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Published in:
Advanced Materials Interfaces

DOI:
[10.1002/admi.201600275](https://doi.org/10.1002/admi.201600275)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Zhou, Q., Wünnemann, P., Kuhn, P. T., de Vries, J., Helmin, M., Böker, A., van Kooten, T. G., & van Rijn, P. (2016). Mechanical Properties of Aligned Nanotopologies for Directing Cellular Behavior. *Advanced Materials Interfaces*, 3(18), [1600275]. <https://doi.org/10.1002/admi.201600275>

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Mechanical Properties of Aligned Nanotopologies for Directing Cellular Behavior

Qihui Zhou, Patrick Wünnemann, Philipp T. Kühn, Joop de Vries, Marta Helmin, Alexander Böker, Theo G. van Kooten, and Patrick van Rijn*

Cells are able to respond to various material surface properties such as stiffness, topology, chemistry, and wettability.^[1–6] They will adjust cellular adhesion properties, proliferation behavior, orientation, protein expression, and also differentiation.^[7–11] Knowledge about the effects of these surface confined properties onto cellular behavior will have a significant impact on the design and development of medical implants as well as scaffold materials for regenerative medicine and tissue engineering.^[3,12–17] Each of the previously mentioned materials properties has been extensively investigated by systematically changing one of these properties and elucidate the response of cells to these changes.^[18–23] Although, these studies have provided many new insights, they mostly tackled a single property, e.g., changing topology features on the model polymer poly(dimethylsiloxane) (PDMS) such as wrinkle or grating structure, altering surface wettability, or using solid materials and hydrogels of different stiffness.^[24–28] PDMS and hydrogels are popular materials because they are frequently used for biomedical applications when properly chosen. As all these stimuli induce cell responses, there is a need for cross-combination

studies to obtain deeper insight, since a surface will always be a combination of properties such as stiffness, topology, and wettability.^[8,29–32]

Here we demonstrate the importance of developing materials which enable investigations toward more complex interfaces, in order to elucidate cellular responses toward combined surface properties. For the system development, PDMS and pHEMA (poly hydroxyethyl) methacrylate hydrogels were chosen as both are already used for biomedical applications such as medical implants and contact lenses. Nanostructured PDMS was translated to pHEMA hydrogels resulting in the same topology but with a different stiffness. These property combinations were expected to trigger different cell responses both macroscopically and on a molecular scale. To the best of our knowledge, for the first time nanotopological effects are combined with mechanical material properties differing over two orders of magnitude in Young's modulus. Results of this approach show that when cells from different tissues with an intrinsically different stiffness are targeted with the intention of inducing tissue directionality and altering protein expression behavior, the surface needs to be tailored with respect to both topology and mechanical properties depending on the cell-type. Development of materials with specific physical parameter combinations along with a diverse number of tissue specific cell-types will facilitate biomedical materials design more rapidly and accurately.

Aligned nanowrinkles on polydimethylsiloxane substrates were used as the hard topological substrate. This substrate was also used as the template to prepare hydrogel wrinkles serving as the soft topological substrates with the same features and dimensions (**Figure 1**). The two different surfaces with similar topology but different stiffness were combined with three human cell-lines, namely osteoblast-like cells (sarcoma osteoblast-like cell-line; SaOs), fibroblasts (skin fibroblast; HSkF) and lens epithelial cells (LEC), to illustrate the different behavior of the cell-types originating from tissues with different intrinsic stiffness. The behavior on structured surfaces was additionally compared to behavior on flat, nonstructured surfaces of the same stiffness (planar PDMS and hydrogel). Combining cells from tissues of different intrinsic stiffness with substrata with similar topologies but differing in stiffness, provided insights into specific behavior toward surface topologies, and it stresses the need to combine surface parameters to derive a better understanding of cells at (bio)interfaces.^[16,33]

The wrinkle structures on the surface of PDMS substrates were induced by applying a uni-directional strain with subsequent surface oxidation via oxygen-plasma treatment.^[34,35] Surface oxidation induced transformation of the PDMS into a silicon oxide layer, which is much stiffer than the untreated PDMS

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DOI: 10.1002/admi.201600275

Figure 1. Schematic approach on the preparation of PDMS nanotopological surfaces and the translation of the nanotopology into soft polymeric hydrogel structures via "hydrogel nanoimprinting lithography."

(Sylgard 184; elastomer:curing agent 10:1 (w:w)).^[36] Untreated PDMS displayed a Young's modulus (YM) of 2.3 MPa (sd.: 0.1 MPa) which was determined by atomic force microscopy (AFM) (Figure S1, Supporting Information). The Young's modulus has been previously used in this context as a measure for the stiffness. Treatment with oxygen plasma for 480 s allowed the modulus (stiffness) to increase to 61.2 MPa (sd.: 3.4 MPa) (Figure S1, Supporting Information). The same oxygen plasma treatment in combination with a unidirectional strain induced by a 30% substrate extension followed by release of that strain after the oxidation, induced the formation of surface wrinkles (Figure 1 and Figure 2A–C). AFM analysis of the wrinkle dimensions obtained after the stretch-oxidation-release procedure revealed

wrinkles with 255 nm (sd.: 11 nm) in amplitude (A) with a wavelength (λ) of 1032 nm (sd.: 57 nm). The oxidized, wrinkled PDMS surface displayed an inhomogeneous distribution of stiffness. On the positions with the highest slope the YM was determined to be ≈ 45 MPa while on top of the wrinkle and on the bottom a YM of ≈ 115 MPa was found. These values deviated from the 61 MPa found for planar oxidized PDMS and are most likely caused by differences in surface contact area of the AFM tip with the PDMS, as the wrinkled PDMS is post-treated with oxygen plasma ensuring a homogenous and full surface oxidation omitting any stiffness and chemical inhomogeneity. The high slopes will also enable contact with the surface from the side of the AFM tip and hence increase the contact area which

Figure 2. AFM images and analysis of the height and mechanical properties of A–C) the PDMS and D–F) hydrogel nanowrinkles. Analysis was performed in PBS buffer to obtain a hydrated gel-wrinkle. The distribution of stiffness is strongly connected to the height position in the case of the PDMS wrinkles. For the gel wrinkles this connection between height and stiffness is less defined. Please note the differences in the images (B,E) and y-axes (C,F) for the Young's moduli (C,F).

most likely was the cause of the lower calculated YM. Although, geometry affected the YM, the values found for structured and nonstructured surfaces were in the same range and therefore considered to be comparable.

For creating soft topologies, imprinting lithography was applied. This is a technique which has been used before to create soft surface confined structures.^[30,37,38] Here the wrinkled PDMS was used as a lithographic template. Imprinting lithography method was performed under inert atmosphere (glove-box) for hydrogels to enhance the degree of polymerization by preventing oxygen-induced termination. Polymerization was performed overnight after which the PDMS substrate was removed. The surface modification of glass with acrylate moieties ensured covalent attachment of the hydrogel layers to the surface enhancing its stability. Before use as a cell substrate, PDMS surfaces were treated with air plasma to render them hydrophilic in order to match the wettability of the hydrogel. Although the hydrogel and freshly activated PDMS have similar wettability (water contact angle, WCA < 10°), the presented surfaces vary in specific chemistry which could have an additional influence. The surfaces have good cytocompatibility and hence can be used for comparison, but future endeavors should also include the development of materials which are able to cover large ranges of physical parameters without affecting the specific chemistry or at least keeping the changes in chemistry to a minimum. Unfortunately, such materials are currently unknown to us.

AFM analysis of the hydrogel wrinkles in the hydrated state displayed features representing those of the PDMS. However, the features of the hydrogel were slightly shallower due to a difference in swelling between the top of the wrinkle and the bottom and the wavelength was decreased most likely due to slight contraction of the gel-layer during polymerization and drying (Figure 2D–F). The dimensions of the hydrated wrinkles are λ : 168 nm (sd.: 24 nm) and λ : 937 nm (sd.: 46 nm). The overall stiffness of the hydrogel was found to be more than two orders of magnitude lower than the PDMS wrinkle substrate. The stiffness of the hydrogel ranged from 300 to 800 kPa and also displayed an inhomogeneous distribution most likely due to combined effects of altered surface contact area of the AFM tip as well as amount of indentation into the hydrogel material (Figure 2F).

Both substrates with the same topology were combined with three human cell-lines. The cells represent a diverse range of tissues which have intrinsically different properties. Osteoblast-like cells (here represented as SaOs) are found in bone tissue, which is very rigid (calcified bone tissue $\gg 1$ GPa) while LEC is found in the lens of the eye, representing a much softer tissue. Fibroblasts are flexible and responsible for the structural framework of tissues and found in connective tissues that can represent quite a broad stiffness range. These three cell-types have been deposited on the structured substrata as well as on flat control substrates of the same composition. The orientation with respect to the surface structuring was determined by visualizing the surface structures underneath the cells (Figure S2, Supporting Information), and relevant protein expression in relation to the used substrate was analyzed along with metabolic activity. To determine the kinetics of cellular behavior, 2 and 5 d cell cultures were investigated. These incubation times

correspond with routine culturing times toward passing the cells.

For the SaOs cells it was initially anticipated that they would respond more to the stiffer structured substrate by adhering stronger and follow the direction of the surface pattern to a higher degree than the soft topology. The initial notion originates from the fact that conventionally they reside in calcified bone. However, before calcified bone is formed, osteoblasts adhere to a collagen matrix.^[39] From microscopy analysis, two striking behaviors were found (Figure 3). First, from the 2 d culture it was seen that the SaOs cells do not align on the stiff nanotopology, while the cells did align efficiently on the hydrogel wrinkles. This was observed not only from the orientation of the actin cytoskeleton but also from the focal adhesion points and even the nuclei. The 2 d culture on PDMS hard (H) nanowrinkle (NW) substrates ($^2H_{NW}$) led to a random orientation (arrow top right of confocal laser scanning microscopy (CLSM) images indicates topology direction). Second, there was a significant difference between the amount of focal adhesions present between hard and soft topology. Apparently, the soft structuring provided better adhesion conditions which would indicate that indeed, as mentioned before, mimicking softer extracellular matrix (ECM) components will influence osteoblasts more than mimicking the actual tissue stiffness. From the control, the 2 d culture on hard, flat PDMS ($^2H_{Flat}$), it became evident that PDMS was not responsible for the lower adhesion effect since the planar substrate displays ample focal adhesion points in a random orientation (Figure 3). This implies that the presence of directional topology on a hard surface actually inhibits proper cell adhesion, which is in line with our previous studies.^[22] The effects of alignment and the amount of focal adhesion were further strengthened and more pronounced after 5 d of culturing. Even though the surface adhesion was limited for the SaOs on the H_{NW} surfaces, it did not inhibit proliferation as a confluent layer is reached after 5 d culturing (Figure 3). For the H_{Flat} surface, the cell number did not increase although the amount of focal adhesions on the flat surface increased over time indicating stronger surface interactions. Although the SaOs cells responded more strongly toward soft nanostructured surfaces, as identified by percentage of aligned cells (Figure 4), at day five there was no statistically significant difference in alignment on the hard ($65.3 \pm 5.6\%$) and soft ($57.8 \pm 5.6\%$) topology. At day five, both structured surfaces displayed confluent and highly aligned cells while both flat surfaces had cobblestone like morphologies and no alignment (Figures 3 and 4). Observing cellular morphologies showed that after 2 and 5 d culturing the SaOs displayed elongated morphology on the hard and soft wrinkle surfaces (Figure 3). Furthermore, a quantitative analysis showed that, after 2 d of incubation, SaOs cultured on the soft wrinkle surface achieved a significant increase in elongation efficiencies ($p < 0.01$) compared to those on the soft flat and hard wrinkle and hard flat surfaces. However, at day five there was no statistically significant difference in elongation efficiencies between the hard and soft topology, similar to the alignment analysis.

The amount of focal adhesions was quantified in order to determine the relative degree of cell adhesion per cell for each of the surfaces. This was done by segmentation of the focal adhesion points and obtaining a binary image, which enables

Figure 3. Confocal laser scanning fluorescence microscopy images of SaOs (top two rows), HSkF (middle two rows), and LEC (bottom two rows) cultures of 2 and 5 d on hard surfaces (flat and wrinkled) and soft hydrogel surfaces (flat and wrinkled). Images shown are for focal adhesion (vinculin; green), actin cytoskeleton (red), extracellular matrix (blue, for HSkF and LEC), nucleus (blue; SaOs and HSkF for $2S_{\text{Flat}}$ and $5S_{\text{Flat}}$). The arrow (top right) indicates the orientation of the nanowrinkles (Figure S2, Supporting Information) while the bar indicates a flat surface. Bottom left indicates substrate used X_Y_Z where X is the number of days for cell culturing, Y depicts hard (H) or soft (S) surfaces and Z notes whether nanowrinkles (NW) were used or a flat surface (Flat).

Figure 4. Macroscopic response of cells toward surface features comparing the wrinkled feature with flat and hard with soft. Cells are considered aligned when cell longitude axis is within 10° of that of the direction of the surface features.

the determination of the effective surface coverage by focal adhesion, and by subsequent extrapolation to the amount of focal adhesion area per cell using previous reported approaches and developed software (Table 1; representative images for focal adhesion analysis shown in Figure S3, Supporting Information).^[40]

The quantification of the focal adhesions of the SaOs cells to the different surfaces showed the strong surface-dependent behavior not only in orientation but also in actual amount of focal adhesion area per cell based on the surface properties related to stiffness and topology. For the time point of 2 d, the focal adhesion area per cell was five times higher for the soft nanowrinkles ($99 \pm 24 \mu\text{m}^2$ per cell) than for the stiffer nanowrinkles ($21 \pm 1 \mu\text{m}^2$ per cell). To a lesser degree, surface structuring also inhibited cell adhesion on stiff surfaces demonstrated by comparing flat stiff PDMS to hard nanowrinkles, $62 \pm 8 \mu\text{m}^2$ per cell versus $21 \pm 1 \mu\text{m}^2$ per cell, respectively. Conversely, surface structuring enhanced cell adhesion on soft surfaces as the focal adhesion on the flat soft was only $37 \pm 7 \mu\text{m}^2$ per cell as compared to the $99 \pm 24 \mu\text{m}^2$ per cell for the structured soft surface.

Fibroblasts have the natural tendency to align themselves parallel to each other in their cell growth. Overall, the alignment in the entire population was random, similar to that of flat PDMS and hydrogel (Figure 3). In the case of the HSkF's for both the hard and soft wrinkles, an alignment was observed in the direction of the nanowrinkle pattern and the alignment increased with longer culture times (Figures 3 and 4). Although both nanostructured surfaces were able to direct the cell orientation, the focal adhesion area per cell after 2 d ($^2\text{H}_{\text{NW}}$; $^2\text{S}_{\text{NW}}$)

was higher for the soft topology ($140 \pm 68 \mu\text{m}^2$ per cell) than for the stiff topology ($46 \pm 2 \mu\text{m}^2$ per cell). The difference between the surface adhesions seemed to be maintained even for 5 d culture at which cell populations had become confluent. However, this difference was more qualitative as there were too many cells to properly assess the focal adhesion area per cell due to proliferation beyond a single cell layer.

The lens epithelial cells did not display any tendency toward alignment as observed in Figures 3 and 4. On none of the surfaces did the cells or the focal adhesion points follow the nanotopology. Furthermore, similar morphologies were obtained on the hard nanotopology surface and on the flat surface. This is also observed from the rather low focal adhesion coverage values and the average focal adhesion area per cell (Table 1). From Figure 3 ($^2\text{S}_{\text{NW}}$) it could even be deduced that the LECs tend to avoid the soft nanostructured surface and prefer the nonstructured surface. There were hardly any cells present on the soft nanostructured surface except on the edges of the printed surface and on a few defective areas where printing had not structured the surface. On the stiff substrate a homogeneous distribution across the surface was observed. This cell-line also supports that nanotopology has the potential to affect cell adhesion.

The comparison between the cells originating from tissues with different intrinsic stiffness in our study indicates that the “soft cell” (cell originating from soft tissue, LEC) is inhibited by the soft topology while in case of the “hard cell” (cell originating from stiff tissue, SaOs) the adhesion is inhibited in combination with the stiff nanotopology as was observed for the SaOs cells. This cannot be definitely concluded, but it does demonstrate the importance of combining different surface features such as topology and mechanics as shown here. Parameter combinations of this kind will provide deeper insights in cell behavior at interfaces, more so than working with isolated single surface parameters.

The aversion of LEC against the soft topology did not inhibit its proliferation. After 5 d culturing, all surfaces were well covered and with an increase in amount of focal adhesion, with the most significant increase for the soft topology (Table 1). Apparently, once the cells had sufficient support from each other, surface adhesion was also stimulated. In addition to increased surface adhesion, the morphology of the cell layer displayed a change in packing and structure. Cell layers formed by LEC have an open structure with large unoccupied areas, as was seen for samples $^5\text{H}_{\text{NW}}$ and $^5\text{H}_{\text{Flat}}$ (Figure 3). The LEC-layer

Table 1. Focal adhesion area of cells on different substrates.

Surface	Foc. Adh. ^{a)} [μm^2 per cell]			
	SaOs		HSkF ^{b)}	
	2 d	5 d	2 d	2 d
H_{NW}	21 ± 1	41 ± 5	46 ± 2	71 ± 29
S_{NW}	99 ± 24	n.d. ^{b)}	140 ± 68	25 ± 2
H_{Flat}	62 ± 8	129 ± 11	n.d. ^{b)}	40 ± 7
S_{Flat}	37 ± 7	41 ± 4	n.d. ^{b)}	57 ± 7

^{a)}The focal adhesion area/cell was determined using the coverage value divided by the number of cells within the covered area; ^{b)}Incubation time of 5 d could not be determined (n.d.) due to overlapping cell layer and hence determining the proper number of cells per area was not possible.

Figure 5. Viability assay (XTT) displaying the metabolic activity of SaOs, HSkF, and LEC on soft and hard surfaces both flat and with aligned nanotopology for 2 and 5 d culture.

covering the soft topology after 5 d culturing (S_{NW}) formed a much denser cell layer which is also generally observed for collagen coated tissue culture polystyrene (TCPS) substrates. The more homogenous packing of the LECs inside the cell layer probably has to do with the extracellular components secreted by these cells. In Figure 3 (S_{NW}) the fibronectin matrix marked in blue showed a more homogeneous distribution as well as an increased amount as compared to the other surfaces. More intercellular interactions allow for a more densely packed layer being formed, which is needed to overcome the unfavorable interactions with the hydrogel wrinkle structures.

In addition to the macroscopic responses such as adhesion and alignment, it was expected that there would also be a significant influence on the molecular level and that metabolic activity and protein expression would be affected by the physical parameters of the substratum (Figures 5 and 6). To this end, metabolic activity (XTT) was quantified and the most relevant protein expressions were qualitatively determined using fluorescence imaging. ECM upregulation was visualized by targeting collagen I, which was compared on the different surfaces for SaOs and HSkF. Alkaline phosphatase (ALP) was analyzed for the SaOs cells indicating mineralization, and alpha-smooth muscle actin (α -SMA) was analyzed for the LEC, which is a strong marker for the mesenchymal transition of epithelial cells toward myofibroblasts and considered a marker for fibrosis.^[41–43]

The results of the XTT assay showed that SaOs cultured on surfaces over a period of 5 d exhibited levels of metabolic activity that were statistically different from SaOs cultured on soft nanostructure. The metabolic activity of HSkF on soft nanotopology was the highest compared to on other surfaces. For LEC, the level of metabolic activity on the hard wrinkled surface was the highest compared to on other surfaces.

The initial hypothesis with respect to osteoblast behavior was that it would either respond to the hard substrates because of the relation with stiff calcified bone or that it would respond to soft as one of the early events is that osteoblasts adhere to collagen before producing mineralized bone tissue. It was identified that osteoblasts respond preferentially initially to soft topologies as it relates more to the initial adhesion on a collagen matrix which was also shown by the alignment efficiency in Figure 4. However, for the alignment there was no difference after 5 d culturing. Collagen production and mineralization are important functions for osteoblasts as it constitutes for

the formation of mineralized bone tissue. Upon investigating the expression of collagen I and ALP some very interesting differences were detected. Both collagen I production and ALP expression were more stimulated on the hard layers after 5 d (Figure 6) whereas initially more collagen I as well as ALP was observed after 2 d on the soft substrates (Figure S4, Supporting Information). In both cases there was more expression on the nanostructured surfaces than on the flat ones. Thus, the behavior of the SaOs cells was clearly affected by both the mechanical properties and the topology of the surfaces.

For fibroblast responses toward the different interfaces, collagen I expression was visualized and most prominent on the soft surfaces, particularly on the flat. The nanostructure did not promote collagen I production on the soft substrate however it did on the hard wrinkles (S_{NW}) as compared to the flat (S_{Flat}).

LEC clearly overexpress α -SMA on the nanostructures but the effect from the hard nanostructured surfaces not nearly as strong as that from the soft nanostructures. This correlates to the observation of initial nonadhesion on the soft nanostructures which may ultimately force the LEC into a different state to cope with the environment. Also when the planar substrates were compared, the soft substrate induced more α -SMA expression than the hard substrates which deviated from the idea that a cell from a soft tissue would behave most naturally on a soft substrate. There was also a large difference between structured and nonstructured surfaces again strengthening the idea that it is vital to identify combined parameter contributions. For both HSkF and LEC (nonhard cell-lines), the soft nanostructure induced events which were prone for fibrosis formation namely upregulation of collagen I and α -SMA for HSkF and LEC, respectively. Hence soft (structured) biointerfaces seem to be unfavorable with respect to potential foreign body responses based on these in vitro assessments.

Designing proper (bio)interfaces which are not only relevant for (bio)medical applications, such as tissue engineering, regenerative medicine, and medical implants, but also for a basic understanding of cell behavior toward different surface confined stimuli is an ongoing endeavor which has no immediate solution.^[12,15,44] However, we surpassed the initial stage where it is known which surface parameters influence the behavior of cells, and it is therefore time to transcend beyond the single parameter studies and increase the complexity.^[6,33] Strong attempts have already been made, but the interactions of alignment studies on a nanotopological scale with increased

Figure 6. Fluorescence imaging of expressed proteins as a consequence of the surface parameters. For SaOs, collagen I (green) and alkaline phosphatase (ALP, green) was analyzed, for HskF, collagen I (green) was analyzed, and for LEC, α -SMA (green) was analyzed. Scale bars are 75 μ m and apply to all images.

complexity are still underdeveloped and highly important to the cell–interface behavior as we have shown here.^[32,33,45] Intrinsically, it is known that different tissues differ in stiffness and that cells respond to stiffness cues.^[6,10,46] Therefore, it is logical to take this into account also when dealing with topological defined surfaces. The system we demonstrated here verifies the behavior of cells obtained from different tissues with intrinsically different stiffness toward surfaces with similar nanotopology but different Young's modulus. This approach can be even further developed by incorporating processes as protein adhesion which is known to be dependent on wettability but has unknown dependence on stiffness, which in turn would also influence cell–interface interactions.

In our study, the initial hypothesis we formulated that SaOs from “hard” tissues would favor stiff nanotopology

(45–115 MPa) and that inversely LEC from “soft” tissues would show this behavior toward soft nanotopologies (0.3–0.8 MPa) reflected in increased focal adhesion area and alignment of the intracellular actin cytoskeleton has been shown to be incorrect. We observed here the inverse behavior which will ultimately influence studies involving stem cells which are most often used in studies concerning wettability and stiffness. Ayala et al. have used acrylate hydrogels in that respect with different wettability and similar stiffness and observed optimal conditions at 60° WCA for adhesion and spreading which was also found by others using monolayers on gold substrates.^[47,48] In recent work, it has been shown that by the addition of a changing stiffness component, the optimum condition differs as well as the overall cell adhesion development over time.^[23,49] As we observed in this study, by clear differences in focal adhesion as

well as overexpression of certain fibrosis related proteins that optimum surface structuring combined with other physical parameters could potentially lower foreign body responses. It has been shown by Pietramaggiore and co-workers that fibrotic response can be manipulated by directing focal adhesion using specifically sized spots of adhesion proteins.^[42] That surface structure can induce similar attributes was shown by Kyle et al. who used different nano- and microstructured surface roughened silicone rubber to modulate foreign body response of fibroblasts.^[50]

Our findings using tissue specific cells could also impact stem cell adhesion and differentiation, especially when topologies and stiffness ranges could be even further broadened. Stem cells will differentiate into a specific cell-lineage. Although the initial adhesion and surface interactions of the stem cells are important, ultimately the surface should also be appropriate for the cell-type/tissue into which they differentiate. Therefore, the presented system will open a new methodological platform into studying more complex nanostructured surfaces combined with mechanical properties. Additionally, the polymer composition can easily be altered to incorporate not only positive and negative charges with a controllable density but also other chemical components such as peptides, providing a platform to increase the biointerface complexity required to initiate the next stage of understanding cell–surface interactions.

Experimental Section

Materials: The polydimethylsiloxane (PDMS) was prepared using a Sylgard 184 elastomer kit obtained from Dow Corning prepared according to their specifications. Chemicals used for the polymerization reaction and surface modification were obtained from Sigma-Aldrich: ammonium hydroxide, ammonium persulfate ($\geq 98\%$), (hydroxyethyl) methacrylate ($\geq 99\%$), N,N'-methylene-bis(acrylamide) ($\geq 99\%$), and N,N,N',N'-tetramethylethylenediamine ($\geq 99\%$). (Hydroxyethyl)methacrylate was purified before use by passing it in the pure form over a basic alumina column. All other chemicals were used as received. Medium and buffers for cell culturing were obtained from AppliChem.

AFM analysis was performed on a Catalyst Nanoscoop V instrument (Bruker, Billerica, MA, USA) with NanoScoop Analysis (also Bruker) as analysis software. The AFM measurements were performed using the PeakForce QNM mode of Bruker with a large amplitude. Bruker SCANASYST-AIR (0.4 N m⁻¹) and NP (0.017 N m⁻¹) cantilevers made from silicon nitride with silicon tips were used and before each measurement the system was calibrated determining the exact spring constant of the tip for the Young's modulus determination.

For confocal laser scanning microscopy, a Leica TCS SP2 system was used (Leica Microsystems Heidelberg GmbH, Germany). For visualization of the focal adhesions a mouse-anti-human-vinculin primary antibody (1:100, Sigma, V9131) followed by a FITC-labeled goat-anti-mouse IgG antibody (1:100, Jackson ImmunoLab, 115-095-146) was used using 488 nm as excitation wavelength; the actin cytoskeleton was stained using TRITC-labeled phalloidin (2 μ g mL⁻¹, Sigma) using 514 and 543 nm as excitation wavelengths; the extracellular matrix protein fibronectin was visualized using rabbit-anti-human fibronectin (1:400, Sigma, F3648) followed by Cy5-labelled donkey-anti-rabbit IgG antibody (1:100, Jackson ImmunoLab, 711-175-152) and 633 nm as excitation wavelength. Finally the nucleus was stained with DAPI (4 μ g mL⁻¹, Sigma) visualized by excitation at 351–364 nm or 405 nm.

Polydimethylsiloxane Wrinkles: Silicone Elastomer Base and Silicone Curing Agent were used in a weight ratio of 10:1 for the preparation of the PDMS substrates. The mixture was poured in a clean Petri dish of

≈ 3 mm thick film and allowed to cure over night at 50 °C after degassing under vacuum. The cross-linked PDMS was cut in pieces of about 2 × 2 cm and mounted on a custom-made stretching apparatus applying uniaxial strain and stretching the substrate by 30% of their original length. After exposure to air plasma for 480 s (0.2 mbar, Plasma Activate Flecto 10 USB) the stress was removed which induces wrinkle formation.

Polymer Hydrogel Wrinkles: Glass microscope slides which were used as a substrate for the printing process, were cleaned and hydrophilized using piranha solution (H₂SO₄/H₂O₂ (30%); ratio 3:1) at 110 °C for 3 h. Subsequently, the slide was washed thoroughly with copious amount of water and modified with 3-(trimethoxysilyl)propyl-methacrylate to enhance the attachment of the polymer layer to the surface. Modification was performed by using a mixture of 75 mL ethanol, 6 mL ammonium hydroxide (30%), and 0.2 mL 3-(trimethoxysilyl)propyl-methacrylate in which the glass slides were kept overnight under constant mild stirring. The modified slides were cleaned afterward using water and ethanol and were subsequently dried under a stream of N₂.

The modified surface was subsequently used in the preparation of the imprinted hydrogel wrinkles. For this a mixture containing the monomer ((hydroxyethyl)methacrylate, HEMA), the crosslinker (N,N'-Methylenbis(acrylamide)), water, and ligand (N,N,N',N'-tetramethylethylenediamine; TEMED) was prepared in molar ratios of: 250 mmol monomer/crosslinker (90:10 mol%), 0.5 mmol ammonium persulfate (APS), 0.125 mmol TEMED, and a water content of 20 vol%. The reaction solution was stored on ice. Upon initiating the printing process 0.5 mmol of APS was added to the reaction solution and 10 μ L was dropped on the modified glass slide. Immediately afterward the wrinkled PDMS master was placed on top and the imprinting system was left at room temperature for at least 24 h before the master was gently removed. Experiments, performed in a glovebox (oxygen content <1%), and all reaction solutions were degassed for 10 min under nitrogen before use.

Cell Culture and Seeding: Three different types of cells were used for the cell adhesion and proliferation studies; SaOs (human osteosarcoma cell-line expressing wild type p53 and Rb, but lacking p16), LEC-B3 cells (Lens Epithelial Cells) and HSKF (Human Skin Fibroblasts -CCD-1122Sk, ATCC CRL2429). Cell-lines were cultured in their respective media; SaOs was cultured in Dulbecco's Modified Eagle Medium (DMEM), LEC was cultured in Eagle's Minimum Essential Medium (EMEM) and fibroblasts were cultured in Roswell Park Memorial Institute medium (RPMI). 10% (v/v) fetal bovine serum was added to all used media.

The substrates on which the cells were deposited were placed in 24-wells microtiter plates and were sterilized by UV radiation for 1 h. For each cell type, a dilution of 4.0×10^4 cells per mL was prepared. From this dilution, 1 mL was added to each of the substrates (gel wrinkles and flat, PDMS wrinkles, and flat oxidized PDMS) for cell adhesion, viability, and differentiation studies. All plates were stored in an incubator at 37 °C and 5% CO₂ for two and 5 d. Medium was exchanged after 2 d.

Cell Adhesion Studies: Cells were stained for analysis by confocal laser scanning microscopy. The cells were fixed using 3.7% paraformaldehyde solution in PBS. Cells were permeabilized in 0.5% Triton X-100 in PBS for 3 min, after which nonspecific background was blocked using 5% fatty-acid free BSA in PBS for 30 min. The first antibodies against vinculin and fibronectin were diluted in 1% BSA in PBS and the specimens were incubated for 1 h, after the incubation period the specimens were washed three times for 5 min in 1% BSA in PBS. The second antibodies were added together with DAPI and TRITC-phalloidin and allowed to incubate overnight at 4 °C. The samples were washed twice in 1% BSA in PBS and once in PBS, all for 5 min. The specimens were kept in PBS at 4 °C until microscopic observation. Cells were observed using confocal laser scanning microscopy. Apart from detecting the fluorescent signals, images were also made in reflection mode in order to visualize the wrinkles.

Image analysis of vinculin was done by using Focal Adhesion Analysis Server (developed by M. E. Berginski, S. M. Gomez), and ImageJ software was used to measure the average area per cell.^[40] Cells were considered aligned if the angle between the long axis and the wrinkle was less than 10°.

Cell Viability Assay: The Cell viability was analyzed using an XTT assay (Applichem A8088). Briefly, on 2 and 5 d culture, 200 μ L of XTT reaction mixture (100 μ L activation reagent and 5 mL XTT reagent for one plate) was added to each well and samples were incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 2 h. The 200 μ L mixtures were added to a 96-well plate and the absorbance at 485 and 690 nm was recorded on the microplate reader. Experiments were performed in triplicate.

Examination of Protein Upregulation: The cell-scaffold constructs from SaOs, HSKF, and LEC were fixed with 3.7% paraformaldehyde solution in PBS for 15 min followed by washing for two times in PBS, permeabilized with 0.5% Triton X-100 in PBS for 3 min and blocked with 5% fatty-acid free BSA in PBS for 30 min to avoid nonspecific binding. Then the SaOs samples were incubated with alkaline phosphatase (ALP; 1:1000, Developmental Hybridoma Bank, B4-78) and collagen type I (COL I; 1:500, Abcam, ab34710) as the primary antibody for 1 h. And the HSKF samples were incubated with collagen type I (COL I; 1:500, Abcam, ab34710) as the primary antibody for 1 h. The LEC samples were incubated with α -smooth muscle actin (α -SMA; 1:100, Sigma, A2547) as the primary antibody for 1 h. The samples were subsequently incubated with FITC-labeled goat-anti-mouse antibody (Jackson Immunolab, 1:100, 115-095-146) as the secondary antibody, DAPI (4 μ g mL⁻¹, Sigma) and TRITC-phalloidin (2 μ g mL⁻¹, Sigma) for 1 h. Then, the samples were washed twice in 1% BSA in PBS and once in PBS, all for 5 min. Cells were observed using a LEICA TCS SP2 CLSM equipped with 40 $\times$ NA 0.80 water immersion objectives.

Statistical Analysis: All data were expressed as mean $\pm$ standard error of at least three samples. Single factorial analysis of variance (ANOVA) was performed to determine statistical significance of the data using Origin 9.0. Tukey's post hoc test was used to analyze differences. A value of $p < 0.05$ was considered statistically significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

Q.Z. is very grateful for financial support of the China Scholarship Council (No. 201406630003). P.v.R. and P.T.K. kindly acknowledge the Graduate School Medical Sciences of the University Medical Center Groningen for funding. The authors thank Dr. Brandon Peterson for proof reading the manuscript.

Received: April 1, 2016

Revised: June 23, 2016

Published online: July 12, 2016

- [1] C. J. Bettinger, R. Langer, J. T. Borenstein, *Angew. Chem. Int. Ed.* **2009**, 48, 5406.
- [2] A. Curtis, C. Wilkinson, *Biomaterials* **1997**, 18, 1573.
- [3] M. P. Lutolf, P. M. Gilbert, H. M. Blau, *Nature* **2009**, 462, 433.
- [4] D. E. Ingber, *Proc. Natl. Acad. Sci. USA* **2005**, 102, 11571.
- [5] D. E. Discher, P. Janmey, Y.-L. Wang, *Science* **2005**, 310, 1139.
- [6] Y. Mei, K. Saha, S. R. Bogatyrev, J. Yang, A. L. Hook, Z. I. Kalcioğlu, S.-W. Cho, M. Mitalipova, N. Pyzocha, F. Rojas, K. J. Van Vliet, M. C. Davies, M. R. Alexander, R. Langer, R. Jaenisch, D. G. Anderson, *Nat. Mater.* **2010**, 9, 768.
- [7] J. M. Dang, K. W. Leong, *Adv. Mater.* **2007**, 19, 2775.
- [8] B. Zhu, Q. Zhang, Q. Lu, Y. Xu, J. Yin, J. Hu, Z. Wang, *Biomaterials* **2004**, 25, 4215.
- [9] T. R. Sodunke, K. K. Turner, S. a Caldwell, K. W. McBride, M. J. Reginato, H. M. Noh, *Biomaterials* **2007**, 28, 4006.
- [10] S. D. Subramony, B. R. Dargis, M. Castillo, E. U. Azeloglu, M. S. Tracey, A. Su, H. H. Lu, *Biomaterials* **2013**, 34, 1942.
- [11] M. J. Poellmann, P. A. Harrell, W. P. King, A. J. Wagoner Johnson, *Acta Biomater.* **2010**, 6, 3514.
- [12] L. E. Freed, G. C. Engelmayr, J. T. Borenstein, F. T. Moutos, F. Guilak, *Adv. Mater.* **2009**, 21, 3410.
- [13] B. J. Papenburg, L. Vogelaar, L. A. M. Bolhuis-Versteeg, R. G. H. Lammertink, D. Stamatis, M. Wessling, *Biomaterials* **2007**, 28, 1998.
- [14] R. Langer, D. A. Tirrell, *Nature* **2004**, 428, 487.
- [15] O. Z. Fisher, A. Khademhosseini, R. Langer, N. a Peppas, *Acc. Chem. Res.* **2010**, 43, 419.
- [16] M. M. Stevens, J. H. George, *Science* **2005**, 310, 1135.
- [17] A. M. Schaap-Oziemlak, P. T. Kühn, T. G. van Kooten, P. van Rijn, *RSC Adv.* **2014**, 4, 53307.
- [18] J. Genzer, R. R. Bhat, *Langmuir* **2008**, 24, 2294.
- [19] J. Genzer, *Annu. Rev. Mater. Res.* **2012**, 42, 435.
- [20] S. Morgenthaler, C. Zink, N. D. Spencer, *Soft Matter* **2008**, 4, 419.
- [21] R. R. Bhat, B. N. Chaney, J. Rowley, A. Liebmman-Vinson, J. Genzer, *Adv. Mater.* **2005**, 17, 2802.
- [22] Q. Zhou, P. T. Kühn, T. Huisman, E. Nieboer, C. van Zwol, T. G. van Kooten, P. van Rijn, *Sci. Rep.* **2015**, 5, 16240.
- [23] P. T. Kuhn, Q. Zhou, T. A. B. van der Boon, A. M. Schaap-Oziemlak, T. G. van Kooten, P. van Rijn, *ChemNanoMat* **2016**, 2, 407.
- [24] S. Huda, D. Pilans, M. Makurath, T. M. Hermans, K. Kandere-Grzybowska, B. A. Grzybowski, *Adv. Mater. Interfaces* **2014**, 1, 1400158.
- [25] M. A. Bucaro, Y. Vasques, B. D. Hatton, J. Aizenberg, *ACS Nano* **2012**, 6, 6222.
- [26] B. K. K. Teo, S. T. Wong, C. K. Lim, T. Y. S. Kung, C. H. Yap, Y. Ramagopal, L. H. Romer, E. K. F. Yim, *ACS Nano* **2013**, 7, 4785.
- [27] J. Lee, G. Khang, J. Lee, H. Lee, *J. Colloid Interface Sci.* **1998**, 205, 323.
- [28] N. D. Evans, C. Minelli, E. Gentleman, V. LaPointe, S. N. Patankar, M. Kallivretaki, X. Chen, C. J. Roberts, M. M. Stevens, *Eur. Cell. Mater.* **2009**, 18, 1.
- [29] E. Lamers, X. F. Walboomers, M. Domanski, J. te Riet, F. C. M. J. M. van Delft, R. Luttge, L. A. J. A. Winnubst, H. J. G. E. Gardeniers, J. A. Jansen, *Biomaterials* **2010**, 31, 3307.
- [30] A. M. Hung, S. I. Stupp, *Nano Lett.* **2007**, 7, 1165.
- [31] J. Xie, M. R. MacEwan, A. G. Schwartz, Y. Xia, *Nanoscale* **2010**, 2, 35.
- [32] V. Brunetti, G. Maiorano, L. Rizzello, B. Sorce, S. Sabella, R. Cingolani, P. P. Pompa, *Proc. Natl. Acad. Sci. USA* **2010**, 107, 6264.
- [33] A. Higuchi, Q.-D. Ling, Y. Chang, S.-T. Hsu, A. Umezawa, *Chem. Rev.* **2013**, 113, 3297.
- [34] M. Pretzl, A. Schweikart, C. Hanske, A. Chiche, U. Zettl, A. Horn, A. Böker, A. Fery, *Langmuir* **2008**, 24, 12748.
- [35] S. Hilt, J. Oltmanns, A. Böker, *Nanoscale* **2012**, 4, 7338.
- [36] M. J. Owen, P. J. Smith, *J. Adhes. Sci. Technol.* **1994**, 8, 1063.
- [37] F. Di Benedetto, A. Biasco, D. Pisignano, R. Cingolani, *Nanotechnology* **2005**, 16, S165.
- [38] D. Chandra, J. A. Taylor, S. Yang, *Soft Matter* **2008**, 4, 979.
- [39] A. I. Caplan, *Cell and Molecular Biology of Vertebrate Hard Tissues*, Wiley & Sons Ltd., Chichester **1988**.
- [40] M. E. Berginski, S. M. Gomez, *F1000Research* **2013**, 2, 68.
- [41] B. Rolf, F. Mooney, B. Zhang, S. Jahnke, S.-J. Le, Y.-Q. Chau, Q. Huang, H. Wang, G. Campbell, J. Campbell, *The fibrotic response to implanted biomaterials: Implications for tissue engineering, regenerative medicine and tissue engineering - cells and Biomaterials*, D. Eberli (Ed.), *InTech*, **2011**, DOI: 10.5772/21790.

- [42] H. Majd, S. S. Scherer, S. Boo, S. Ramondetti, E. Cambridge, W. Raffoul, M. Friedrich, B. Pittet, D. Pioletti, B. Hinz, G. Pietramaggiore, *Biomaterials* **2015**, 54, 136.
- [43] S. Lamouille, J. Xu, R. Derynck, *Nat. Rev. Mol. Cell Biol.* **2014**, 15, 178.
- [44] B. Weng, J. Diao, Q. Xu, Y. Liu, C. Li, A. Ding, J. Chen, *Adv. Mater. Interfaces* **2015**, 2, 1500059.
- [45] L. Yang, L. Zhang, T. J. Webster, *Adv. Eng. Mater.* **2011**, 13, B197.
- [46] J. Swift, I. L. Ivanovska, A. Buxboim, T. Harada, P. C. D. P. Dingal, J. Pinter, J. D. Pajerowski, K. R. Spinler, J.-W. Shin, M. Tewari, F. Rehfeldt, D. W. Speicher, D. E. Discher, *Science* **2013**, 341, 1240104.
- [47] R. Ayala, C. Zhang, D. Yang, Y. Hwang, A. Aung, S. S. Shroff, F. T. Arce, R. Lal, G. Arya, S. Varghese, *Biomaterials* **2011**, 32, 3700.
- [48] L. Hao, H. Yang, C. Du, X. Fu, N. Zhao, S. Xu, F. Cui, C. Mao, Y. Wang, *J. Mater. Chem. B* **2014**, 2, 4794.
- [49] G. Mohan, N. D. Gallant, *J. Biomed. Mater. Res. A* **2015**, 103A, 2066.
- [50] D. J. T. Kyle, A. Oikonomou, E. Hill, A. Bayat, *Biomaterials* **2015**, 52, 88.