that are between 1–100 nm [103, 104]. Several studies have reported improved *in vitro* osteoblast adhesion on the surfaces of nanophase materials created from a wide range of materials including ceramics [105, 106], metals [105, 107, 108], polymers [109, 110], and composites [111, 112]. This effect has been shown to be due to improved protein adsorption characteristics [113, 114] that are a consequence of their surface energy properties [103]. However, to date only a few *in vivo* studies have been performed to test the effect of nanophase materials on integration in bone tissue. Nanostructured Al_2O_3 topographic features applied to machined implants promoted a greater bone-specific gene expression in tissues adjacent to implants after implantation in rabbit tibias and an associated increase in bone-to-implant contact and torque removal [115]. Similarly, a greater amount of new bone formation was observed in contact with a tantalum-based scaffold coated with nanohydroxyapatite (nano-HA) than on scaffolds coated with conventional HA [103]. The results obtained on

nano-HA were inconsistent and appear to be dependent on the implantation method. Nano-HA increased bone formation when no gap existed between bone and implant but failed to enhance bone formation in a gap-healing model [116, 117].

3.2 Organization of topography

As previously discussed, when cells are cultured on an anisotropic topography such as a groove, cells orientate themselves along the axis of the groove because of the contact guidance phenomenon. Also, when the surface consists of an organized array of pillars the cells are able to identify this organization and orient themselves in the orthogonal direction [41]. Normally, however, it is difficult to define the topographic organization for implant surfaces prepared by classical process (plasma spraying, sandblasting...) that display rather isotropic surfaces. As discussed in part 1 of this paper, numerous approaches have been proposed to solve the problem of how to define the degree of organization on a surface. It is the current authors experience that the parameters that best describe the organization are those that are correlated with long-term adhesion on the implant surface, for example, (see Fig. 3) the Order parameter that describes with the highest relevance the adhesion of human osteoblast cells on electro-eroded surfaces presented in [51] and [118]. The study of cell organization at the nanometre scale has been recently reported [119]. hMSCs were shown to differentiate better on surfaces presenting slight disorder of 120 nm diameter nanopits (displaced square placements) compared to a hexagonal, square, or even random placement. Their differentiation was comparable to the one obtained with an osteogenic medium containing stimulative factors such as dexamethasone and L-ascorbic acid. This confirms that cells are sensitive to surface organization at both the nanoscale and microscale and that they prefer relatively disordered surfaces. However, these experiments were done with bone-derived cells that have *in vivo* a relatively disorganized environment. Other cells such as keratocytes, fibroblasts, or endothelial cells that have rather organized environment in their tissue of origin have shown a different behaviour *in vitro* [120].

The validity of the results obtained *in vitro* need to be confirmed *in vivo* since the key factor for using *in vitro* studies is an improved implant surface. *In vivo*, it has been shown that the roughness of implants permits the interlocking of bone tissue by increasing the bone-to-implant contact length as well as

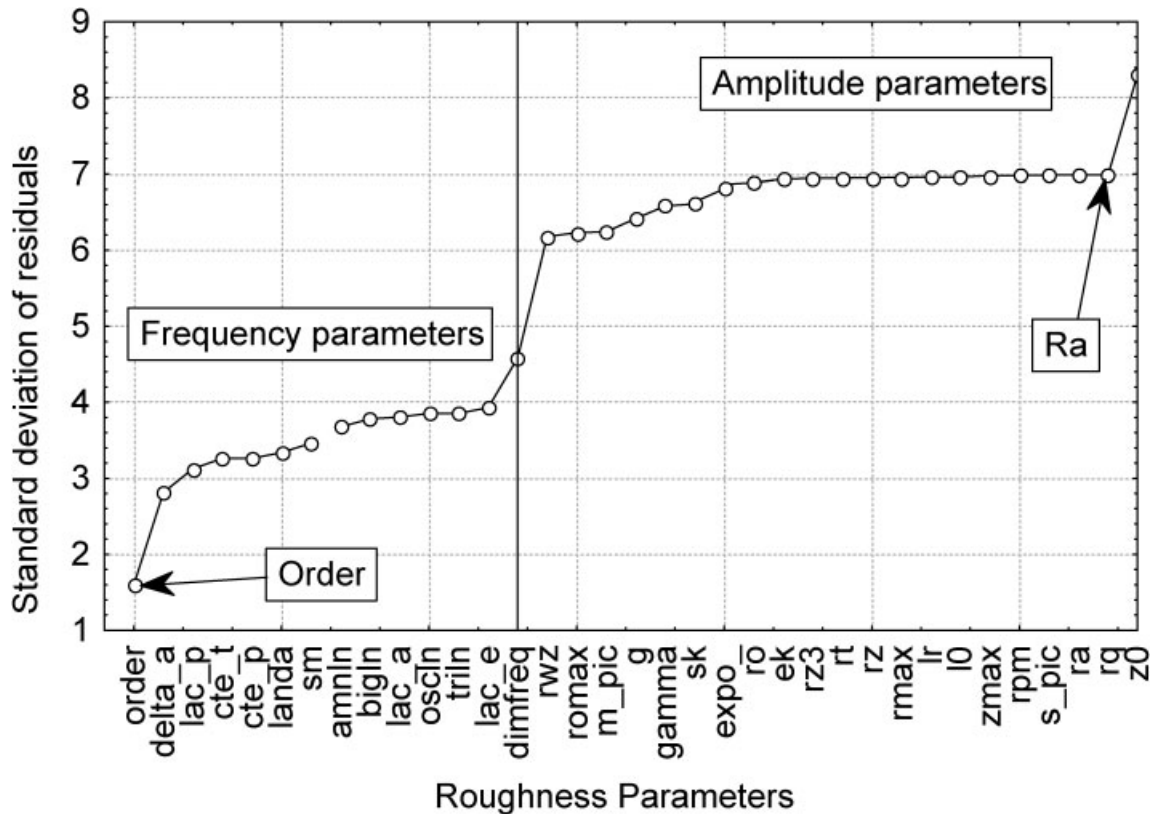


Fig. 3 The classification of the 35 roughness parameters in terms of the adhesion power that represents the long-term adhesion. X-axis: roughness parameters divided in 14 frequency parameters (left of the dotted line) and 21 amplitude parameters (right of the dotted line). Y-axis: standard deviation of the residuals obtained from the statistical correlation analysis between each roughness parameter and adhesion power. The lower the standard deviation of the residuals the higher the correlation with adhesion power (reprinted from [51])

biomechanical integration with bone [121]. In a systematic review of the literature on the effect of implant surface roughness on bone response and implant fixation, Shalabi *et al.* [122] concluded that almost all papers reported an enhanced bone-to-implant contact with increasing surface roughness and also that a significant relation was found between push-out strength and surface roughness.

However, the influence of the organization of the surface topography at different scales on implant integration *in vivo* remains an open topic. To the best of the current authors' knowledge, the only work dedicated to a comparison of surface topography is the paper of Göransson and Wennerberg [123] who considered isotropic and anisotropic implant titanium surfaces with similar roughness levels. In their study they failed to demonstrate any difference in the integration to bone of the two implant surfaces during the first 3 months after implantation.

4 RESPONSE OF CELLS TO SURFACE CHEMISTRY

The tailoring of surface chemistry, as discussed in part 1 of this paper, is now possible thanks to new techniques such as self-assembled monolayers (SAMs), molecular grafting, polymer brushes, polymer gradients, etc. However, these techniques have been developed on glass or silicon substrates and are not easily transferred to bone implant surfaces. Using these techniques, it was shown that the *in vitro* adhesion of cells was favoured on moderate to highly hydrophilic substrates. In particular using SAMs, it was shown that the differentiation of osteoblasts was higher on hydrophilic substrates (OH- and NH₂-terminated SAMs) than on hydrophobic substrates (COOH- and CH₃- terminated SAMs) [124]. This was also confirmed *in vivo* with thicker fibrous capsules formed around implants coated

by CH₃-terminated SAMs compared to COOH- and OH-terminated SAMs [125]. Gradients of polymers with variable surface energy have been prepared and used to demonstrate that surface energy influences adhesion, spreading, and proliferation of human fetal osteoblasts and to confirm that these cells prefer moderate hydrophilic positions in the gradient [126]. Chemical gradients were recently developed using plasma polymers [127]. A mixed gradient from hydrophobic polymer plasma (hexane) to a hydrophilic plasma polymer (allylamine) was produced and used to confirm that adhesion and proliferation of fibroblasts are higher on the hydrophilic part of the gradient [127].

In pioneering work on non-model surfaces Schakenraad *et al.* [128] demonstrated on 13 different polymers and glass substrates that the spreading of human fibroblasts reached its maximum for an intermediate surface energy. Using a polymer/calcium phosphate composite, the zeta potential was found to influence osteoblastic cell differentiation; however, no direct relation was found between attachment and proliferation of osteoblasts on biomaterials and the surface energy of substrates [129]. *In vitro* studies that compared osteoblastic cell adhesion and proliferation on different biomaterials with almost the same finish treatment have been performed. They showed better results on the more hydrophilic materials that presented a higher protein adsorption [130]. It was subsequently shown that human osteoblasts are able to identify and respond to slight modifications in surface chemistry [131, 132]. Notably, it was demonstrated that the intracellular signalling of human osteoblasts was modified on titanium implanted with Mg or Zn ions [133]. Hallab *et al.* [23] compared various materials with controlled surface energy and surface roughness and reported that a roughly parabolic relationship existed between fractional polarity (polar component divided by total surface energy) and cellular adhesion strength. The highest adhesion strength was again obtained for intermediate fractional polarity. Using a variety of biomaterials, Liu *et al.* [134] concluded that the adhesion of hFOB1.19 human osteoblasts was delayed and attenuated on hydrophobic surfaces. From these studies it can be concluded that in general osteoblast cells adhere and proliferate better on moderate to highly hydrophilic substrates and this probably explains the observed higher adsorption of proteins such as fibronectin.

Pioneering *in vivo* work has shown that implanting CoCrMo to create different surface energy states resulted in closer tissue adhesion at high-surface-

energy implants whereas a separation existed between tissue and the low-surface-energy implants [135]. Most *in vivo* studies on the influence of implant surface chemistry have been performed using titanium-based implants. Numerous treatments including acid etching [101, 136–138], electrochemical oxidation [139–141], UV treatment [142, 143], and plasma immersion/ion implantation [144] can be used to modify the titanium surface chemistry. Electrochemical oxidation methods have been extensively used to modify the surface chemistry by adding ions such as calcium [145], sulphur [139], phosphorus [139], or magnesium [141] to the native titanium oxide layer covering the implant. This approach leads to implants with one half presenting a turned native oxide surface and the other being a test surface. After implantation in the cortical tibial bone of rabbits, the osseointegration of the implants was studied using histomorphometry and by measuring the removal torque value. The osseointegration of the modified titanium surface was found to be systematically higher than the unmodified surface [139, 141, 145]. Even if, as is usually the case, the electrochemical treatment used to modify the surface chemistry also modified the surface roughness, it was stated in these papers that the authors believed that their results were more likely the result of the effect of changes in the surface chemistry and that a biochemical bonding occurred that facilitated a rapid and strong integration of implants particular at earlier healing periods [141].

Interesting results have been obtained in clinical dental practice using titanium implants treated by sandblasting with large grits of 0.25–0.50 mm followed by acid etching with HCl-H₂SO₄ (SLA surfaces) [136, 138, 144]. Even if the osseointegration of these implants was good [138, 146–148], several studies have considered the modification of the SLA surfaces in order to achieve faster intimate implant bone bonding. The treatment of SLA surfaces by CO₂ ion implantation [144] or H₂O₂/HCl [137] improved their osseointegration. A very interesting modification of the SLA surface in order to enhance surface free energy and hydrophilicity of SLA implants has been proposed by Rupp *et al.* [149]. These modified SLA implants (modSLA) are produced in the same way as SLA implants but after the acid etching procedure they are rinsed in NaCl and stored in an isotonic NaCl solution under a protective N₂ atmosphere to preserve the chemically active stage until implant placement. The *in vitro* results obtained using modSLA show that the osteoblasts grown on the modified surfaces exhibit a more differentiated phenotype

[150, 151] and *in vivo* results have confirmed the higher osseointegration and a decrease of the healing time in clinical applications [146, 152]. The efficiency of the modSLA implants has been explained in terms of a homogenous wetting of the complete surface without formation of an air/water interface; thereby facilitating the interaction of the implant surface with organic molecules such as proteins adsorbed from blood [136]. For more information on the potential of modSLA implants interested readers are referred to the recent review by Schwarz *et al.* [153]. Aita *et al.* [142] UV treated machined and acid etched titanium surfaces and demonstrated that the UV treatment favoured implant osseointegration. Aita *et al.* [143] explained this observation in terms of the elimination of oxygen-containing hydrocarbons from the surface leading to a higher adsorption of proteins from the body fluids. The following section discusses the role of adsorbed or grafted proteins on a cell's response to materials.

5 ROLE OF ADSORBED OR GRAFTED PROTEINS ON A CELL'S RESPONSE TO MATERIALS

5.1 Adsorbed proteins

As previously discussed, the surfaces that the cells interact with are nearly always coated with proteins adsorbed from the biological fluids. The way in which these biomolecules are adsorbed and the conformation they adopt will 'condition' how the cells recognize them using their integrins. As previously discussed, modifications of the surface topography or chemistry influence the surface energy that in turn changes the way the proteins are adsorbed. Proteins on the surface are different in terms of quality, quantity, and conformation, all messages that the cells are able to decipher using their integrins [154].

The surface chemistry and surface energy influence the protein adsorption and they are modified by the adsorbed proteins. The surface energy of 13 different polymer substrates has been shown to have no influence on human fibroblast adhesion after the serum proteins were adsorbed on the surfaces [128]. SAMs are convenient tools to study this phenomenon. Lee *et al.* [155] demonstrated that the fibronectin binding efficiency on CH₃-, NH₂-, COOH-, and OH-terminated SAMs can be directly correlated with the adhesion of erythroleukemia cells and that fibronectin adsorbed on COOH-terminated SAMs interacts more with $\alpha 5\beta 1$ integrins than fibronectin adsorbed on OH-terminated SAMs. This suggests

that surface charges, and in particular negative charges, are also a significant factor for protein adsorption and presentation to integrins. It is generally thought that hydrophilic substrates adsorb lower levels of proteins and induce less modification in their conformations [156, 157]. Thus, proteins retain a more active conformation on hydrophilic substrates than on hydrophobic substrates. However, the adsorption of albumin is larger and faster on hydrophobic substrates [158–161]. The Vroman effect allows the replacement of a rapidly adsorbed protein with a low affinity for a surface, such as albumin, by a protein with a higher affinity, such as fibronectin. When albumin and fibronectin are mixed, the albumin adsorbs on hydrophobic surfaces whereas fibronectin adsorbs on hydrophilic surfaces [160]. Cooperation can also exist between different proteins. The presence of albumin is known to prevent conformational changes in adhesive proteins such as fibronectin and permit their interaction with integrins by their RGD sites [160].

It is known that HA and more generally calcium phosphate materials adsorb large amounts of fibronectin and vitronectin from serum and that the proteins are adsorbed in a conformation that promotes the binding of hMSCs [162]. This is one of the events that take place at the interface between a bioactive ceramic and the surrounding biological environment [163]. For bioactive materials such as calcium phosphate ceramics, bioactive glass, and glass-ceramics the following processes can occur.

1. Dissolution from the ceramic.
2. Precipitation from solution onto the ceramic.
3. Ion exchange and structural rearrangement at the ceramic-tissue interface.
4. Diffusion from the surface boundary layer into the ceramic.
5. Solution-mediated effects on cellular activity.
6. Deposition of either the mineral phase or the organic phase without integration into the ceramic.
7. Deposition with integration into the ceramic.
8. Chemotaxis to the ceramic surface.
9. Cell attachment and proliferation.
10. Cell differentiation.
11. ECM formation [163].

This sequence of events is considered to be the driving force for the high osseointegration capacity of bioactive materials since the first four processes create a stable and highly topographically complex surface at the submicron scale with which bone bonding can occur [164].

In addition to this submicron surface, microporosity in the ceramics can also significantly influence cell function. Microporous (pores with a diameter $< 10 \mu\text{m}$) HA, the beta phase of tricalcium phosphate (βTCP), and biphasic phosphate ceramics (BCP) can in specific conditions have an osteoinduction capacity when implanted alone (without hMSCs) in heterotopic sites i.e. in extra-osseous sites [165–168]. There exists an optimal specific surface area and a minimum threshold in the microporosity below which bone will not be induced. Additionally, the dissolution capacity of the ceramic is also an important factor in a material's osteoinduction behaviour since osteoinduction is higher with BCPs than with HA and significantly delayed in biomaterials that initially do not contain calcium phosphate i.e. alumina and titanium. The mechanism for this osteoinduction capacity is not well elucidated but it has been postulated that the precipitation of apatite that occurs in bioactive materials could be accompanied by the co-precipitation of growth factors such as bone morphogenic proteins from the body fluids which in turn could recruit cells to differentiate into the osteogenic lineage [166, 168]. However, it is important to note that this osteoinduction is a transient phenomenon that disappears in time and has not been consistently observed for all animal types.

In addition to the adsorption of proteins from the biological fluids, the cells themselves synthesize their own ECM on the substrate. Several papers in the literature highlight the point that the organization of the ECM reflects the cell layer organization [21, 58, 169]. Moreover, it was recently shown using confocal microscopy that a pre-adsorption of fibronectin on rough titanium substrates resulted in an irregular pattern with a higher amount of protein on the peaks than in the valleys. This was attributed to the physicochemical heterogeneity of the rough surfaces [169]. The fibronectin fibrils synthesized by the MG63 cells were overlaid on top of this pre-adsorbed fibronectin layer and did not penetrate into the topography by more than half of the maximum peak-to-valley distance [169].

5.2 Functionalization of surfaces with proteins or peptides

As previously discussed the modification of surface topography or surface chemistry of titanium implants induces an increased protein adsorption from biological fluids and an increase in the cell adhesion on their surface [143]. Another approach to

improve cell adhesion on implants is to immobilize adhesive proteins or peptides on their surface. Type-I collagen has been shown to significantly increase bone growth and bone-to-implant contact after covalent grafting on smooth and rough pure titanium substrates [170, 171]. Coating a titanium surface with the GFOGER collagen-mimetic peptide significantly improved the peri-implant bone regeneration and osseointegration compared to a surface modified with type-I collagen [172].

Similarly, grafting the $\alpha 5\beta 1$ -integrin-specific FN fragment FNIII₇₋₁₀ onto a titanium implant significantly increased osseointegration compared to RGD-functionalized and unmodified titanium surfaces [173]. The effect of coating titanium implants with RGD peptides has been widely studied and in general the osseointegration of the RGD-coated implants has been reported to be enhanced compared to an uncoated titanium surface [6, 174–177]. However, when compared to titanium surfaces coated with either collagen or collagen-chondroitin sulphate, the RGD-coated titanium appears to be less efficient for osseointegration [178, 179].

The possibility of accelerating osseointegration of HA coatings [180] or sintered HA disks by using a RGD coating has been explored. The RGD coating appears to be either inefficient [180] or to significantly inhibit the osseointegration of the implants [181]. These results were interpreted as being caused by the RGD peptides preferentially binding to the integrins which thus were no longer available for binding to the adhesion proteins adsorbed on the HA from the biological fluids. Since the integrin–RGD complexes have a lower efficiency for cell survival than integrin–adhesion protein complexes, the osseointegration of RGD-coated HA implants is lower than that of the uncoated HA. Thus, it appears that for materials with a high adsorption potential for adhesion proteins from biological fluids, the coating or grafting of RGD peptides is not advisable.

6 RELATIVE INFLUENCE ON CELLS OF SURFACE TOPOGRAPHY AND SURFACE CHEMISTRY

It is very difficult to isolate the relative influences of surface topography and chemistry on the cells. In a pioneering study by Britland *et al.* [182] model surfaces presenting simultaneous parallel and orthogonal topographic (micro-grooves) and adhesive guidance (aminosilane tracks) were investigated and it was reported that the adhesive response consistently dominated the topography response. Nebe *et al.* [183] compared the influence of roughness and

surface energy of polymeric and metal surfaces on fibroblast adhesion and concluded that surface energy was more important than surface roughness in determining cell adhesion and proliferation [23]. A correlation was found between cell adhesion and surface free energy as well as surface roughness. However, the latter correlation was visible only when the substrates were divided into two classes of materials with either a high or low surface energy. This finding suggests that the influence of roughness on cell adhesion strength may be secondary to the surface energy for high surface energy materials (metals). On nickel-titanium alloys, Ponsonnet *et al.* [25] observed that the free energy of the surface was a dominant factor on cell adhesion and alkaline phosphatase activity whereas cell proliferation was modulated by roughness (this effect increasing during cell culture) and by chemistry (this effect remaining stable over time) together. However, Wirth *et al.* [27] observed that total protein content and cell morphology were independent of both these parameters. Nebe *et al.* [183] also performed systematic correlation studies and demonstrated that several cellular parameters describing adhesion, spreading, integrin expression, proliferation, and differentiation were quantitatively correlated with material parameters describing surface roughness or electrochemical surface characteristics. The surface roughness amplitude (R_a) correlated with cell adhesion, $\beta 1$ integrin focal contact length, cell spreading, and cell proliferation but not with the cell differentiation (gene expression of bone sialoprotein). Additionally, most of the electrochemical parameters correlated with cellular parameters describing adhesion, $\beta 1$ integrin focal contact length, and cell spreading. Additionally, the corrosion resistance (R_{corr}) correlated negatively with adhesion and bone sialoprotein expression i.e. when R_{corr} increased the two cellular parameters decreased. The correlation of cellular and

material surface parameters has also been used by the current authors to define the main parameters that influence cell response. The short-term and long-term adhesion levels of human osteoblasts on metallic substrates with different topographies were compared before and after coating with a thin (a few nanometres) gold-palladium layer, in order to compare substrates with the same surface chemistry. Short-term adhesion was mainly influenced by surface chemistry (by the presence of the coating) whereas long-term adhesion was mainly influenced by surface topography (roughness amplitude and surface morphology) [22, 83, 184, 185]. Logically, proliferation is influenced by both factors since it is related to both short-term and long-term adhesion (Table 1). These studies on metallic materials were reproduced on ceramic materials. In this case, the sensitivity of cells to topography and chemistry was dependent on the individual material. On β -TCP, the main parameter was the surface chemistry that negatively affected the initial cell adhesion but positively affected the proliferation and differentiation. On HA, the main factor was the surface topography that increased cell differentiation but lowered proliferation [28].

7 CONCLUSIONS

Knowledge of the complexity of cell-material interactions is vital for future developments in biomaterials and tissue engineering; however, there is still a long way to go before a clear understanding is achieved, as illustrated in this review. A lot of factors both on the cellular and on the material side influence these interactions and must be controlled systematically during experiments. On the cellular side, it was highlighted that the cell phenotype must be as close as possible to the phenotype of the cells

Table 1 Summary of the influence of material parameters on cell response. Material: material of substrates (pure titanium, titanium alloy Ti6Al4V, stainless steel 316L). Roughness: roughness amplitude ($R_a = 0.85 \mu\text{m}$ or $R_a = 2.35 \mu\text{m}$). Process: process used to produce topography (electro-erosion, sandblasting, polishing, machining, acid etching). Coating: coating of samples by electro-sputtering of a few-nanometre-thick gold-palladium layer. Short-term adhesion: number of cells adhered after 1 day of culture. Long-term adhesion: strength of the cell-material interface formed during 21 days of culture quantified by the adhesion power parameter. Proliferation: number of cells after 21 days of culture (for more details see [20])

	Material	Roughness	Process	Coating
Short-term adhesion	Yes, with process	No	Yes	Yes
Long-term adhesion	No	Yes, with process	Yes	No
Proliferation	No	No	Yes	Yes

Short-term adhesion is significantly influenced by surface chemistry; long-term adhesion is significantly influenced by surface topography; proliferation is influenced by surface topography and surface chemistry.

used for *in vivo* implants. The use of cell lines issued from tumours must be avoided in the future notably for studying the influence of surface topography. The reproducibility of techniques used to quantify cellular response must be controlled in order to allow the comparison of results from different laboratories. With the same objective, as seen in part 1 of this review, the surface of implants need to be rigorously characterized and defined in terms of several complementary and pertinent parameters. Detailed knowledge of the physicochemical characteristics of the surface will allow their correlation with the parameters that characterize the biological response. This approach will allow a detailed understanding of the response of bone cells and tissue to implant materials and allow the process to be optimized. Finally, an effort should be made to apply this approach to implants after *in vivo* implantation to validate if the knowledge acquired on cell-material interactions *in vitro* is applicable to the *in vivo* case.

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