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Nanotopographical modification: a regulator of cellular function through focal adhesions

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Abstract

As materials technology and the field of biomedical engineering advances, the role of cellular mechanisms, in particular adhesive interactions with implantable devices, becomes more relevant in both research and clinical practice. A key tenet of medical device design has evolved from the exquisite ability of biological systems to respond to topographical features or chemical stimuli, a process that has led to the development of next-generation biomaterials for a wide variety of clinical disorders. In vitro studies have identified nanoscale features as potent modulators of cellular behavior through the onset of focal adhesion formation. The focus of this review is on the recent developments concerning the role of nanoscale structures on integrin-mediated adhesion and cellular function with an emphasis on the generation of medical constructs with regenerative applications.

Keywords

Focal adhesions; Biomaterials; Nanotopography; Cell signaling

This review highlights the importance and development of the physiomechanical processes that regulate early cell-biomaterial interactions and the influence of nanoscale topographical modification on integrin-mediated cellular adhesion. As materials technology and the field of tissue engineering advance, the role of cellular adhesive mechanisms, in particular the interactions with implantable materials, becomes more relevant in both research and clinical practice.

Biomaterials are never truly inert, being at best biotolerable. The cell-substratum interface functions as more than a simple boundary of definition between the host and an implanted device; instead, it presents primary cues for cellular adhesion and the subsequent induction of tissue integration. Indeed, the cytocompatibility of a material can be assessed in vitro by observing the viability and biofunctionality of cells at the substratum interface, paving the way for in vivo studies into device functionality. The range of materials currently designated as biomedically useful and their lack of biofunctionality reflects an increasing need for biomimetic constructs but also indicates the challenges present within the field. In particular a need exists to create truly biocompatible devices and ultimately to control the interactions that occur at the cell-substratum interface.

A key tenet of medical device design has evolved from the exquisite ability of biological systems to respond to topographical features or chemical stimuli, a process that has led to the

development of next-generation biomaterials. Recently published in the journal Science are the prerequisites for third generation biomaterials; not only should they support the healing site (as first-generation biomaterials), but they should be bioactive and possibly biodegradable (as second-generation biomaterials) and they should influence cell behavior in a defined manner at the molecular level.[1] The synthetic surfaces encountered by endogenous cells following implantation usually possess an imposed topography from the fabrication processes, perhaps uncharacterized or unknowingly derived from the methods of manufacture.[2] Indeed, at the molecular level, truly smooth surfaces are an ideal almost impossible to reproduce accurately on a functional device. Microscale roughness may or may not be formed intentionally; however, micron-sized topography has been shown to have an essential role in the induction of cell adhesion and subsequent changes in cellular function.[3–5]

An increased knowledge of the extracellular environment, the topographical and chemical cues present at the cellular level, and how cells react to these stimuli has resulted in the development of functionalized surfaces via topographical modification with an aim to regulate cell attachment and subsequent cellular function. Although microscale topography significantly modulates cellular behavior in vitro, an important consideration in material biophysical modification is the observation that cells in vivo make contact with nanoscale as well as microscale topographical features. Also, whereas single cells are typically tens of microns in diameter, the dimensions of subcellular structures—including cytoskeletal elements, transmembrane proteins, and filopodia—tend toward the nanoscale. Furthermore, extracellular supporting tissues also typically present an intricate network of cues at the nanoscale, composed of a complex mixture of nanometer-size (5–200 nm) pits, pores, protrusions, and fibers,[6,7] suggesting a regulatory role for these structures in vivo.

The use of lithographic and etching techniques derived from the silicon microelectronics industry has facilitated investigations into the intricate role of nanoscale topography on all aspects of cellular behavior—importantly, cellular (including bacterial) adhesion, activation, and differential function. The focus of this review is on recent in vitro studies considering cellular interactions with fabricated nanoscale topographies, with an emphasis on the modulation of integrin-mediated cellular adhesion and how nanotopographical modification may influence cellular function.

Regenerative medicine

The American National Institutes of Health describe regenerative medicine as a rapidly growing multidisciplinary field involving the life, physical, and engineering sciences that seeks to develop functional cell, tissue, and organ substitutes to repair, replace, or enhance biological function that has been lost due to congenital abnormalities, injury, disease, or aging. The successful development of this technology requires intellectual and practical expertise from engineers and biological scientists alike, as well as clinicians to voice a need for new technologies and to help translate this basic science into clinical solutions (Figure 1). Since the inception of tissue engineering over 20 years ago in reference to the endothelial-like membrane that had adhered to the surface of a polymethylmathacrylate ophthalmic prosthesis.[8] the field of biotolerable materials or “biomaterials” has seen consistent growth with a steady introduction of new ideas and productive branches.[9] As knowledge of the mechanisms and the predictive outcomes of specific diseases advances, so too does the range of potential therapeutic targets for tissue-engineered constructs; aiding in providing practical solutions for both traumatic defects and degenerative diseases.

It is estimated that at least 20 million people in the United States have undergone implantation of an exogenous material or device.[10] The repertoire of biomaterials that are currently being used and/or investigated for regenerative medical purposes is constantly being revised and

updated; however, biocompatibility (suitability of a particular material for a particular in vivo application) is inherently tissue-specific and may vary from site to site.[11] Thus far, however, successful outcomes have been reported for the use of tissue-engineered constructs in the treatment of a diverse number of degenerative disorders including diabetes mellitus.[12] rheumatoid arthritis,[13] and degenerative heart disease.[13] The medical and surgical cost of treating device failure or implant-associated infection can average up to US $50,000 per patient.[14] Clearly, to negate revisional surgery and improve long-term implant function it is necessary to enhance device integration by modulating cell adhesion and function while reducing the foreign body response.

Cell-biomaterial interactions

Cell-substrate interactions can be regarded as the defining factors of a biomaterial performance in vivo, ultimately determining the long-term performance of a device in situ. This is particularly true of biomaterials designed to provide mechanical stability, which rely on tissue adhesion and ingrowth for continued function. Fibrous encapsulation is known to occur with both metal[15] and polymeric constructs.[16] This is characterized by the diminished adhesion of tissue-specific cells and commonly the presence of a fluid-filled void between the tissue and implant. This reduced biocompatibility may have many causative origins; however, a frequent outcome is diminished implant integration followed by destabilization, along with an inhibition of tissue regeneration and repair as well as an increase in the potential for infection.[17]

The recruitment of immunological cells to a site of implant involves a complex cascade of immune mediators, including various cell types, soluble signaling molecules, and cell-cell interactions. Previous studies have made it clear that the macrophage is the dominant cell in the foreign body response. Once adhered to an implanted material single macrophage cells fuse through a complex series of events to form multinucleated giant cells; this response is accompanied by the recruitment of fibroblasts and fibrous tissue formation. The adherence of giant cells to a biomaterial surface is correlated to the release of enzymes (e.g., esterases, lipases) and other bioreactive intermediates that can degrade and cause a loss of implant function. It follows that the regulation of cellular adhesion or selective adhesion of specific cellular phenotypes is crucial to regulate optimal tissue-specific integration while preventing inflammatory cell recruitment and scar tissue formation.

Conversely, inert materials may be successfully employed for applications in which protein and/or cellular interaction may reduce device functionality. For example, mitral valve replacement or arthroplastic procedures making use of expandable stents both require minimal protein adsorption and cell adhesion to prevent device failure and patient morbidity. Moreover, an ideal outcome to such procedures would be characterized by the neogenesis of functional tissue by regulated cellular adhesion, proliferation, and differentiation of specific cell types.

In vitro studies indicate that endogenous proteins become rapidly adsorped to a material surface in response to surface free energy,[18,19] providing a structural framework on which cellular adhesion may initiate. Modern implants make use of chemical and topographical modification to regulate cellular adhesion,[20,21] differentiation, and de novo tissue deposition.[22–24] For example, surface electropolishing[25] and drug coating[26] are commonly employed in the fabrication of arterial stenting devices to reduce platelet adhesion and the adhesion of circulating progenitor cells,[27,28] major contributors in neointimal hyperplasia and device failure. Surface functionalization via chemical or topographical modification has improved upon the so-called biocompatibility of first-generation materials by regulating the in vivo interactions that mediate the foreign body response. In particular, recent developments in small technologies encompassing the generation of micro-and nanoscale structures have been successfully translated into the development of second generation implantable materials, and

have been shown to enhance biomaterial compatibility through the induction of selective cellular and protein adhesion.

Adherent cells are complex, self-sustaining units[29] that require extracellular matrix (ECM) anchorage to proliferate and undergo differential function.[30] Cells actively probe the physical properties of the ECM; their contractile machinery facilitating the formation of polarized "lamellipodia" (Figure 2, A)[31–33] and fine hairlike protrusions termed "filopodia" (Figure 2, B), structures which gather spatial, topographical, and chemical information from the ECM and/or material surface.

Initial cell tethering and filopodia exploration is followed by lamellipodia ruffling,[34] membrane activity, and cellular spreading. With time endogenous matrix is secreted by the cells, and matrix assembly sites form on the ventral plasma membrane. Once cell-receptor ligation has occurred with an ECM protein motif, a signaling-feedback pathway initiates integrin receptor clustering at the plasma membrane and adhesion plaque protein recruitment.[35] It can be reasoned that this reduction in cellular migration, the formation of mature adhesion sites, and the onset of ECM synthesis are processes indicative of the onset of terminal cellular differentiation in adherent cells.

The focal adhesion

One well-studied process of cellular adhesion involves the activation and recruitment of α-and β-chain transmembrane proteins termed integrins.[36] These receptors bind specifically to motifs located on ECM molecules (e.g. the RGD tripeptide motif found in fibronectin, vitronectin, and laminin[37]) via their globular head domains and form discrete supramolecular complexes that contain structural adaptor proteins, such as vinculin, talin, and paxillin.[34,38,39] Ligand binding in itself alters integrin conformation and affinity, and, in the case of multivalent ligands, integrin clustering. With increased integrin recruitment, these early cell-matrix contacts form anchoring focal complexes at the lamellipodium leading edge that are reinforced intracellularly to form larger focal adhesion plaques upon increased intra and/or extracellular tension (Figure 2, C).

The regulation of focal adhesion formation in adherent cells is highly complex and involves both the turnover of single integrins and the reinforcement of the adhesion plaque by protein recruitment. It follows that focal adhesions emerge as diverse protein networks that provide structural integrity and dynamically link the ECM to intracellular actin filaments (Figure 3), directly facilitating cell migration and spreading through continuous regulation and turnover. Furthermore, in combination with growth factor receptors, these adhesive clusters initiate signaling pathways and regulate the activity of nuclear transcription factors—processes crucial to cell growth, differentiation, and survival, as will be discussed below.

Ward and Hammer developed a model of adhesion strengthening,[40] which predicts large increases in adhesion strength following increased receptor clustering and adhesion size, marked by an elongation of the adhesion plaque. This process is believed to be due to an increase in tension at the adhesion site, because focal adhesion size has been shown to be proportional to the force applied to it by the cell.[41] This indicates that adhesion sites act as mechanosensors[29] that form additional contact points with the underlying substratum in response. Preceding focal adhesion reinforcement a tightly regulated series of temporospatial events occurs, mediating integrin clustering in an anisotropic manner in the direction of force.[42] This integrin clustering has a discrete lateral spacing that lies in the realm of 15—30 nm[43] and, as will be discussed, is a key indicator of the mechanisms involved in the nanofeatures-mediated perturbation of focal adhesion formation.

Nanotopography and focal adhesion formation

That material topography and in particular nanoscale features can affect cell behavior and integrin-mediated cell adhesion is evident from studies with fabricated topographical features. Nanotechnology aims to create and use structures and systems in the size range of about 1–500 nm covering the atomic, molecular, and macromolecular length scales. A range of methods exists for the generation of topographical nanoscale features, including chemical vapor deposition, polymer phase separation, colloidal lithography, photolithography, and electron beam lithography (EBL), to name but a few. For a full review of the methodology for nanoscale fabrication technology in 2006 see Norman and Desai.[44]

The general protocols for nanomanufacturing require high resolution and throughput coupled with low cost. With respect to biological investigations, nanotopographies should occur across a large surface area (ensuring repeatability of experiments and patterning of implant surfaces), be reproducible (allowing for consistency in experiments), and preferably, be accessible (limiting the requirement for specialized equipment).[45] The extent to which nanotopography influences cell behavior within an in vitro environment remains unclear, and investigation into this phenomenon is still ongoing. A question being asked in the field of medical device manufacture is whether nanofeatures offer any relevant stimuli to the cellular component of the immediate tissue in vivo and, if so, whether implants could be fabricated to include these topographical structures. It follows then that fabricated model surfaces with defined topographies are of great experimental importance in engaging with such issues in vitro and, further, may facilitate early studies examining the cellular reaction to nanostructures in vivo.

The processes that mediate the cellular reaction to nanoscale surface structures, however, are not well understood and may be direct[46] (a direct result of the influence of the surface topography) or indirect (where the surface structure has affected the composition, orientation, or conformation of the adsorbed ECM components).[47,48] Of particular interest is the temporo-spatial reorganization of the cell cytoskeleton and of focal adhesion formation in response to nanofeatures,[3,49] parameters that have already been established as important mediators of mechanotransductive processes[50] and differential gene expression.[51] Initiation of the adhesive process, however, is dependent on integrin interactions with the substratum and the topographical regulation of cell adhesion, a process that seems to be dependent on the symmetry and spacing as well as the x, y, and z dimensions of the topographical nanofeatures.[52,53] Studies with defined arrays of bound RGD fragments indicate that integrin-substratum interactions are disrupted when the integrin spacing is in the range of 70–300 nm, and that an integrin spacing of less than approximately 60–70 nm is required for protein recruitment to the focal adhesion.[54] Hence it can be inferred that decreasing the nanofeature spacing to less than 60–70 nm or increasing this distance to the submicron range facilitates integrin clustering, thus restoring focal adhesion formation.

Effects of nanoscale protrusions on focal adhesion formation

Nanoprotrusions and raised topographical features have been reported within the ECM in a large number of tissues.[55–58] Studies of cell adhesion on nanoscopic protrusions have increased greatly with the development of novel fabrication techniques, which provide robust, high-throughput methods for the fabrication of topographical features ranging from the submicron to the lowest resolution features obtainable with current technology—approximately 5–10 nm.[59] The fabrication of nanoprotrusions has been achieved using various methods including colloidal lithography,[60] polymer phase separation,[61] anodization,[62] and EBL.[63] Of these, the first three methods provide a relatively rapid technique for fabricating random or semirandom nanoprotrusions, whereas EBL can be employed to fabricate highly reproducible ordered nanopatterns.

A common theme of cellular adhesion on nanoscale protrusions is the observation of a decrease in cellular adhesion with increasing nanoprotrusion height.[62] Studies thus far indicate the restrictive nature of nanofeatures measuring >70 nm in height, whereon focal adhesion formation is perturbed (Table 1). Recent studies point to a reduction in focal adhesion size[64,65] on these nanoprotrusion substrates, and that the changes in focal adhesion density stem from the innate ability of surface protrusions >70 nm in height to inhibit protein reinforcement at the focal adhesion site.[66]

Recently studies assessing cellular adhesion on 95 nm-high protrusions have demonstrated that fibroblasts adhesion is reduced on features of this size.[65,67] More specifically, it has been shown that cells initially undergo increased cytoskeletal organization and filopodia formation when compared with cells cultured on flat controls but that this initial attachment phase is short-lived and fibroblasts begin to dedifferentiate and undergo anoikis (adhesion-mediated apoptosis) as a result of reduced adhesion and cellular spreading. Similarly, Berry et al. showed that three-dimensional constructs with phase-separated polymer features of a similar dimension also reduced adhesion in bone marrow–derived osteoprogenitor populations.[68]

Reducing the height of nanoprotrusion features to <50 nm has been shown in numerous cell types to return the frequency of focal adhesion formation to that of cells cultured on planar controls, with accompanying upregulations in proteins critical to cytoskeletal dynamics.[69] We have investigated the feasibility of modulating the adhesion and behavior of $STRO^+$ mesenchymal stem cells (MSCs) on surfaces containing 45 nm-high “islands” manufactured by polymer phase separation (Figure 4, A). Here focal adhesion frequency in primary human cortical osteoblasts was comparable to cells cultured on planar substrates; however, $STRO^+$ MSCs were shown to upregulate the synthesis of osteospecific proteins critical for bone formation.[51] Lim et al. have further demonstrated the increased incidence of mature adhesion plaque formation in osteoblasts cultured on nanoislands that approach 11 nm in height.[65] It can be inferred that the effects of feature height on integrin clustering are disruptive at heights >70 nm and that features with z-dimensions <70 nm are insufficient to disrupt integrin clustering.[62] Indeed, substrates possessing nanoisland with heights <70 nm are reported to increase cellular adhesion[70] and enhance cellular spreading by providing tactile stimuli. Perturbation of integrin clustering on nanoprotrusion arrays with heights >70 nm is related heavily to feature width and density; however, this disruption of cell adhesion is observable in many cell types on a wide variety of polymeric substrates fabricated by differing methods.[52,71–74]

The ability of raised features to prevent cellular contact with the basal “planar” substrate and to reduce cellular adhesion is dependent on protrusion diameter and density. Although nanoprotrusion height is critical in the regulation of focal adhesion formation in vitro, feature diameter and the edge-edge spacing dictate whether adherent cells become exclusively localized to the feature apexes or contact the basal substrate. When protrusion height and density are sufficient to prevent cell contact with the planar basal substrate, parameters still unknown, the influence of the nanoprotrusion diameter and edge-edge spacing become the defining factors in the regulation of integrin clustering and focal adhesion formation.[62]

To facilitate integrin clustering in cells suspended on a nanoprotrusion array, the feature diameter must exceed 70 nm. This has been verified by multiple studies making use of pillar arrays >400 nm in height. Here the nanoprotrusion height and density was sufficient to isolate cells from the underlying planar substrate. Reducing the pillar diameter to <70 nm and increasing the edge-edge distance to 300 nm markedly reduced cellular adhesion,[75,76] again indicating that an interprotrusion distance of >70 nm inhibits focal adhesion formation at the bridging site between two adjacent nanoprotrusions. This was identified by Sjostrom and colleagues, who noted a reduction in cellular spreading and focal adhesion formation when

skeletal stem cells were cultured on nanopillar arrays with an edge-edge spacing approaching 70 nm.[62] For a schematic explanation see Figure 5.

Effects of nanoscale pits on cell adhesion

As with nanoscale protrusions, the fabrication of high-resolution and high-symmetry nanopit topographies has benefited greatly from the advent of high-resolution writing techniques such as EBL and dip-pen nanolithography. Yet less ordered topographies can be fabricated via self-organization techniques, such as polymer phase separation to rapidly produce large-area nanotopographic pit substrates for assessing the cellular response to these features.

Nanopores are identified as common constituents of tissues in vivo, notably basement membrane of the cornea,[77] the aortic heart valve.[7] and the vascular system,[78] and may be implicated in the regulation of cell behavior and function. Pitted topographies have been shown to produce differing effects on cellular adhesion in vitro, depending on pit diameter and the spacing and symmetry of pit positioning.[49,79]

Currently the majority of experimental evidence indicates that the spacing and density of nanopit features are as influential as the feature dimensions on focal adhesion formation when in the nanoscale (Table 2). Studies indicate that cells can respond significantly to small changes in the order of nanopit spacing and that modulating the order of pit conformation significantly affects both cellular adhesion and cellular function.[46,80] It seems that introducing a degree of disorder or increasing the interpit area facilitates focal adhesion formation and subsequent cellular spreading.

Highly ordered arrays of 120 nm-wide nanopits, in both hexagonal and square conformation patterns, significantly reduce cell adhesion by directly modulating filopodial formation[81] and preventing focal adhesion reinforcement[79] indicating the ability of cellular populations to gather spatial and topographical signals from nanoscale pits. Moreover, it is reported that focal adhesion formation on nanoscale pit arrays occurs between the nanopit features at the interpit region,[51,82] suggesting that sites of focal adhesion can be facilitated or restricted by modifying the planar interpit area (Figure 4, B). The conformation of ordered nanopit substrates may also dictate parallel or perpendicular adhesion formation and perturb the radial peripheral focal adhesion formation observed during early cell spreading.

It has been reported that the dorsal (and also probably the ventral) surface of the focal adhesion has a corrugated dorsal surface formed by filamentous structures spaced by an average of 127 nm and protruding by 10 to 40 nm over the interadjacent areas.[83] It may be that these dimensions place a limit on the minimum nanopit depth, which may perturb adhesion formation by direct means. Thus, as with nanoprotrusions it can be proposed that nanopit topographies act to perturb focal adhesion formation by disrupting integrin activation and clustering.

This has been demonstrated in numerous studies, whereby arrays possessing an interpit area of <300 nm reduce cellular adhesion. A study by Lim et al. concluded that greater cell adhesion and increased integrin expression occur when topographic features have <10-to 20-nm-scale z-axis dimension (height or depth), and that this occurs despite topographic shapes (island or pit). Also, this effect deteriorates when nanofeatures reach a height or depth of <100 nm, again indicating the perturbing effects of nanopits and pores on cell adhesion when within the 70-to 300-nm z-axis range (Table 2).[35,84] Similarly, the effects of pit diameter on focal adhesion formation was recently demonstrated in a study by Park and colleagues with hollow TiO_2 nanotubes. It was shown experimentally that a central tube lumen of <30 nm with a maximum at 15 nm provided an effective length scale for accelerated integrin clustering and focal adhesion formation, and that this length scale strongly enhanced cellular activities in MSCs compared with smooth TiO_2 surfaces. Conversely, increasing the size of the central lumen to

>70 nm in these vertically aligned nanorods significantly reduced cellular adhesion.[85] Furthermore, the expression of multiple focal adhesion–associated proteins is increased on pits of this diameter.[35] For a schematic explanation see Figure 6.

Effects of nanoscale grooves on cell adhesion

Nanogrooved topographies consisting of alternating grooves and ridge features differ from both nanoprotrusions and nanopits in that they produce very predictable effects on cellular morphology— which, it can be argued, are directly related to cellular alignment through contact guidance.[86] Common methods of nanogroove fabrication include EBL,[87] photolithography,[88] and direct laser irradiation,[89] which may be employed to yield anisotropic substrates with varying feature widths and depths.

A key fabrication tenet of nanogroove substrates for the study of cell-interface interactions is that of biomimetic ECM design, an attempt to mimic the topographical cues imparted by the fibrous nature of ECM. ECM components include both individual fibril elements, which have been reported to measure <20–30 nm in diameter in vasculature basement membrane,[78] and fibril bundles, which range from 15 to 400 μm in diameter in tendon tissue.[90] Key to this is that nanogrooved surfaces may induce enhanced tissue organization and facilitate active self-assembly of ECM molecules to further mediate cell attachment and orientation. Indeed, the elongated morphology and alignment induced by grooved substrates may resemble the natural state of many cell populations in vivo and is observed to occur in a wide range of cell types, including fibroblasts,[91] osteoblasts,[92] nerve cells,[93] and MSCs,[88] which respond profoundly to grooved substrates and have been shown to upregulate the expression of components of the ECM[94] as well as proteins central to cellular adhesion[95] and the transduction of mechanical forces.[96]

As with the topographies discussed above, nanogroove features seem to influence directly the formation of focal adhesions in cells cultured in vitro, by simultaneously providing vertical ledges which disrupt integrin binding as well as topographically planar areas, which facilitate integrin binding. These modulate protein adsorption and integrin binding and furthermore also influence the orientation of focal adhesion formation,[97,98] Focal adhesion alignment occurs following filopodial extension within the topographical grooves (Figure 2, B), resulting in both adhesion proteins and actin filaments becoming aligned parallel to groove direction (Figure 4, C).[99]

At present no clear conclusions have been reached about the absolute dimensions required for cellular and focal adhesion alignment; most likely this process is cell-specific and dependent on whether the cell is isolated or has established contact with adjacent cells.[100] It is probable that an interplay between groove pitch and groove depth regulates adhesion alignment, yet recent studies indicate that groove depth is the more influential.[100,101] A pivotal study by Crouch et al. investigated anisotropic cell behavior in human dermal fibroblasts with respect to the aspect ratio (depth to width) of gratings. Human dermal fibroblasts were found to increase their alignment and elongation with increasing aspect ratios. Whereas aspect ratios as small as 0.01 induced significant alignment (60%), the maximum aspect ratio required for 95% alignment was 0.16 (ref.[102]).

Studies show that cellular cytoskeletal and adhesion complex alignment is generally more pronounced on patterns with ridge widths between 1 and 5 μm than on grooves and ridged topographies with larger lateral dimensions,[6,98,103,104] and that cells cultured on grooves with nanoscale widths produce focal adhesions that are almost exclusively oriented obliquely to the topographic patterns.[105] This occurs predominantly on topographical ridges as opposed to grooves, effectively limiting the length of focal adhesions formed perpendicular to the groove orientation. Thus, it arises that grooved nanoscale topographies can influence both the adhesion

direction as well as adhesion reinforcement. Indeed, we have recently reported that nanogroove arrays influence both focal adhesion frequency and orientation, and have correlated this to changes in progenitor cell differentiation.[106]

Importantly, studies thus far suggest that contact guidance is not initiated on groove depths below <35 nm[107] or ridge widths <100 nm (Table 3).[101] Similarly, contact guidance or a modulation in focal adhesion formation is not initiated on anisotropic grooved topographies with feature widths significantly greater than that of the cellular diameter. Such topographies, it can be argued, are essentially planar areas separated by a topographical step that neither perturb integrin activation and clustering nor offer an increased surface area to facilitate focal adhesion formation.

Nanotopography and cellular function

It is becoming increasingly clear that epigenetic modulation of cellular function induced by mechanical and topographical cues has a central role in the regulation of differential behavior, a property that can be exploited in the fabrication of implantable materials to direct cellular differentiation and enhance construct biocompatibility. Cellular mechanotransduction relies on the ability of proteins of the focal adhesion to change chemical activity state when physically distorted, converting mechanical energy into biochemical energy by modulating the kinetics of protein-protein or protein-ligand interactions within the cell. However, little is known about the effects of topographical modification on cellular function or the role of nanoscale features on integrin-mediated activation of adhesion proteins and downstream signaling pathways.[50] The ability of proteins to reside in both the activated and quiescent states, and to shuttle between the cytoplasmic and nuclear compartments, is of key importance in intracellular signaling and the central mechanism behind altered gene expression, as mediated by cellular adhesion.[108]

The integrin-dependent signaling pathways are mediated by nonreceptor tyrosine kinases,[109] most notably focal adhesion kinase (FAK), which is constitutively associated with the β-integrin subunit. FAK localizes at focal adhesions or early focal complexes and can influence cellular transcriptional events through adhesion-dependent phosphorylation of downstream signaling molecules, thus controlling essential cellular processes such as growth, survival, migration, and differentiation.[110–112] Extensive evidence has shown that FAK is activated in response to both the ECM and soluble signaling factors, suggesting that the FAK family may be at the crossroads of multiple signaling pathways that affect cell and development processes. Integrins are also important signal transduction molecules in their own right, activating multiple signaling cascades including Ras and p38 mitogen-activated protein kinase, calcium channels, and mechanosensors.[113]

The extracellular signal-regulated kinases (ERK) 1 and 2 (refs.[114,115]) are members of the mitogen-activated protein kinase pathways and are activated in adherent cells by FAK to act as a mediators of both cellular differentiation[116] and survival.[112] Studies with MSC populations and primary human osteoblasts indicate that FAK-mediated ERK1/ERK2 signaling is an important modulator of osteospecific and adipospecific differentiation,[117] implying that topographical modification of an orthopedic construct may be a viable strategy to regulate both cellular adhesion and subsequent osteospecific differentiation. Indeed, nanotopographical modification that induces an increase in integrin-substratum interaction and cellular spreading has been shown to upregulate the expression of FAK and ERK1/ERK2 in osteoprogenitor cells.[51,62] Furthermore, both ERK1/ERK2 signaling and focal adhesion formation is decreased in MSC populations cultured on topographical features that approach 100 nm in height.[85] One obvious advantage of this osteodifferential response in progenitor populations is that of in creased implant stability and a reduction in repeat surgery.

As well as acting to promote differential function in adherent cells, it seems that surface features also induce a significant response in nonadherent cell types. Although several studies have reported on the effects of nanotopographical structures on immune cell activation,[118–120] the mode of signal transduction remains unclear. Emerging data suggest that the proteins involved in adhesive processes in cells of the immune system are analogous to those found in focal adhesions in adherent cells,[121–123] and that leukocyte binding to ECM components can induce FAK-mediated immune cell activation,[124] phagocytosis, [125] and chemokine-mediated migration.[126] Although the immune response is tightly regulated by the complex interplay of events and interactions between its constituent cells, preliminary studies suggest that implantable materials could be fabricated with nanoscale structures to modulate the immune response, and that as in adherent cell types, this may be through FAK-mediated activation of critical signaling pathways.

Recent work from several laboratories also points to the importance of FAK in influencing the angiogenic response by mediating the synthesis of vascular endothelial growth factor[127,128] and modulating the activity of FAK.[129] In particular, the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ have been reported to specifically regulate the Ras-ERK pathway in endothelial cells,[130] and it has been reported that downstream ERK1/ERK2 phosphorylation is important for the enhanced chemotactic response of vascular smooth muscle cells to fibroblast growth factor.[131,132] Importantly, fibroblast growth factor synthesis and angiogenesis have also been shown to be upregulated on nanophase materials.[129]

Because a diverse variety of signals affect cellular differentiation, it seems very likely that no single signaling pathway is responsible for regulating adhesion mediated cell function. Rather, a network of signaling pathways is probably at work, and FAK is at the helm of integrating these signaling activities. As well as differentiation through ERK signaling, autophosphorylation of FAK initiates the formation of dynamic molecular complexes that contain numerous signaling proteins (e.g., Src, p85 regulatory subunit of phosphatidylinositol-3-kinase, phospholipase Cγ, Grb-7, and Shc).[22] These pathways may be activated to differing degrees by different integrin-ECM interactions, influencing cellular function.

Thus, nanotopography can be considered an important mediator of both cellular adhesion as well as differential function, acting to impart changes in cellular behavior through the modulation of focal adhesion reinforcement and protein interaction kinetics. Furthermore, it may be feasible to enhance the in vivo response to a biomaterial construct by implementing nanoscale modification to regulate cellular differentiation, the immunological response, and angiogenesis (Table 4).

To summarize, nanostructures have been shown to induce significant modulation of focal adhesion formation, cytoskeletal development, and cellular spreading, changes that are subsequently transduced to signaling pathways, affecting functional differentiation through integrin-specific signaling pathways. It would seem that topographical disruption of focal adhesion formation in cellular populations is mediated directly through the perturbation of integrin activation and clustering, a phenomenon that has been shown experimentally to be dependent on nanotopographical features of critical dimensions and density. Nanoscale protrusions disrupt the lateral spacing of integrin clustering, and activation of focal adhesion proteins when feature dimension are less than 70 nm and feature spacing lies in the 70-to 300-nm range. Integrin clustering and the anisotropic elongation of the adhesion plaque is restored as substrate features approach the micron scale. Conversely, the inverse is true on nanoscale pit topographies; here a pit diameter and depth <70 nm facilitates sufficient integrin clustering for focal adhesion reinforcement.

Grooved substrates can be seen as an anisotropic collection of alternating nanopits (grooves) and nanoprotrusions (ridges) and, as such, provide alternating planes for focal adhesion formation. Although it has not been verified experimentally, it seems sensible that reduction in the lateral dimensions of the ridge structures to <70 nm and increase in the groove widths to 70–300 nm will bring about perturbation of integrin clustering and disruption of focal adhesion formation.

As well as disrupting focal adhesion formation, nanofeatures are also reported to increase focal adhesion formation in adherent cells, the mechanisms responsible would seem to be based on an interplay between two promoting and perturbing mechanisms. First, an increase in the inter-feature spacing of nanoprotrusions effectively increases the number of available integrin binding sites conversely the opposite is true of nanoscale pit topographies. Introducing nanoscale features with lateral dimensions >300 nm provides no perturbation to focal adhesion formation if the feature height/depth is <70 nm, yet increases the total surface area over which an adherent cell can establish cell-substratum contacts, effectively increasing integrin-ligand interactions. Second, nanoprotrusion features with edge-edge spacing or vertical dimensions of <70 nm do not perturb focal adhesion formation but may act to trap proteins of the ECM that provide integrin binding motifs, again increasing the interactions between transmembrane integrins and substratum-bound proteins. Again the opposite is true for nanopit topographies, which see an increased potential for protein capture in the absence of diminished integrin binding when interfeature spacing is >70 nm.

It is also known that enhanced cellular function can be induced by nanotopographical modification in the absence of increased focal adhesion frequency, and that signaling pathways crucial for cellular differentiation can be initiated by a diverse range of nanoscale features. It has earlier been observed that the formation of elongated rather than numerous focal adhesions is important in osteospecific differentiation and that adhesion elongation relies on enhanced integrin clustering. With focal adhesion reinforcement, increased FAK is recruited and subsequently activated to initiate downstream signaling cascades. Conversely, when focal adhesion frequency is reduced to that of sparse focal complexes, such mechanosensitive signaling events are reduced. This balance between mature focal adhesion formation and related cell signaling seems to be critical in MSC differentiation.

Future perspectives

The exact mechanisms involved in integrin clustering and focal adhesion formation are still being investigated; however, recent studies indicate that the focal adhesion protein talin makes a determining contribution to adhesion disruption through nanotopographical features. Although the structure of talin and its precise interactions at the focal adhesion plaque are still unknown, it is accepted that this protein provides the link between the transmembrane integrin heterodimer and the contractile apparatus of the cell, and it is the conformation and number of integrin-binding domains of this molecule that dictate the critical spacing of bound integrins required for focal adhesion activation. Another proposal is based on integrin clustering and the forces needed for protein reinforcement. It seems likely that focal adhesion growth is a function of intracellular force—a parameter governed by initial integrin clustering. Integrin clustering in cells cultured on disruptive nanofeatures can only occur at the interfeature areas, effectively limiting the cluster sizes and the early forces that may be generated, essentially perturbing focal adhesion formation and FAK activation.

With a growing number of studies indicating that topographical modification of the cell-substrate interface is a significant regulator of cellular adhesion and function, we may see modified biomaterials in clinical use in the near future. In particular, biodegradable devices may be functionally modified to control cellular interactions, with an aim to enhancing tissue

regeneration. The next stage, then, in the evolution of biomaterial design may rely on the topographical modification of advanced materials that have been fabricated to include a bioactive component, with an aim to regulating cellular adhesion and differentiation followed by controlled construct resorption.

One important outcome of the mounting data relating cell adhesion to nanoscale features is the development of smart multiphase materials for a specific regenerative application. It can be proposed that optimal tissue regeneration can be induced by selective cell adhesion/activation —an ideal that may be achieved by the inclusion of discrete surface nanofeatures on implantable materials. Indeed, a preliminary study by Dalby et al. reports on the use of nanotopographical features to induce selective adhesion of endothelial over fibroblasts and blood components.[133]

The fabrication of complex three-dimensional biomedical devices to include nanoscale features, however, is a complicated process associated with low reproducibility and represents a major challenge in the field of next-generation biomaterials. However, sophisticated modeling and production methods of small devices, in particular replica and injection molding, are advancing the field of nanofabricated biomaterials. It follows, then, that new technologies arising particularly from the microelectronic and plastics industries will indirectly facilitate the production of next-generation biomedical devices.

The findings presented within this review identify the cellular response to topographical features in vitro and indicate that topographical modification can be employed to regulate adhesion in vivo at the cell-device interface; furthermore, the critical dimensions required for integrin disruption have been identified. It follows, then, that topographically modified devices may enhance the differential function of endogenous cellular populations, have critical implications for tissue repair, and possess the potential for future clinical translation.

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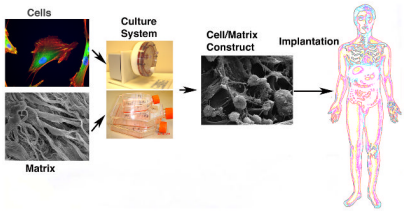

Figure 1.
The central dogma of tissue engineering. Cells are isolated and combined in vitro with a suitable scaffold system. Culture systems are used to encourage cellular infiltration and proliferation before being transplanted to a site of disease or compromise.

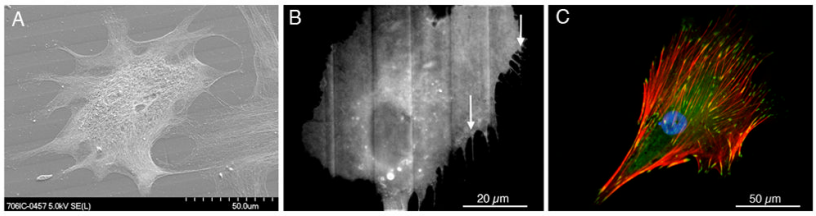

Figure 2.
Cell-substrate interactions and focal adhesion formation. (A) Adherent cells form dynamic actin-rich extensions during the process of cellular spreading and migration and (B) probe the underlying (grooved) substratum with fine filopodial extensions (arrows) from the leading and trailing free edge. (C) Adherent cells maintain cellular integrity through a dynamic network of contractile actin stress fibers (red) that terminate in focal adhesion plaques (green), molecular complexes that intimately connect the cytoskeleton with the extracellular matrix.

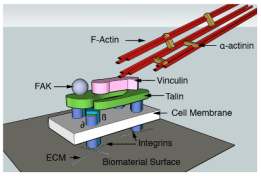

Figure 3.

A simplified overview of the molecular interactions occurring at the focal adhesion. Focal adhesions are macromolecular structures that serve as mechanical linkages of the cell cytoskeleton (F-actin) to the extracellular matrix (ECM), and as biochemical signaling hubs involved with the transmission of external mechanical forces to changes in cell function through the regulated interactions of focal adhesion associated signaling molecules.

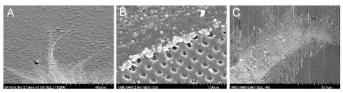

Figure 4.

Nanoscale topographical features influence cellular spreading and focal adhesion formation. (A) Nanoprotrusion with microscale x-y dimensions and a z dimension >70 nm increases cellular spreading. Nanoisland topography increases cellular spreading by providing tactile stimuli. (B) Immuno-gold labeling of focal adhesions (electron-dense clusters) in adherent cells allows the visualization of cell-substratum interactions. Nanoscale pits >70 nm in diameter perturb integrin clustering forcing adhesion formation to occur at the interpit regions. (C) Focal adhesions as visualized by scanning electron microscopy and immuno-gold labeling indicate that grooves with z dimensions down to a minimum 30–40 nm can induce adhesion alignment to the groove orientation.

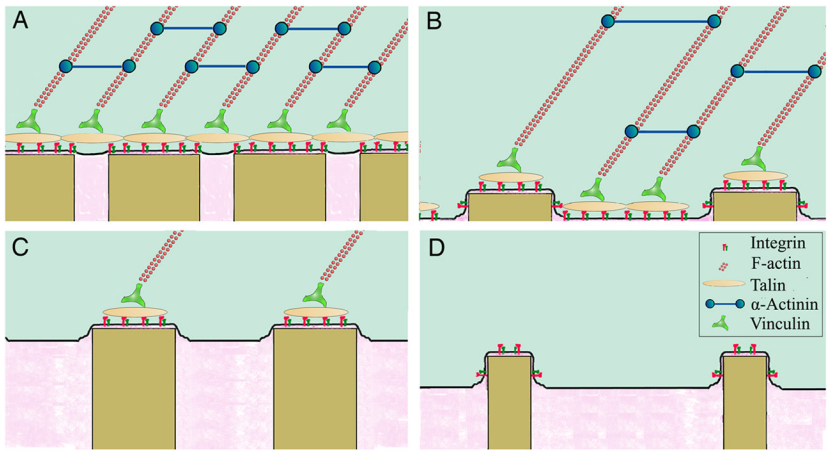

Figure 5.

The influence of nanoscale protrusions on focal adhesion formation and reinforcement. (A) Integrin clustering and focal adhesion reinforcement is unaffected on nanoscale protrusions with a critical spacing of <70 nm and a nanoprotrusion diameter of >70 nm. (B) Increasing the interfeature spacing to the submicron scale facilitates cell-basal substratum interactions below a feature height of <70 nm. (C) Conversely, increasing the feature height restricts integrin binding to the planar basal substrate and restricts focal adhesion formation to the feature apexes. (D) Integrin clustering and cellular adhesion is greatly perturbed on nanoscale protrusion with a feature diameter of <70 nm and an interfeature distance >70 nm.

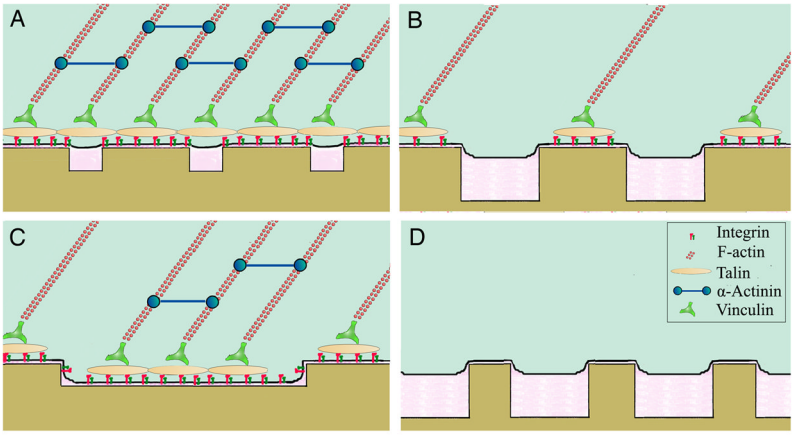

Figure 6.

The influence of nanoscale pits on focal adhesion formation and reinforcement. (A) Integrin clustering and focal adhesion reinforcement is unaffected on nanoscale pits with a diameter of <70 nm irrespective of pit depth. (B) Increasing the pit diameter to >70 nm perturbs integrin clustering when the z dimensions of the pits exceed <100 nm. (C) Conversely, increasing the pit x-y dimensions and reducing the z dimensions facilitates integrin clustering and focal adhesion formation on the basal planar surface and at the base of the pits. (D) Integrin clustering and cellular adhesion is greatly perturbed on nanoscale pits with a feature diameter between 70 and 300 nm and an interpit separation of <70 nm.

Table 1

The influence of nanoscale protrusions on cellular adhesion. Cellular adhesion is decreased on structures measuring <70–100 nm in height, or when the feature diameter is <70 nm. Conversely, studies show an increase infocal adhesion formation when cells are cultured on nanofeatures with lower aspect ratio features with an interfeature or feature diameter >70 nm.

Ref.	Cell type	Chemistry	Pitch	Diameter	Height	Adhesion modulation
[64]	Human foreskin fibroblast	Poly(L-lactic acid)	590 nm	500–550 nm	250–300 nm	Increased adhesion formation
[67]	h-TERT fibroblast cells	poly(styrene)	1.67 μm±0.66 μm	0.99 μm±0.69 μm	95 nm	Reduced cell adhesion
[68]	Human Bone marrow stromal cells	poly(styrene)/poly(n-butyl methacrylate) blend	-	0.99 μm±0.69 μm	90 nm	Reduced adhesion formation
[72]	Human foetal osteoblastic cells	Poly(lactic acid)/poly(styrene) blend	-	-	15–45 nm	Increased adhesion formation
[71]	Human mesenchymal stem cells	Ti	40 nm	28 nm	15 nm	Increased adhesion formation
[69]	h-TERT fibroblast cells	poly(styrene)/poly(bromo styrene) blend	527 nm	263 nm	13 nm	Increased adhesion formation
[65]	Human foetal osteoblastic cells	poly(styrene)/poly(bromo styrene) blend	527 nm	263 nm	11 nm	Increased adhesion formation
[71]	Human mesenchymal stem cells	Ti	40 nm	28 nm	15 nm	Increased adhesion formation

Table 2

The influence of nanoscale pits on cellular adhesion. Cellular adhesion is decreased on nanopit arrays possessing x-y and z dimensions of <70–100 nm. Focal adhesion formation is also reduced in cells cultured on nanopit arrays with a pitch <70 nm. Conversely, adhesion is reported to increase when topographical structures exceed or do not meet these critical feature dimensions.

Study	Cell type	Chemistry	Pitch	Width	Depth	Adhesion modulation
[138]	hematopoietic stem cells	TIO_2	15 nm	15 nm	1.5 μm	Increased adhesion and viability
[139]	Corneal epithelial cells	Silicone	400 nm	Variable	350 nm	Increased adhesion
[84]	Osteoprogenitor cells	poly(carbonate)	300 nm Hexagonal array	120 nm	100 nm	Decreased adhesion formation
[86]	h-TERT fibroblast cells	poly(methyl methacrylate)	300 nm Hexagonal array	120 nm	100 nm	Decreased focal and fibrillar adhesion formation
[140]	Corneal epithelial cells	poly(acrylic acid)	Variable	100	50 nm	Increased adhesion
[35]	Human foetal osteoblastic cells	poly(L-lactic acid)/pS blend	Variable	400 nm	45 nm	No modification
[35]	Human foetal osteoblastic cells	poly(L-lactic acid)/pS blend	Variable	90 nm	14 nm	Increased adhesion
[141]	h-TERT fibroblast cells	poly(caprolactone)	200 nm	75 nm	-	Decreased adhesion formation

Table 3

The influence of nanoscale grooves on cellular adhesion. Nanogroove substrates modulate adhesion orientation as well as regulating adhesion frequency. Studies indicate that substrates with a groove pitch b35 nm and a depth <70 nm do not initiate contact guidance in most cells.

Study	Cell type	Chemistry	Pitch	Depth/Height	Adhesion modulation
[109]	Human corneal epithelial cells	Silicon dioxide	400 & 4000 nm	70 & 1900 nm	Oblique Adhesion formation
[109]	Human corneal epithelial cells	Silicon dioxide	800–2000 nm	550–1150 nm	Parallel adhesion formation
[142]	Human osteoblasts	poly(methyl methacrylate)	10 μm	300 nm	Reduced focal adhesion frequency
[6]	Human corneal epithelial cells	Silicon dioxide	400 nm	250 nm	Adhesions restricted to the tops of the ridges
[76]	Human foreskin fibroblasts	Silicon dioxide	230 nm	200–300 nm	Increased Integrin β_3, Adhesion alignment
[143]	Rat aortic endothelial cells	Titanium oxide	750 nm	150 nm	Increase adhesion
[105]	Rat dermal fibroblasts	poly(styrene)	100 nm	35 nm	Reduced alignment and adhesion

Table 4

The influence of nanoscale features on cellular function. GFAP, glial fibrillary acidic protein; MAP2, mitogen-activated protein 2. Nanotopographical modification induces functional changes in a wide variety of cell types. Thus far, nanoscale pits have shown to upregulate ERK1/ERK2 and FAK signaling in osteoblastic cells. Nanoprotrusions enhance the synthesis of osteospecific protein and fibroblast growth factor in osteoprogenitor and endothelial cells, respectively. Nanogrooves have been shown to induce neurone differentiation in mesenchymal stem cells and the upregulation of proteins concerned with proliferation in canine kidney cells.

Topography	Study	Cell type	Chemistry	Serum proteins	Width	Pitch	Depth/Height	Functional modification
Nanoscale pits	[144]	Human osteoblasts	poly(methyl methacrylate)	10% fetal bovine serum	120 nm	300 nm	100	Upregulation in ERK 1/2 signalling
	[35]	Human fetal osteoblastic	poly(L-lactic acid)/poly(styrene) blend	10% fetal bovine serum	500 nm	variable	29 nm	Increase in FAK phosphorylation
Nanoscale protrusions	[62]	Human osteoprogenitor cells	Ti	10% fetal bovine serum	28 nm	40 nm	15 nm	Upregulation of osteospecific proteins - Osteopontin and osteocalcin
	[133]	Bovine postcapillary venular endothelial cells	hydroxyapatite	10% fetal bovine serum	200 nm	variable	40 nm	Increased synthesis of fibroblast growth factor
Nanoscale grooves	[97]	Human mesenchymal stem cells	Poly(dimethylsiloxane)	substrates coated with bovine collagen	350 nm	700 nm	350 nm	Upregulation of neuronal markers - MAP2 and GFAP
	[100]	Canine kidney cells	poly(styrene)	10% fetal bovine serum	350 nm	350	40 nm	Upreulation of cyclin D1 and keratin 18