CHAPTER FOUR

DOUBLE LINEAR GRADIENT BIOINTERFACES FOR DETERMINING TWO-PARAMETER DEPENDENT STEM CELL BEHAVIOR

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ABSTRACT

Two-parameter contributions of stiffness and wettability display altered hBM-MSCs behavior as compared to solely investigating the wettability of the biointerface with a homogenous stiffness. The importance of double parameter influence is identified by using newly developed double linear wettability-stiffness gradients. The interfaces show that substrate stiffness can influence cell adhesion and spreading both positively and negatively in combination with wettability. This effect is validated by decoupling the stiffness gradient using a wettability gradient composed of a monolayer on glass ensuring a homogenous stiffness.
4.1. INTRODUCTION

In the fields of regenerative medicine and tissue engineering it is pivotal to have full control over cellular behavior such as adhesion, spreading, migration, proliferation and differentiation. Scaffold biomaterials are therefore designed and modified in such a way that cell behavior can be controlled. Often this can be achieved by mimicking the natural environment e.g. creating an extracellular matrix-like (ECM) environment with appropriate chemical, topology and mechanical properties.

It is well-known that cells respond to their micro-environment in terms of chemical composition, mechanical properties and structural features either on 2-dimensional surfaces or within 3-dimensional networks. Most frequently targeted parameters on biomaterial surfaces are wettability, stiffness and topology, which have been shown to influence cell adhesion, spreading, morphology and differentiation as well as inducing specific gene-activation and hence influencing protein expression. To this end, various biointerfaces have been designed to investigate cell behavior. Designs of these include: 1) surface structuring in the form of nanopillars, microgrooves and aligned fibers; 2) wettability: where surfaces are chemically altered to change their water affinity; 3) stiffness: in which often the internal cross-linking density of polymer networks cause a change in mechanical properties (Young’s Modulus); and 4) chemical factors: tethering of bio-active moieties like peptides or other small signal molecules.

Many of these properties cannot be obtained in the bulk without affecting the overall function of the biomaterial. Hence surface manipulation in the form of surface chemistry and coatings offer possible solutions. Although the bulk is shielded from cellular interactions, it still affects the behavior of cells as often only nanometer-sized molecules, in some cases sub-nanometer, are being used for modifications like monolayers. Therefore, often combinations of both bulk and surface properties need to be identified.

To obtain a high degree of control over cellular behavior, the influence of wettability, stiffness and topology needs to be well-understood. Many different studies as shown above have investigated parameter manipulation for directing cellular behavior with regenerative medicine and tissue engineering applications in mind. Although it has been realized that surface properties of a biomaterial are highly important, it is not precisely known how combined effects influence cell behavior. General approaches for determining cell behavior often use one particular parameter, such as wettability, stiffness or topology. Occasionally, gradients are used but still targeting only one property, while biomaterials have at least three combined basic properties as mentioned above. With the use of individual substrates, previous studies showed that property combinations indeed matter by using fixed parameter combinations which often include different types of biomaterials and thereby introducing additional chemical variables. Therefore it is important to minimize the number of different materials used in such a study. For this reason, silicone rubber (PDMS; polydimethylsiloxane) is used in this study as it represents a clinically used biomaterial and therefore identified parameters are potentially translational towards medical implants.

A problem with altering the surface of a biomaterial is that often several parameters are altered at the same time and that specific combinations may affect cellular behavior more than the individual parameters would do themselves. To elucidate the influence of surface properties on cells, gradients are time/cost efficient tools compared to single
substrate analysis of parameter combinations\cite{5,8}. Wettability-induced cell responses\cite{14} combined with stiffness influences\cite{37} can provide deeper insights into surface parameter combination effects on cellular behavior at interfaces. It is known that wettability is important for surface attachment and cell viability, which are strongly connected. Earlier work generally displays that intermediate wettability (water contact angle (WCA): 50-70°) is often preferred by the cells but this has never been combined with changing other parameters which also influence cellular behavior.\cite{38–41} Both wettability and stiffness as a surface characteristic have been shown to influence differentiation behavior of stem cells although, stiffness conventionally has a stronger influence as also reflected from the vast amounts of performed studies.\cite{8,42–47}

Here we developed a double linear gradient based on PDMS, using a single step shielded air plasma treatment which generates upon oxidation a more hydrophilic and stiffer material. The degree of oxidation dictates the stiffness and the hydrophilicity\cite{48} which is gradually varied by controlling the doses of plasma reaching the surface using the shielding approach. The generated gradient spans across 2 cm (Figure 1a/b). In a separate experiment, the stiffness component was decoupled by producing a similar wettability gradient on glass, obtained via a silanization reaction using trichloro(propyl)silane followed by the same shielded plasma treatment with retention of stiffness across the developed gradient (Figure 1a/b). The decoupling provides insights in the influence of a stiffness component in addition to only the influence of wettability. Additionally, using PDMS and modified glass ensures that the overall chemistry is kept very similar as it provides similar oxidation products. This ensures that the observed cell responses are not due to highly different surface bound chemical functionalities, which are known to influence cell behavior.\cite{49} Comparing the linear wettability gradient with uniform stiffness to the linear, coupled wettability-stiffness double gradient showed that human bone marrow derived mesenchymal stem cell (hBM-MSC) behavior is highly influenced by introducing the second physical component. Both surface adhesion and subsequent spreading were followed over time.

4.2. GENERATION OF GRADIENTS

In order to test the combinatorial effect of wettability and stiffness of a surface towards hBM-MSC in an efficient way, a surface which contains different combinations of both parameters is needed. A linear double gradient in wettability and stiffness is a surface which fulfills these requirements. The treatment of PDMS with air plasma oxidizes the top layer of the surface making it hydrophilic and stiff.\cite{50} This was used to create a linear double gradient in stiffness and wettability on PDMS. By shielding the sample with a right angled triangular prism mask during the plasma treatment, the oxidation intensity varies from one side to the other (Figure 1a). The open side is highly oxidized and therefore hydrophilic and stiff (30° WCA and 85 MPa), whereas the closed side is only slightly oxidized and therefore hydrophobic and soft (94° WCA and 7 MPa) (Figure 1b). It has to be noted that in many more fundamental oriented studies substrates in the low kPa range are used rather than the more clinically relevant implant materials. During oxidation a surface with continuous change of both parameters is created by using the shadow-effect resulting from the shape of the mask (Figure 1a). The hydrophobic glass was treated in a similar way as the PDMS to create the wettability gradient but with uniform stiffness. In addition to the gradient, PDMS was also used completely oxidized or non-modified as
control surfaces. The same was done for the glass, so that for each sample the extreme situation of full exposure or no exposure to oxygen plasma could be compared to the gradient (Figure 1a).

By correctly choosing the oxidation parameters, a linear PDMS double gradient surface was created in only one minute. The completely oxidized PDMS had a WCA of 20° and a modulus of 90 MPa and the non-oxidized control a WCA of 118° and a modulus of 3 MPa. This shows that the gradient represents a significant range of situations going from oxidized to non-oxidized on a single substrate. The 2 cm wettabibility gradient on glass ranged from a WCA of 121° on the closed side to a WCA of 31° on the open side of the mask. Data of both wettability and stiffness for PDMS and wettability for glass were fitted using a Boltzmann equation, wettability for glass was fitted using a linear equation (data not shown).

Figure 1. a) An overview scheme showing the generation process of the gradient samples and the layout of the different used surfaces. The graph displays the development of the different physical properties of both, the PDMS double gradient as well as the glass single gradient. b) Plotted are the Young's modulus, grey symbols corresponding to the right y-axis and the WCA, black symbols corresponding to the left axis. The squares represent the PDMS double gradient and the stars represent the glass wettability gradient.

4.3. Macroscopic Cell Behavior

As expected, the Young’s modulus of glass did not alter significantly over the length of the sample (range of 550 MPa to 590 MPa). Here it has to be noted that the “real” value for glass is most likely higher, since these values represent a minimum value and literature reports glass stiffness in the GPa range. Glass was used as a non-compressible material for calibration and hence does not provide an accurate value but only a minimum however, it clearly shows that the relative change across the gradient is only marginal as compared to the PDMS. The hydrophilic control of glass had a zero WCA and the hydrophobic glass a contact angle of 138°. Also this gradient represents a large number of situations in between both extremes.
In order to identify the adhesion behavior of stem cells with respect to the wettability and stiffness of the substrates, hBM-MSCs were seeded on both the gradient substrates and controls. The adhesion and spreading were analyzed after 3 and 24 hours. A detailed overview of the macroscopic cell behavior was obtained by analyzing the fixed and stained cells on all surfaces with an automated microscope (TissueFAXS), which allowed to observe the surfaces as a whole (Figure 2). For this, the nuclei and F-actin of the cells were stained with DAPI in blue and TRITC-phalloidin in red, respectively (Figure 2).

The overview images of the wettability/stiffness gradient on PDMS and the wettability gradient on glass clearly display more cell spreading after 24 hours than after 3 hours on both types of gradients. Another observation is that for both, PDMS and glass substrates, independent of the incubation time, the hydrophilic controls induce more cell spreading than the hydrophobic controls. This coincides with recently reported experiments.\[13,14,16,34,35\]

For fair comparison of the different gradients, it has to be noted that the marked areas on the glass gradients (white dashed lines) represent the same range of wettability as the full sample of the PDMS gradient. This difference in wettability range originates from both the higher starting water contact angle of the glass substrate and the slightly different gradient preparation conditions. Within the marked areas it is clearly visible, that for the initial attachment after 3 hours the PDMS sample shows a higher number of cells, whereas their spreading does not differ significantly. This situation changes for the later time point of 24 hours, where the differences become more apparent and cells spread more on PDMS. Apart from the fact, that the cell distribution on both samples is different, the
hBM-MSCs on glass form clusters of multiple cells. This is not the case for PDMS and indicates a non-optimal environment for the cells. It has to be noted that all gradients and control substrates were used directly after formation and no additional coatings such as collagen and fibronectin were applied or any other treatments were performed. For the more detailed analysis using confocal fluorescence microscopy (Figure 3), the clustered areas as observed in figure 2 were avoided to properly assess the focal adhesion and cell spreading area.

4.4. CELL ADHESION AND SPREADING

For glass the density of cells across the substrate seems to follow a simple trend of increasing cell numbers with increasing wettability. For the PDMS this trend is not visible. In fact, the highest cell count can be found at a distance of 1.2-1.6 cm from the closed end, which coincides with a WCA of 44° to 37° and 22 to 38 MPa, respectively. On occasion, control experiments in tissue culture styrene well-plates displayed higher cell counts in the middle of the well. Since the middle of the well always coincides with the middle of the sample, the behavior of hBM-MSCs on the glass gradient is deviating since it never has a higher density in the middle. It can still be seen, that the distribution on PDMS changes significantly from 3 hours to 24 hours whereas for the glass the same trend was observed for both time points. These differences can be attributed to the difference in Young’s modulus of the samples, as wettability and chemistry of both samples are very similar.

To obtain deeper insights in the effect of wettability and Young’s modulus towards the adhesion of stem cells, the focal adhesion points of the cells were also stained using an antibody against vinculin and both cell spreading and adhesion were analyzed using confocal laser scanning microscopy (CLSM). From this higher resolution analysis, cell shape, area per cell and focal adhesion area per cell were determined (Figure 3a).

The CLSM images of the hBM-MSCs show a clear difference between the two incubation times for both gradient surfaces in area per cell and focal adhesion area per cell. The general trend can be intuitively recognized in the representative images of the stem cells on the different positions of the gradient surfaces. After an incubation time of 3 hours the stem cells still have a rounded shape, even though they already started adhering to the surface. After 24 hours their shape is more elongated, which is typical for this kinds of cells (Figure 3a). From comparing these images, it can also be confirmed that the hydrophilic control is beneficial over the hydrophobic one for all conditions.
Figure 3. a) Representative CLSM images of hBM-MSCs on the PDMS double gradient and the glass wettability gradient as well as of the controls. Above the position a schematic of the mask indicates the direction of the gradient. b-e) In the lower part of the picture, 3 dimensional plots of the average area per cell (red) and focal adhesion area per cell (green) dependent on the wettability and Young’s modulus of the samples are shown for the different incubation times. The cubes represent the glass gradients whereas the spheres represent the PDMS gradients. In addition, in the 3D plots a projection of the data on the XZ plane is shown to visualize the effect of wettability towards cell response more clearly and these 2D plots have additionally been added to the supporting information (SI3). The Young’s modulus is plotted on a logarithmic axis for clarity.

Investigation of the area per cell and the focal adhesion area per cell show similar trend as for the macroscopic behavior. As expected, the area per cell increases going from the 3-hour incubation to a 24-hour incubation (Figure 3b and c). Also a regular increase in spreading area was observed. With decreasing WCA (increasing wettability) the area per cell increases, going from about 500 µm²/cell for the hydrophobic side of the surface up to 2900 µm²/cell for the hydrophilic side for both the glass and the PDSM gradients. No significant difference was observed between PDMS and glass, indicating that the Young’s modulus does not have a strong influence on the early stage spreading. After longer
incubation times (24 hours) the area per cell seemed to be influenced by the stiffness of the surface. Even though the development for adhesion across the gradient for glass and PDMS after 24 hours is very similar, from WCAs of 100° and lower, the PDMS displays a higher area per cell (Figure 3c). Stem cells on PDMS with WCA of 20° have an area of 4820 µm²/cell whereas on glass an area of 3040 µm²/cell was determined. For the hydrophobic side of the substrates, no difference between the two conditions was observed and soft and stiff induced similar spreading. This clearly points out that not only the WCA and stiffness individually have a great impact on hBM-MSC spreading, but that the two parameters interact.

In addition to the spreading, also adhesion was analyzed in more detail. From the focal adhesion area per cell, conclusions about the quality of adhesion of the hBM-MSCs can be drawn. A significant difference to the cell spreading can immediately be identified by looking at the general trends of hBM-MSC behavior on both gradients. The focal adhesion area per cell does not seem to follow the same correlation with WCA as the area per cell does. Especially for the 3 hour substrates an optimal focal adhesion area per cell was identified on areas with WCA of 20° and 35° for glass and PDMS, respectively. A lower WCA beyond the optimum leads to a lower focal adhesion area per cell which is significant for PDMS (data not shown). For the hydrophobic areas of the substrates with a WCA of 120°, hBM-MSC grown for 3 hours on glass shows a higher focal adhesion area per cell with 35 µm²/cell than on PDMS with 11 µm²/cell. This is not the case for the hydrophilic area, where the hBM-MSCs on PDMS have highest focal adhesion area per cell of 225 µm²/cell compared to glass with 150 µm²/cell. The same behavior is observed for the 24-hour incubation. Here the focal adhesion area per cell at a wettability of 120° increased to 30 µm²/cell on PDMS compared to 57 µm²/cell on glass whereas it is 257 µm²/cell for PDMS and only 181 µm²/cell on glass at the optimal condition.

Although the trends of the focal adhesion area per cell and the area per cell differ from each other with respect to wettability, here it is also shown that the influence of wettability is not the same for samples with different Young’s moduli. This clearly shows again, that a simple wettability gradient does not provide the answer towards cell behavior when transferring wettability to other materials of different stiffness (data not shown). Very striking is that in case of focal adhesion area per cell after 3-hour incubation, the curves for glass and PDMS intersect. Hydrophobic and soft has a lower focal adhesion area per cell than hydrophobic and stiff. At around 80° WCA and 10 MPa Young’s Modulus (PDMS) the focal adhesion area per cell becomes larger than for the 80° WCA and 550 MPa Young’s Modulus (glass). After 24-hour incubation time, there is still an intersection between both curves but it has shifted towards 55° WCA and 18 MPa Young’s Modulus (PDMS) and 55° WCA and 550 MPa Young’s Modulus (glass). This development indicates that combined surface parameters not only influence cell behavior in general but also combined parameters over time will follow different behavior patterns. This shows that it is important to not only determine one cell stage but monitor cell behavior over time.
4.5. CONCLUSION

In conclusion, combinations of physical surface parameters need to be investigated in order to obtain a better perspective on how hBM-MSCs and most likely cells in general behave at material interfaces. The importance of multi-parameter identification has been shown here by the use of a linear double gradient in wettability and stiffness compared to a single wettability gradient with uniform stiffness. This approach of using two physical surface cues in a controlled high-throughput fashion has not yet been utilized before and future endeavors are expected to go into the directions of orthogonal gradients as has been shown before using RGD-adhesion peptide gradients combined with porosity gradients. The different behavior of stiff and soft substrates of comparable wettability with similar surface chemistry showed that it is necessary to identify the combinatorial physical cues of the samples in great detail before making conclusions. Surface gradients have shown to be powerful tools to test the cellular response to these physical cues yielding a large amount of conditions using a minimal number of samples and thus allowing high-throughput sampling. In order for it to be a true high-throughput approach also the analysis of gradient surfaces has to be coupled to a high-throughput analysis method such as done here using an automated microscopy setup (TissueFAXS). Unfortunately, the high-throughput is still limited and does not provide the details yet as obtained using CLSM. It has been shown, that spreading measured in area per cell and adhesion measured in focal adhesion area per cell follow different paths with a different optimal wettability depending on the samples stiffness. In general, it showed that for both measures for high WCAs (hydrophobic) a hard surface is beneficial for the cells whereas for low WCAs (hydrophilic) a softer surface shows improved behavior. Even though hBM-MSCs are potent cells with differentiation capabilities towards a range of cell types, this property was not tested here. Nevertheless, since the adhesion of a cell to a surface is an early event, it can be assumed that the quality of adhesion influences the differentiation behavior of stem cells which can be modulated with both stiffness and wettability. The investigation here has been limited to the macroscopic behavior rather than going into depth of molecular mechanisms including gene transcription, protein expression and differentiation to focus on the details of material properties and how combinations are perceived by cells rather than single properties. The clinical relevance here is high as in particular silicone rubber (PDMS) is used in many different biomedical applications as scaffold, tubing and implant coatings. Future investigations will include molecular aspects of cell behavior as well as further gradient design entailing independent parameter control to yield even more combinations of which the previous mentioned example of RGD-gradient with porosity gradient in an orthogonal fashion is an interesting approach. However, combination of physical cues in a similar fashion requires more careful design. All endeavors entailing such gradient approaches should be combined with high throughput analyses methods to maximize screening efficiency.
4.6. EXPERIMENTAL SECTION

**Preparation of double gradients on PDMS:** Polydimethylsiloxane (PDMS) substrates were prepared using a commercially available elastomer kit from Dow Corning. Sylgard-184A and Sylgard 184B were mixed in a mass ratio of 10:1 according to suppliers’ information. Precisely 30 g of the viscous mixture were poured into a 12 cm x 12 cm squared polystyrene petri dish, to ensure equally thick samples. The mixture was put in a vacuum oven and a pressure of approximately 200 mTorr was applied to remove the gas from the PDMS in order to avoid bubble formation. The mixture was cured at 70 °C ambient pressure overnight. A circular cutting tool was used to cut the crossed linked PDMS in the desired size. The PDMS pieces were stored in a covered petri dish at ambient conditions until use. In order to obtain a double gradient patterned surface, the PDMS samples were treated with a shielded plasma approach. PDMS samples were put on a glass slide for support. A triangle shaped metal mask with an angular aperture of 30° was put in the middle part of the PDMS sample. One outside part was covered during plasma modification and the other outside part was uncovered for the completely hydrophobic or hydrophilic control respectively. The covered PDMS samples were always placed in the exact same way in the plasma oven to ensure reproducibility. For Plasma modification, air plasma generated by a plasma oven by “Diener electronic” model atto was used. The gas flow was adjusted in such a way that the pressure in the oven was stable at 30 mTorr. The modification was performed at 50% intensity for 1 minute. PDMS double gradient samples were used immediately after formation for cell experiments.

**Preparation of wettability gradients on glass:** In order to produce wettability gradients on glass samples, the glass was firstly cut into 2 cm x 0.8 cm big samples and cleaned with air plasma for 20 minutes at a stable pressure of 120 mTorr and 100% intensity, to ensure complete oxidation. The samples were immediately placed into a desiccator containing a vial containing 50 µL (0.06 g, 0.34 mmol) Trichloro(propyl)silane (98%, Sigma Aldrich). Vacuum was applied overnight to modify the glass surfaces in order to yield hydrophobic surfaces. Afterwards, these samples were treated similar to the PDMS samples using the same plasma oven and the same metal mask. The plasma treatment was performed at a pressure of 40 mTorr for 10 seconds at 20% power. Hydrophobic and hydrophilic controls were created using untreated hydrophobic and fully oxidized hydrophobic glass respectively. PDMS double gradient samples were used immediately after formation for cell experiments.

**Sample characterization:** The wettability of all samples was measured using a home build tensiometer. For all measurements 2 µL droplets of fresh milli-Q water were used in the sessile drop method. The Young’s modulus was analyzed using a AFM model CatalystBruker, Billerica, MA, USA) with nanoscope V as software. All measurements were performed in QMN (quantum-mechanical nano mapping) mode with a large amplitude using Bruker SCANASYST-AIR cantilevers made from silicon nitride with silicon made tips.
Cell adhesion studies: hBM-MSC (Poietics™ Normal Human Bone Marrow Derived Mesenchymal Stem Cells, p7, Lonza) were used for the cell adhesion studies. The growth medium consisted of Dulbecco's MEM Alpha Medium (1x) + GlutaMAX, 10% (v/v) fetal bovine serum and 0.1% (v/v) ascorbic acid 2-phosphate. The cells were incubated at 37 °C and 5% CO₂. All circular PDMS samples (Ø 20 mm) were sterilized with 70% ethanol prior to use. Afterwards hBM-MSCs were seeded onto the samples in a density of 3 × 10⁴ cells/well in a 12-well plate for cell adhesion. The samples were transferred into an incubator at 37 °C and 5% CO₂ for 3 or 24 hours.

Cell analysis: To quantify cell size and adhesion, the hBM-MSCs were fixated with 3.7% paraformaldehyde in PBS for 20 minutes. For the immunostaining, the primary antibody against vinculin (clone hVin-1, Sigma, 1:100) was used in combination with a secondary FITC-labeled goat-anti-mouse antibody (Jackson Immunolab, 1:100). In addition, DAPI and TRITC-phalloidin were used to stain the cells nuclei and F-actin respectively. Cells were observed using a LEICA TCS SP2 CLSM equipped with a 40 × NA 0.80 water immersion objective. Additionally, the nuclei and F-actin were observed using TissueFaxes®, with a Zeiss AxioImager Z1 Microscope System (Tissue-Gnostics GmbH, Vienna, Austria) in a 10x magnification. The complete samples were scanned and combined together using the Tissue-Gnostics software. Image analysis of vinculin was done by Focal Adhesion Analysis Server and ImageJ software was used to measure the average area per cell.[54]

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LITERATURE

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