Restricted cell functions on micropillars are alleviated by surface-nanocoating with amino groups

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Summary statement
Several medical implants fail due to insufficient tissue contacts. Biomaterials with spiky surfaces induce phagocytic processes. Plasma polymer nanocoating is able to alleviate unfavorable cell physiological reactions on microtopographies.

Abstract
The topographical and chemical surface features of biomaterials are sensed by the cells, affecting their physiology at the interface. On titanium we recently discovered an attempted caveolae-mediated phagocytosis of the sharp-edged microstructures by osteoblasts. This active, energy-consuming process resulted in decreased osteoblastic cell functions, e.g. extracellular matrix proteins. However, chemical modification with plasma polymerized allylamine (PPAAm) was able to amplify osteoblast adhesion and spreading, resulting in better implant osseointegration in vivo. In the present in vitro study we analyzed whether this plasma polymer nanocoating is able to attenuate the microtopography-induced changes of osteoblast physiology. On PPAAm we found a higher cell interaction with the geometrical micropillars already after 30 min, and a less distinct reduction in the mRNA expression of collagen type I, osteocalcin and fibronectin after 24 h of cell growth. Interestingly, the cells are more active and sensitive on PPAAm-coated micropillars and react with an impressive calcium ion mobilization after stimulation with adenosine triphosphate (ATP). These results highlight the importance of the osteoblasts establishment of cell-surface contact for the manifestation of their cell function.

Keywords: Cell-material interaction; Osteoblasts; Calcium mobilization, mRNA expression, Microtopography; Plasma polymer nanocoating
Introduction

Cells are sensitive to their underlying micro- and nanotopography; in particular topographical features on microscale level offer cues that cause diverse cell responses. These responses range from altered adhesion behavior, resulting in changed integrin expression as well as signaling to a disturbed cell function (Luethen et al., 2005; Staehlke et al., 2015; Moerke et al., 2016). Because of steadily increasing human lifespans and the associated rising requirements placed on body parts, temporary and permanent orthopedic implants are gaining more and more relevance. An orthopedic implant should establish a mechanically solid interface with complete fusion of the material within the native bone tissue. This process is called osseointegration and can determine the successful ingrowth of the implant into the native bone, which is achieved by accelerating the onset and the rate of immediate osteoblastic cell attachment as well as proliferation (Bacakova et al., 2011). Therefore, implant surface design with its topographical as well as chemical properties, has a huge impact on osseointegration [3–6]. Implant materials have to fulfill certain requirements concerning corrosion resistance and biocompatibility. Titanium is an inert and biocompatible material and therefore commonly used as an orthopedic implant material (Bacakova et al., 2011). Osseointegration is enhanced on rougher titanium surfaces rather than on smooth ones, but it is also accompanied by changes in the cell physiology, such as the integrin expression if the titanium surface features sharp edges (Luethen et al., 2005; Bacakova et al., 2011). New implant design strategies pursue the development of new bioactive surfaces to evoke cellular responses which promote osseointegration (Wennerberg and Albrektsson, 2009); these include the coating of the surface to change its chemistry. In this case, coating with polymerized allylamine (PPAAm), which was deposited by a physical, low-pressure plasma process, led to an improved osseointegration of titanium implants in vivo (Gabler et al., 2014) which may be caused by the enhanced cell adhesion and spreading investigated in detail in vitro (Rebl et al., 2012; Finke et al., 2007; Kunz et al., 2015). PPAAm is a nanometer-thin, positively charged amino-functionalized polymer layer rendering the surface
more hydrophilic (Finke et al., 2007). Regular geometric micropillar topographies with the
dimension of 5 µm in pillar length, width, height and spacing (P-5x5) were used as artificial
surfaces, extending the work of stochastic surface models with the advantage of constant
and recurring topography variables (Luethen et al., 2005). Previous studies showed that
osteoblastic cells mimic the underlying geometrical micropillar structure with their actin
cytoskeleton, and we recently discovered an attempted caveolae-mediated phagocytosis of
each micropillar beneath the cells (Moerke et al., 2016). Characteristic for this process was
the dot-like caveolin-1 (Cav-1) protein and cholesterol accumulation on the micropillar
plateaus after 24 h. Cav-1 and cholesterol are the major components of caveolae and are
essential for the formation and stabilization of the caveolar vesicles (Parton et al., 2013).
Caveolae are a special form of cholesterol and sphingolipid-enriched plasma membrane
subdomains, called lipid rafts, distinguish themselves via the containment of the caveolin-1
protein. These special plasma membrane domains are involved in various cellular
processes, including phagocytosis (Parton et al., 2013; Pelkmans and Helenius, 2002). The
attempted caveolae-mediated micropillar phagocytosis was accompanied by increased
intracellular reactive oxygen species (ROS) production, reduced intracellular adenosine
triphosphate (ATP) levels and a higher mitochondrial activity (Moerke et al., 2016). A
consequence of this energy-consuming process was the reduction of the osteoblast marker
production; these are extracellular matrix (ECM) proteins involved in the generation of new
bone tissue, e.g. collagen type I (Col1) or fibronectin (FN). As a result, the cells on the
micropillars showed diminished osteoblast cell function, which was shown as well on
stochastically structured, corundum blasted titanium with spiky elevations (Moerke et al.,
2016). This indicates that the given surface microtopography also strongly affects the cell
physiology in a negative sense if surface characteristics are sharp-edged. In this study we
wanted to shed light on the question of whether a chemical surface modification such as
PPAAm, which has a positive impact on cell spreading, adipose-derived stem cell
differentiation (Liu et al., 2014) and osseointegration, can alleviate this microtopography-
induced negative cellular outcome.
Results and Discussion

Nanocoating and surface characteristics:
To chemically functionalize a biomaterial surface the deposited nanolayer should have a homogenous distribution. Therefore, a surface characteristic using X-ray photoelectron spectroscopy (XPS) to detect the elemental surface composition is mandatory. The density of the amino groups NH$_2$/C of the plasma polymerized allylamine (PPAAm) nanolayer was about 3% and the film thickness ~25 nm due to the plasma deposition time of 480 s. After the PPAAm coating, no Ti or Si components were found on the surface (Fig. 1). The homogenous PPAAm coating renders the surface more hydrophilic with water contact angles for PPAAm-coated Ti versus untreated Ti (planar) of 48° and 78°, respectively (Finke et al., 2007; Liu et al., 2014), and creates positively charged surfaces (zeta potential of PPAAm-coated Ti versus untreated Ti with +7.7 mV and -3.4 mV, respectively) (Finke et al., 2007).

Nanocoating and cell morphology:
The micropillars were coated with PPAAm to enhance the cell-material contact by increasing the surface-occupying cell area. We observed earlier, that the PPAAm coating promotes the initial cell spreading after seeding the cells and increased the cell mobility (Finke et al., 2007; Rebl et al., 2016). The PPAAm coating has proved to be a cell adhesive layer and osteoblasts can overcome topographical restrictions, e.g. guidance due to machined grooves (Rebl et al., 2012; Rebl et al., 2016). We observed that the osteoblastic cells try to internalize these surface-fixed micropillars (Moerke et al., 2016) and hypothesized that cells possibly increase their cell-surface contact, because this cell-surface contact is essential for the maintenance of the osteoblast specific cell function (Saldana and Vilaboa, 2010).

In Figure 2, the enhanced cell spreading after PPAAm-coating is impressively to be seen. The scanning electron microscopy (SEM) images show widely spread-out cells reaching the bottom of the microtopography already after 30 min on PPAAm-coated pillar surfaces. On
the uncoated micropillars, the cells exhibited a more spherical form and sit on a maximum of four pillars, whereas on PPAAm, the cells covered more than four pillars. After 24 h of cultivation, the changes were no longer as drastic as those seen after 30 min. The osteoblasts on the PPAAm-coated micropillars were still more spread out, with the micropillars more imprinted into the cells. Enhanced spreading as well as molding into the topography was also observed on stochastic surfaces after PPAAm coating, e.g. on machined Ti (Rebl et al., 2012; Finke et al., 2007).

**Cells’ caveolin-1 and actin filaments:**

Characteristic for the attempted caveolae-mediated phagocytosis on microstructures is a clear dot-like clustering of Cav-1 accompanied with accumulated, short filaments of actin on the micropillar plateaus. To be sure that this is a common phenomenon of cells on micropillars we also observed the Cav-1 distribution in human primary osteoblasts, human primary fetal osteoblasts as well as the osteoblastic cell lines Saos-2 and U-2Os and found the same accumulated pattern (Moerke et al., 2016). The question arose, whether the osteoblasts still express the Cav-1 clusters and the clustered actin filaments on the pillar tops after coating with PPAAm. Immunofluorescence labeling reveals less Cav-1 cluster formation on the P-5x5 + PPAAm (**Fig. 3**), which is comparable with Cav-1 on uncoated micropillars after the longer cell cultivation up to 96 h (Moerke et al., 2016). Cav-1 was found to be more concentrated around the pillar walls and in regions of the pillar edges after the 24 h growth period.

The actin cytoskeleton was accumulated in a ring-like fashion around the PPAAm-coated micropillars after 24 h (**Fig. 4A**), similar to the cells on the uncoated micropillars not earlier than 72 up to 96 h cultivation time (Moerke et al., 2016). The correlative light and electron microscopy (CLEM) is a method to demonstrate the cell components, e.g. the actin filaments as observed in our experiments, in precise positions to the sample topography, the micropillars. Thus, it is impressively to be seen that the pillars are enveloped by the actin filaments (**Fig. 4B**). Comparing our MG-63 cells with human primary osteoblasts we
recognized earlier the same accumulation of the actin filaments on pillared microstructures except that primary cells have additionally some longer actin fibers (Moerke et al., 2016). Plasma membrane reorganization works in concert with the underlying actin cytoskeleton to mediate morphological changes and cell-surface contacts (Head et al., 2014). Actin remodeling is especially sensitive to signals that are generated at the membrane-cytoplasm interphase (Janmey and Lindberg, 2004). The actin reorganization at the micropillar topography was shown to be characteristic for an attempt at phagocytosis. Our previous study revealed a high energy requirement (ATP loss 1.45-fold) for the cell's attempted phagocytosis of every single pillar in a fixed position (Moerke et al., 2016). This leads to the question: why do the cells undergo the nuisance of pillar phagocytosis when this is accompanied by high energy requirements and negative cellular outcome? A possible explanation for this cell behavior would be the fact that osteoblasts are attachment-dependent cells which want to ensure the highest cell-surface contact. The maintenance of the osteoblastic function relies on this contact; therefore a certain cell-surface contact is needed for adequate osteoblastic cell function (Saldana and Vilaboa, 2010).

Surfaces, such as the geometrical micropillars as well as rough corundum-blasted Ti, which is a commercially used orthopedic implant design, offer the osteoblasts insufficient surface interaction area and induce internalization processes of the surface features [3]. These lead to impaired osteoblast cell function as well as biomaterial-tissue interactions in the first phase of osseointegration. A coating such as PPAAm, which enhances the cell attachment and spreading and the interaction with the topography, was shown to reverse these topography-induced negative effects and may also be beneficial for the improvement of commercially used rough surfaces.
Gene expression:

In our earlier experiments we observed a significantly increased Cav-1 gene expression on the micropillars, likely due an attempt to phagocytize every individual post (Moerke et al., 2016). The PPAAm coating influenced this process such that the Cav-1 gene expression showed only a trend toward elevation (Fig. 5B).

The osteoblast marker gene expression was drastically reduced after 24 h on uncoated micropillars due to the attempt to phagocytize these fixed structures, as revealed in Moerke et al. (2016). The subsequent question was if the PPAAm coating, with its advantage of enhanced cell-material contact, is able to positively influence the osteoblast marker expression. The uncoated micropillars led to significantly reduced gene expression of collagen-I (Col1), fibronectin (FN) and osteocalcin (OCN) (Fig. 5A), whereas after PPAAm coating these genes showed only a trend toward decrease or no difference to the planar reference. However, the gene expression of alkaline phosphatase (ALP) was significantly diminished on uncoated as well as PPAAm coated micropillars (Fig. 5A). ALP is involved in the mineralization of the bone ECM. This leads to the assumption that the ALP gene expression is not caused by lessened cell-surface interaction, but more by the phagocytic process itself. If a process requires high energy demands, such as phagocytosis, other energy expenditures must be abated, e.g. the expression of proteins, such as ALP, which are redundant in case the cells produce less ECM or are occupied with the establishment of sufficient cell-ECM contact (Ataullakhanov and Vitvitsky, 2002). In particular it is to be taken into account that high energy demands are placed on osteoblasts not only during phagocytosis, but also during the production of the ECM, by expressing and secreting ECM proteins (Komarova et al, 2000). However, in experiments with polished titanium (Ti) (roughness average Ra = 0.19 µm) the PPAAm coating promoted the ECM expression of MG-63 cells, as is seen by the middle-to-late-stage differentiation-related ALP- and bone sialo protein (BSP)-mRNA (Nebe et al., 2007).
Cell adhesion mechanism:

An important question remains: how does the PPAAm nanocoating enhance the initial cell spreading? The pericellular matrix substance hyaluronan (HA) plays a key role in initial interface interactions and is essential for the first encounter of cells with the substrate (Fig. 6) (Zimmermann et al., 2002; Finke et al., 2007; Nebe and Luethen, 2008). HA is a high molecular weight (nearly 7000 kDa) linear polysaccharide originally found in soft tissue that consists of N-acetyl-D-glucosamine and D-glucuronic acids, responsible for the negative charge of the molecule (Chopra et al., 2014). The glycosaminoglycan HA is produced by the cells itself which causes a negatively charged spherical shell, e.g. completely enveloping chondrocytes with 4.4 µm in thickness as measured by Cohen et al. using SEM (Cohen et al., 2014). Nebe et al. (2008) showed that a positively charged PPAAm-coating on a Ti surface could boost the initial cell adhesion, including fast formation of adaptor proteins in focal adhesions. They hypothesized that a positively charged surface provided the basis for HA-mediated attachment and spreading. HA mediates the immediate adhesion of osteoblasts to the material surface within seconds, and the first contact is hampered on different surfaces, e.g. titanium or even collagen-I, if HA is cleaved by the enzyme hyaluronidase (600 U) (Fig. 6) (Nebe and Luethen, 2008). For instance, the cell adhesion (5 min) is significantly reduced by about 5-fold if cells have to adhere to pure, polished Ti without their HA coat, and still reduced 1.7-fold on a collagen-I coated surface[21].

In our study the energy consumption could unfortunately, not be measured due to the characteristics of the PPAAm nanolayer. PPAAm exhibits a slight autofluorescence, especially in the green channel. This would interfere with quantitative immunofluorescence analysis of these structures. Additionally, the PPAAm coating does not allow the trypsinization of the cells in a vital state from the micropillared surfaces due to its high cell-attractive characteristics as measured earlier by a spinning disc device. MG-63 cells on PPAAm were 1.5 times more resistant to shear stress compared to cells on uncoated
titanium alloys (Ti6Al4V) (Gabler et al, 2014). For this reason, further experiments, such as determination of the cellular cholesterol amount, intracellular ATP as well as reactive oxygen species (ROS) generation could not be investigated in relation to uncoated micropillars. Instead, we observed the calcium dynamics in vital cells.

**Calcium signaling:**

In vital osteoblasts on PPAAm we could identify highly active cells by analysis of the calcium mobilization capacity. The basal level of intracellular calcium ions (0-180 s) was already significantly higher in osteoblasts established for 24 h on P-5x5 + PPAAm than on P-5x5 with mean fluorescence intensity values (MFI) of 42.03 ± 0.05 and 26.18 ± 0.14 (mean ± s.e.m.), respectively (Fig. 7A). It is impressively to be seen, that these 24 h-adherent cells on PPAAm react intensively after ATP stimulation with a high increase of intracellular calcium ions. The difference in the calcium dynamics of fluo-3 stained cells on P-5x5 + PPAAm (MFI peak at 188 – 246 s: 96.45 ± 5.36; maximum MFI value 130.35 ± 13.11) is significant compared to cells grown on P-5x5 (MFI peak at 188 – 246 s: 44.43 ± 1.84; maximum MFI value 53.36 ± 10.78) (mean ± s.e.m.) (Fig. 7A). The same cellular reactivity was found in human primary osteoblasts (HOB) after 24 h cultivation: a significantly higher calcium mobilization capacity on P-5x5 + PPAAm (MFI peak at 188 – 246 s: 91.81 ± 1.13, maximum MFI value 100.46 ± 6.47) compared to HOB cells on P-5x5 (MFI peak at 188 – 246 s: 66.62 ± 2.52, maximum MFI value 82.49 ± 7.89).

After 30 min of cell cultivation on P-5x5 + PPAAm, MG-63 osteoblasts showed not only a more spread morphology compared to uncoated pillars as shown in Fig. 3 (30 min) but also a significantly higher calcium mobilization after stimulation with ATP (Fig. 7B) (P-5x5 + PPAAm: MFI peak at 188 – 246 s: 81.40 ± 2.89, with maximum MFI value 107.30 ± 40.90; P-5x5: 49.3 ± 0.99, with maximum MFI value 53.88 ± 19.1). Already the basal calcium signal in 30 min-adherent cells is increased on P-5x5 + PPAAm (MFI mean ± s.e.m.: 55.34 ± 0.14) but not significant vs. P-5x5 (MFI mean ± s.e.m.: 37.78 ± 0.13) in direct statistical comparison using multiple t-test (Fig. 7B). Staehlke et al. (2016)
demonstrated earlier the negative influence of the cubic pillar topographies on calcium signaling and dynamics compared to a planar Ti surface. Here, we could display that cells on these pillars coated with an additional plasma-nanolayer (PPAAm) with positively charged amino groups can overcome the negative influence of the pillared topography and increase their activity despite the sharp edged surface structure. This may also contribute to the positive outcome in cell function parameters typical for osteoblasts, e.g. collagen-I and osteocalcin (see Fig.5). The local physical microenvironment can not only modulate cell shape and mechanics (Bellas et al., 2014), but also the activity of cells as shown here for intracellular Ca^{2+} levels. Although cells on pillars feel the same microtopography (range 5 µm) the additional nanolayer (PPAAm) is decisive to trigger the cells in a more active state.

We could shed light on how plasma polymer nanocoatings can strongly alleviate the restrictions of a microtopography concerning cell functions and cell signaling. This could have importance for implant surface design and production in cases where mechanical aspects of an implant have to be considered.

**Conclusion**

The present study confirms the hypothesis of the osteoblasts wanting to create the highest cell-surface contact to maintain their osteoblast-specific function, displayed by the production and secretion of ECM proteins. Moreover, this analysis demonstrates that chemical surface modification with a plasma polymer nanolayer is able to attenuate the microtopography-mediated cell alterations. This PPAAm-coating process could contribute to improved cell acceptance of rough surfaces with extraordinary topographies, e.g. corundum-blasted titanium for mechanical stability of orthopedic implants.
Materials and methods

Titanium (Ti) surfaces

Periodically microtextured samples (size 1 cm²) with a regular cubic pillar geometry on the surface having a dimension of 5 x 5 x 5 µm in width x length x height and 5 µm in spacing (P-5x5) were used (Fig. 8). As controls plane wafers (Ref) were employed. The samples were fabricated by deep reactive-ion etching (DRIE) (Center for Microtechnologies ZFM, University of Technology Chemnitz, Germany) on silicon (Si) wafers and coated with an additional 100 nm titanium (Ti) layer, as reported before (Staehlke et al., 2015; Moerke et al., 2016).

Plasma polymer nanocoating and surface characterization

Plasma polymerized allylamine (PPAAm) functionalization was performed in a low-pressure plasma process reactor V55G (plasma finish, Germany, V = 60 l) in a two-step procedure with the precursor allylamine (Rebl et al., 2012). The plasma deposition time for the pulsed plasma process was 480 s gross. The elemental chemical surface composition was determined by high-resolution scanning X-ray photoelectron spectroscopy (XPS). The Axis Ultra (Kratos, UK) ran with the monochromatic Al Kα line at 1486 eV (150 W). The spot size for high resolution measurements was limited to 250 µm. Spectra were recorded at a pass energy of 80 eV (Finke et al., 2007).

Osteoblast cell culture

The human osteoblast-like cell line MG-63 (American Type Culture Collection ATCC®, CRL1427™) was used throughout the experiments. MG-63 cells have an integrin subunit profile similar to primary human osteoblasts and were considered applicable for studying initial cell attachment processes to surfaces (Czekanska et al., 2012). In our own experiments, we found similarities in the formation of β1- and β3-integrin receptors in MG-63 osteoblasts grown on structured titanium (e.g. glass-blasted, corundum-blasted) which is
comparable with human-derived primary osteoblasts (Luethen et al., 2005). The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies GmbH, Darmstadt, Germany) with 10% fetal calf serum (FCS) (Biochrom FCS Superior, Merck KGaA, Darmstadt, Germany) and 1% gentamicin (Ratiopharm GmbH, Ulm, Germany) at 37°C in a humidified atmosphere with 5% CO₂. Human primary osteoblasts (HOB, C-12720, PromoCell GmbH, Heidelberg, Germany) were cultured in osteoblasts growth medium with SupplementMix (PromoCell) and 1% antibiotic-antimycotic (Anti-Anti 100x, Life Technologies). In the experiments, 15,000 cells/cm² were seeded onto the samples placed in NUNC 4-well dishes (Thermo Fisher Scientific, NUNC GmbH & Co. KG, Langenselbold, Germany). Before use, the uncoated Ti arrays were washed in 70% ethanol for 10 min and rinsed three times in phosphate buffer solution (PBS) (Sigma Aldrich, Munich, Germany).

**Real time quantitative PCR for mRNA expression**

Total RNA was isolated after 24 h cell growth on the samples using a NucleoSpin® RNA II kit (Macherey-Nagel GmbH & Co KG, Dueren, Germany) that includes the elimination of any genomic DNA by DNase (Macherey-Nagel) treatment. The purity and quantity of the resulting RNA were determined via measurement of the absorbance at 280 nm and 260 nm with the Nano Drop 1000 (Thermo Scientific). 50 ng of total RNA was used for first strand cDNA synthesis using Superscript® II Reverse Transcriptase and Random Primers (Invitrogen AG, Carlsbad, CA, USA). The real time quantitative polymerase chain reaction (RT-qPCR) was performed using TaqMan® Universal PCR Master Mix and TaqMan® gene expression assays for alkaline phosphatase (ALP) (Hs00758162_m1), caveolin-1 (Cav-1) (Hs00971716_m1), collagen type 1 (Col1) (Hs0016404_m1), fibronectin (FN) (Hs00900054_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs00900054_m1), and osteocalcin (OCN) (Hs01587813_g1) (all Applied Biosystems by Life Technologies GmbH, Darmstadt, Germany) following the manufacturer’s instructions. The TaqMan® PCR assay for each target gene was performed in triplicates of 4 independent experiments. The PCR was performed with RT-PCR Applied Biosystem 7500 and the data
were collected and analyzed by the 7500 System SDS Software (Applied Biosystems). Each expression was calculated relative to GAPDH (Δ Ct) and then relative to the references (ΔΔ Ct).

**Immunofluorescence staining**

MG-63 osteoblastic cells were cultured on the Ti arrays for 24 h, washed three times with PBS and then fixed with 4% paraformaldehyde (PFA) (10 min; room temperature, R.T.) (Sigma-Aldrich). After washing three times with PBS, the cells were permeabilized with 0.1% Triton X-100 (10 min, R.T., Merck), washed again three times with PBS and blocked with 2% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS (30 min, R.T.).

For actin filament staining, cells were incubated with phalloidin coupled with tetramethylrhodamine (TRITC) (5 µg/ml in PBS, Sigma-Aldrich). Nuclei were stained for 10 min with Hoechst 33342 dye (1 mg/ml; Sigma Aldrich, 1:2000 in PBS).

Caveolin was stained with the polyclonal rabbit anti-caveolin-1 (New England Biolabs GmbH, 1:400) as primary antibody for 1 h at R.T. followed by the secondary antibody anti-rabbit-IgG-AF488 (Life Technologies, diluted 1:200 in PBS) applied at R.T. for 30 min. The samples were embedded with Fluoroshield™ mounting media (Sigma-Aldrich).

**Confocal laser scanning microscopy (LSM)**

Fluorescent cells were observed using an inverted confocal laser scanning microscope LSM 780 (Carl Zeiss AG, Oberkochen, Germany) with a ZEISS oil immersion objective (C-Apochromat 63). For image acquisition the ZEISS Efficient Navigation (ZEN) 2011 SP4 (black edition) software was used. All images were displayed as three dimensional z-stacks: 13 stacks with an interval of 1 µm. High-resolution images of the actin cytoskeleton in figure 4B (insert) were recorded with the LSM 410 (Carl Zeiss AG), equipped with a 63x oil immersion objective (1.25 oil/ 0.17). For visualization of the corresponding substrate region, the reflection mode was used and the pillars were false colored in green.
**Correlative light and electron microscopy (CLEM)**

For correlative microscopy, the fluorescence images of the actin stained, non-embedded samples were acquired with the upright microscope AxioScopeA1 (Carl Zeiss) equipped with a 63x water objective. The calibration of the samples and image overlay was performed with the software AxioVision vers. 4.8.2 (tool: shuttle and find). Calibration of the samples was accomplished by using the edges of the sample as point of reference. Afterwards the samples were prepared for scanning electron microscopy as described in the corresponding paragraph.

**Scanning electron microscopy (SEM)**

Cells were grown for 30 min, or 24 h on the samples, washed with PBS three times and then fixed with 2.5% glutardialdehyde (Merck KGaA, Darmstadt, Germany) for 1 h at R.T., dehydrated through a graded series of ethanol (30, 50, 75, 90 and 100% for 5, 5, 15, 10 min and twice for 10 min, respectively) dried in a critical point dryer (K 850, EMITECH, Taunusstein, Germany) and then samples were sputtered with gold for 100 s (layer ca 20 nm) (SCD 004, BAL-TEC, Wetzlar, Germany). Scanning electron microscopy (SEM) observations were performed with a field emission (FE)-SEM (ZEISS Merlin VP compact, Carl Zeiss AG). The recovering of the desired areas for correlative microscopy was performed with the software AxioVision vers. 4.8.2 (tool: shuttle and find) after sample calibration.

**Calcium ion (Ca\(^{2+}\)) imaging in vital osteoblasts**

Fluo-3 staining of suspended cells: Trypsinized MG-63 cells were washed in PBS (+ Ca/Mg) and afterwards stained with the calcium ion (Ca\(^{2+}\)) indicator fluo-3 acetoxymethylester (AM, 5 µM, Life Technologies Corporation, Eugene, Oregon, US) in hypotonic (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffer (Staehlke et al., 2015). Subsequently, the osteoblasts were collected in isotonic HEPES-buffer and seeded on the micropillars (with/without PPAAm). After 30 min cultivation the samples were turned down in ibidi µ-dish
Fluo-3 staining of adherent cells: After 24 h cultivation of MG-63 cells on micropillars (with/without PPAAm), the cells were marked with fluo-3 AM as described above. For the analysis the osteoblasts were cultured in isotonic HEPES-buffer (Staehlke et al., 2015) as described for suspended cells.

Vital cell observations: The analysis of intracellular Ca\(^{2+}\) mobilization was performed on the confocal microscope LSM 780 (Carl Zeiss) equipped with an argon-ion laser (exc. 488 nm) using Zeiss 40x water immersion objective (C Apochromat, 1.2 W Korr M27). The measurement of the mean fluorescence intensity (MFI) of the global Ca\(^{2+}\) signal was carried out by Zen2011 (SP4, black) software (Carl Zeiss) with the mode “time series”. Images (512x512 pixels) from at least 3 to 4 (30 min and 24 h, respectively) independent experiments à 10 cells per time point (for 240 cycles) were measured at full pinhole (13.5 µm section) and the same settings. During recording of the time series over 240 cycles (2 s each) adenosine 50-triphosphate (ATP, 10 µl, SERVA Electrophoresis GmbH, Heidelberg, Germany) was added after the 90th cycle for stimulating the intracellular calcium. The evaluation of fluorescence signals of defined areas of individual single cells over time was carried out by ZEN2 (blue edition, Carl Zeiss) using the function “mean ROI”.

**Statistical analysis**

Statistical analyses were carried out with GraphPad Prism5 software (GraphPad Software Inc., La Jolla, CA, USA). Results are presented in box plots with medians, quartiles and an interquartile range (IQR) ± 1.5 x IQR. Data analyses were performed using the Mann-Whitney U test. For Ca\(^{2+}\) analyses the multiple t-test was used in addition and the time dependent data are presented as mean ± standard error of the mean (s.e.m.). P-values < 0.05 were considered to indicate significant differences.
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Competing interests

The authors declare no competing or financial interests.
References


Figure 1: Surface characterization of the material substrates via X-ray photoelectron spectroscopy. Note that after PPAAm functionalization, Ti and Si are not visible indicating a homogenous N-containing layer. (XPS, Axis Ultra, Kratos).
**Figure 2: Spreading of MG-63 osteoblasts on micropillars.** Note that after 30 min on uncoated samples (P-5x5) the cell is restricted to 4 pillars, whereas on plasma polymer-coated pillars (P-5x5 + PPAAm) the cell area is increased impressively. This advantage in the initial phase of occupying a surface is nearly adapted after 24 h. (Scanning electron microscopy, ZEISS FE-SEM Merlin VP, first row 3,000x magnification, bars 4 µm; second row 1,000x magnification, bars 10 µm).
Figure 3: Cell membrane component caveolin-1 (Cav-1) of osteoblasts on micropillars.

Note the less cluster formation for Cav-1 on plasma polymer-coated pillars (P-5x5 + PPAAm) in contrast to control samples (P-5x5) after 24 h. (Confocal microscope LSM 780, Carl Zeiss; z-stacks, magnified views: 5x zoom of the marked area with dashed lines, 10x zoom with continuous lines; bars: left = 20 µm, 5x zoom = 5 µm, 10x zoom = 2 µm).
Figure 4: Actin cytoskeleton of osteoblasts on micropillars. (A) On plasma polymer-coated pillars (P-5x5 + PPAAm) the actin cytoskeleton is organized in a typical ring-like structure (arrows) compared to the more clustered phenotype on controls (P-5x5) after 24 h. (Confocal microscopy, LSM 780, Carl Zeiss, z-stacks, bars left 20 µm, middle 5 µm, right 2 µm). (B) Correlative light and electron microscopy (CLEM) as method to demonstrate the cell components – the actin filaments – in precise positions to the sample structures – the micropillars. Insert: The reflection mode of the LSM allows indicating the pillars (green, false color) which are enveloped by the actin filaments (red). (FE-SEM Merlin, bar 10 µm; insert LSM 410, bar 5 µm).
Figure 5: Relative mRNA expression of osteoblasts after 24 h on micropillars. (A) Note that the osteoblast marker proteins alkaline phosphatase (ALP), collagen type I (Col1), fibronectin (FN) and osteocalcin (OCN) all are restricted on uncoated controls (P-5x5), which are alleviated by the plasma polymer nanolayer (P-5x5 + PPAAm), beside ALP. (B) Caveolin-1 (Cav-1) as membrane component and involved in phagocytic processes is adapted to the planar reference if the pillars are coated with the plasma polymer. All: Ref values normalized on 1, n = 4, Mann-Whitney U test, *: P < 0.05.
Figure 6: Schema: importance of the cells’ hyaluronan coat on initial cell spreading. Left: cell areas of normal osteoblasts. Right: cell areas of hyaluronidase (HAdase) treated osteoblasts. Normal cells can spread well after 30 min on pure titanium, whereas HAdase treated cells are decelerated in their surface occupation process. (Note: schema according to an original SEM image of MG-63 osteoblasts on glass-blasted Ti, HAdase 600 U treatment, (method see Finke et al., 2007).
Figure 7: Intracellular Ca$^{2+}$-mobilization after ATP-stimulation (180 s) in vital MG-63 osteoblasts on micropillars. (A) Influence of the plasma polymer nanolayer (P-5x5 + PPAAm) on the time dependent calcium signal after ATP stimulation of 24 h adherent cells. Note the impressive significant increase in intracellular Ca$^{2+}$ on P-5x5 + PPAAm. In addition, the basal calcium level is already significantly higher compared to the uncoated control (P-5x5). (B) Influence of the plasma polymer nanolayer (P-5x5 + PPAAm) on the time dependent calcium signal after ATP stimulation of 30 min adherent cells. The increase in intracellular Ca$^{2+}$ on P-5x5 + PPAAm is clearly to be seen and significant in the range of 244 – 316 s. (Fluo-3 stained cells, LSM 780, Carl Zeiss, Zeiss 40x water immersion objective, ZEN2011 software, 3 independent experiments à 10 cells per time point = 240 analyses, multiple t-test, mean ± s.e.m.).
Figure 8: (A) Schematic illustration of the deep reactive ion etching process for the generation of micropillar topography with $5 \times 5 \times 5 \, \mu m$ in width x length x height. (B) Scanning electron microscopy (SEM) images of the planar reference (Ref) and the micropillars (P-5x5) (bars 5 $\mu m$) with schematic side view (bar 20 $\mu m$).