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Substrate micropatterns produced by polymer demixing regulate focal adhesions, actin anisotropy, and lineage differentiation of stem cells

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Abstract

Stem cells are adherent cells whose multipotency and differentiation can be regulated by numerous microenvironmental signals including soluble growth factors and surface topography. This study describes a simple method for creating distinct micropatterns via microphase separation resulting from polymer demixing of poly(desaminotyrosyl-tyrosine carbonate) (PDTEC) and polystyrene (PS). Substrates with co-continuous (ribbons) or discontinuous (islands and pits) PDTEC regions were obtained by varying the ratio of PDTEC and sacrificial PS. Human mesenchymal stem cells (MSCs) cultured on co-continuous PDTEC substrates for 3 days in bipotential adipogenic/osteogenic (AD/OS) induction medium showed no change in cell morphology but exhibited increased anisotropic cytoskeletal organization and larger focal adhesions when compared to MSCs cultured on discontinuous micropatterns. After 14 days in bipotential AD/OS induction medium, MSCs cultured on co-continuous micropatterns exhibited increased expression of osteogenic markers, whereas MSCs on discontinuous PDTEC substrates showed a low expression of adipogenic and osteogenic differentiation markers. Substrates with graded micropatterns were able to reproduce the influence of local underlying topography on MSC differentiation, thus demonstrating their potential for high throughput analysis. This work presents polymer demixing as a simple, non-lithographic technique to produce a wide range of micropatterns on surfaces with complex geometries to influence cellular and tissue regenerative responses.

Graphical Abstract

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Disclosures

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Keywords

Mesenchymal stem cells; topography; micropatterns; polymer demixing; differentiation

1. Introduction

Central to the development of biomaterials for use in regenerative medicine is their ability to regulate cell behavior. Human mesenchymal stem cells (MSCs) are a promising cell source for tissue engineering due to their autologous availability and ability to differentiate into cell types present in connective tissues [1]. Additionally, MSCs suppress the local immune response [2] and thus are being used for several clinical applications including wound healing [3] and tissue regeneration [4]. To further advance the clinical and industrial use of MSC-based therapies, biomaterials that instruct MSCs to retain their stemness or differentiate into cell types present in various tissues need to be developed. MSCs are regulated by a confluence of outside-in and inside-out signals emanating from microenvironmental cues including soluble factors [1], components of the extracellular matrix (ECM) [5], mechanical signals [6], and topography [7–9]. Since topography influences the cell response independently of substrate chemistry, techniques that modify surface texture can be generalized to other polymers [10], and more importantly to modulate the effect of surface chemistry on cell behavior.

There are numerous studies in which the role of cell morphology in regulating stem cell behavior have been investigated. For instance, McBeath et al. showed that MSCs patterned onto larger islands amenable to cell spreading preferentially differentiated into osteoblasts, whereas cells on smaller islands that restricted cell spreading differentiated into adipocytes [11]. Building upon these findings, Kilian and coworkers showed that patterning MSCs onto islands of the same size but into shapes that favor actinomyosin contractility resulted in increased osteogenic differentiation in bipotential adipogenic/osteogenic induction medium [9]. In addition to cytoskeletal remodeling induced by cell shape, substrate topography ranging from nano- to meso-scales can also regulate intracellular morphology and cell behavior [12–14]. Since the seminal studies of Curtis and Wilkinson [7] as well as Chen et al. [15], other groups have investigated the influence of surface topography on cell behavior. For example, MSCs on aligned fibers exhibit increased intracellular cytoskeletal alignment, more pronounced focal adhesions, and osteogenic differentiation when compared to cells on randomly-oriented fibers [16]. It is clear that various aspects of topography including lateral spacing, feature height, and anisotropy all play an important role in determining cell attachment, proliferation, and differentiation profiles.

Although topography plays a significant role in cell behavior, practical approaches to patterning surfaces with reproducible topographies onto complex shapes remains a challenge. 3D printing, microcontact printing, and lithographic techniques are widely used to produce precisely controlled surface features [17,18], but these approaches require expensive equipment and are ill-suited to pattern fabricated devices with complex surface geometries [17,18]. An alternative and more practical approach is one that involves the phase separation of two immiscible polymers [19], a technique first used by Dalby and coworkers to elicit different cell behaviors [20].

Here, we used polymer demixing to create discrete and gradient micropatterns onto flat substrates as a platform to investigate the effects of topography on stem cell behavior. Poly(desaminotyrosyl-tyrosine carbonate) (PDTEC), a member of a library of tyrosine-derived polymers used in several biomedical devices [18] and polystyrene (PS) were used as the substrate and sacrificial polymers, respectively. By varying the ratio of PDTEC and PS, we created co-continuous and discontinuous PDTEC micropatterns and used these topographies to study their influence on MSC morphology and their ability to regulate MSC differentiation.

2. Materials and Methods

2.1 Polymer synthesis and characterization

PDTEC was synthesized and purified as described previously [18,19]. Molecular weight was determined by gel permeation chromatography (GPC). Dimethylformamide (DMF) was used as a mobile phase, and the molecular weight (M_w) as determined relative to polystyrene standards was 167 kDa. The glass transition temperature (T_g) of the polymer, as determined using Mettler DC 823 differential scanning calorimeter (DSC) was 96.5 °C. PS (M_w = 350,000 Da) was purchased from Sigma-Aldrich.

2.2 Fabrication of surface micropatterns

Micropatterns were fabricated using phase separation in a mixture containing substrate (PDTEC) and sacrificial (PS) polymers. Spin coating conditions were optimized from a set of trials with changing polymer concentrations (1 to 5 wt %) and spin coating speeds (1000 to 4000 RPM). Viscosity was too low or high at the 1 and 5 wt % extremes, respectively. Radial force also played a role in the distribution and formation of PDTEC micropatterns. At 1000 RPM PDTEC did not disperse evenly (PDTEC clumps appeared in middle of the cover slips). Based on these trials, most of the samples reported here were prepared from a 2 wt % solution by spinning at 4000 RPM for 30 s.

Polymer solutions (~ 70 μ l) prepared in tetrahydrofuran (THF) with different PDTEC and PS ratios were spin coated onto 12 mm glass coverslips to obtain thin (~200 nm) films of phase-separated PDTEC and PS. PS was then removed using cyclohexane, resulting in the desired PDTEC micropatterns featuring co-continuous (ribbons) or discontinuous (islands, pits) regions, depending on the starting PDTEC:PS ratios. By varying PDTEC:PS ratios, discrete micropatterns were obtained and labeled “Dx”, where “x” corresponds to the PDTEC w/v fraction such that D0 corresponds to an uncoated substrate (0% PDTEC:100%

PS) and D100 corresponds to a uniform PDTEC film (100% PDTEC:0%PS). Using this scheme, three distinct micropatterns were created: (i) co-continuous ribbons (D40, D60), (ii) discontinuous PDTEC islands (D20), and (iii) discontinuous pits (D80). Micropatterns produced using D40 and D60 formulations both had ribbon-like features, and the cell responses were similar; therefore, only data from D40 will be presented. D100 also served as a non-patterned PDTEC control surface. Micropatterned glass coverslips were vacuum-dried overnight to remove residual cyclohexane and stored in a desiccator until use.

Micropattern gradients were fabricated by dip coating microscope slides into polymer solutions. The glass slide was attached to the arm of a dip coater (KSV Nima, Biolin Scientific, Finland). Briefly, a 90 mL of 0.5% (w/v) PDTEC solution in THF was prepared. Next, 450 mg of PS pellets were placed at the bottom of the beaker and the solution was continuously stirred as the glass slide was gradually lifted out of the solution at a rate 1 mm/min. Using this scheme, as more and more PS got into solution, the ratio of PDTEC to PS continuously decreased, and newly dried layers with gradually increasing PS ratio formed onto the upward moving glass slide. The speed of the glass slide was adjusted so that at time $t = 0$ s, polymer films formed were entirely PDTEC and non-patterned (reminiscent of D100), and at the end of the dip coating at time $t = 50$ min, the deposited polymer produced ribbons with 1:1 PS:PDTEC w/v in solution following completely dissolution of the PS pellets in THF. The glass slides were then immersed in cyclohexane to remove PS leaving behind the patterned PDTEC substrate on the glass slide. To remove residual cyclohexane, the substrates were vacuum dried for 3 days, and stored in desiccator for 2–3 days.

Prior to cell seeding, substrates were plasma treated with an oxygen plasma chamber (600 mTorr, 100 W) for 3 minutes. This step was performed to attain uniform adsorption properties and to assist in cell adhesion. Post-plasma treatment the samples were also sterilized with UV light for 900 s and washed thrice with PBS.

2.3 Characterization of surface micropatterns

Micropatterns were characterized using optical microscopy (OM; Nikon Optiphot-Pol), scanning electron microscopy (SEM; Amray 18302), and atomic force microscopy (AFM; Park Systems NX-10). Optical micrographs were used to determine the spacing (d) of micropattern features and AFM images were obtained in contact mode (PPP-CONTSCR 5M probe, force constant 0.2 N/m. 25 kHz) to determine the height of the micropatterns.

2.4 Cell culture

Human MSCs from three donor lots (8004L, 7078, 7071L) were obtained from Texas A&M University (College Station, TX). For each donor lot, MSCs were expanded in T-75 flasks in a humidity-controlled environment under 5% CO₂ and 37 °C and fed every 3 days with growth medium (basal culture condition, BA) supplemented with 16.7% fetal bovine serum (FBS) and 1% v/v penicillin-streptomycin (Gibco). MSCs were received at passage 1, used for up to four passages, and subcultured upon reaching 80% confluence.

To assess the effects of topography on MSC morphology and differentiation, MSCs were cultured on micropatterns in bipotential adipogenic/osteogenic (AD/OS) induction medium. Bipotential AD/OS induction medium was prepared by combining Lonza MSC AD and OS

media in a 1:1 ratio. For optimal adipogenic differentiation, MSCs were cultured in Lonza AD induction medium for 3 days followed by AD maintenance medium for 1 day. As such, bipotential AD/OS induction medium consisted of a 1:1 ratio of OS and AD induction media for 3 days followed by a 1:1 ratio of OS and AD maintenance media for 1 day. OS induction, AD induction, and AD maintenance media were constituted per the manufacturer's instructions.

2.5 Cell staining

To assess cell morphology, after 3 days in culture samples were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 minutes. Samples were then blocked and permeabilized with a 0.1% Triton X-100 (Sigma, St. Louis, MO) / 5% normal goat serum (MP Biomedicals, Solon, OH) solution in phosphate-buffered saline (PBS; Lonza) for 30 minutes. After two washes with blocking buffer (5% NGS in PBS), primary phosphorylated focal adhesion kinase (pFAK, ab81298; Abcam, Cambridge, UK) antibody in blocking buffer at a 1:200 ratio was added overnight at 4 °C. After three 15-minute washes in blocking buffer, secondary antibody (Alexa Fluor; Invitrogen, Carlsbad, CA) was added at a 1:200 dilution for 2 hours at room temperature, followed by three 15-minute washes in blocking buffer. To label the actin cytoskeleton, cells were stained with Alexa Fluor 488 phalloidin (Invitrogen) per the manufacturer's instructions. Lastly, samples were counterstained with 1 µg/ml (4',6-diamidino-2-phenylindole) (DAPI; Sigma) in PBS.

To assess differentiation, after 14 days in culture samples were fixed with 4% paraformaldehyde for 10 minutes and stained for alkaline phosphatase (ALP) (fast blue RR; Sigma) and intracellular triglycerides (lipids) (AdipoRed; Lonza) per the manufacturer's instructions. Samples were then counterstained with 1 µg/ml DAPI to identify ALP- and lipid-positive cells.

2.6 Analysis of single cell morphology, actin anisotropy, focal adhesion length, and differentiation

All cell analysis was conducted with high-resolution confocal images (Zeiss LSM 480). Morphological features (area, aspect ratio) were quantified using ImagePro 7.0 (Cybernetics). Actin anisotropy was computed by manually defining cellular ROIs on phalloidin-stained cells using the FibrinTool plugin for ImageJ as described by Boudaoud and coworkers [20]. To assess focal adhesion maturation, the major axis of at least 20 focal adhesions (phosphorylated focal adhesion kinase, pFAK) per cell were measured using the measurement feature in ImageJ.

To assess stem cell differentiation, after 14 days of culture cells were fixed and stained with DAPI (nuclei), AdipoRed (lipids) and fast blue (alkaline phosphatase, ALP). On discrete micropatterns (cover slips), MSCs were identified as ALP or lipid positive by counting overlaying nuclei in the confocal images. On micropattern gradients (glass slides), 18 selected regions (6 rows along the length of the slide - direction of the gradient, and 3 columns across the gradient) were imaged by confocal microscopy. For each region, mean gray value of fast blue or AdipoRed stains were measured on ImageJ and normalized to DAPI.

2.7 Statistics

Statistical analysis of morphometric parameters included analysis of variance (ANOVA) with Tukey's honest significance difference (HSD) post-hoc method. Differences were considered significant for $p < 0.05$, and error bars indicate the standard error of the mean (SEM).

3. Results and Discussion

3.1 Fabrication and characterization of discrete micropatterns

Three distinct PDTEC micropatterns were deposited onto glass substrates using the polymer demixing method, followed by removal of PS as described in the Methods section (Figure 1a). Lower PDTEC fraction in the starting PDTEC:PS polymer mixture (D20) yielded discontinuous PDTEC islands. As the starting fraction of PDTEC is increased (D40, D60), a co-continuous morphology of PDTEC formed ribbons appeared. At higher PDTEC fractions (D80) discontinuous features resembling the inverse of D20 micropatterns formed, with pits in lieu of islands in an otherwise continuous PDTEC film (Figure 1b). SEM micrographs and quantification show no meaningful differences in the co-continuous ribbon micropatterns formed with D40 and D60 formulations (Figure S1). Optical micrographs show the transition from islands to ribbons and to pits with decreasing PDTEC concentration (Figure 1b). There is an increase in the pattern spacings (d) of PDTEC micropatterns with increasing PDTEC fraction and were $16 \pm 1 \mu\text{m}$, $48 \pm 5 \mu\text{m}$, and $151 \pm 13 \mu\text{m}$ in D20, D40, and D80, respectively (Figure 1c). AFM images (Figure S2) show the heights of these features to be $\sim 200 \text{ nm}$, close to the optimum value required to elicit topography-induced cellular responses [21]

3.2 Micropatterns induce changes in actin anisotropy and focal adhesion size

MSCs cultured on flat surfaces readily differentiate towards adipogenic and osteogenic lineages if cultured under well-defined medium formulations [1]. We were interested in studying how topography influences morphology and differentiation in the presence of media that can steer MSCs to different lineages. As such, MSCs were cultured on micropatterns featuring co-continuous or discontinuous PDTEC regimes (Figure S3) in the presence of bipotential adipogenic/osteogenic (AD/OS) induction medium. This design allows one to discern the role of the topography on lineage restriction above the base-line effects that enable lineage progression along either lineage.

After 3 days in culture, representative images of single MSCs in each condition stained with actin do not feature any observable differences between the groups (Figure 2a). Quantification of MSC cell spread area (A) and aspect ratio (AR) in these conditions corroborated qualitative observation (Figure 2b, 2c). Interestingly, we did not observe a significant change in area and aspect ratio although previous findings reported the effects of topography on these metrics [25]. For example, MSCs on lamellar surface topographies exhibited an increase in cell aspect ratio and surface area with a concomitant increase in osteogenic differentiation, while cells in hexagonal surface topographies exhibited reciprocal cellular morphology favoring adipogenic differentiation [25]. It is possible that these trends are not present in this study due to differences in feature dimensions ($\sim 200 \text{ nm}$ height versus

~20 mm) or moduli (~1.5 GPa versus 150 kPa) between the studies. Additionally, although the PDTEC ribbons (D40) are co-continuous, they are not as highly oriented and aligned as in other systems, such as fibrous scaffolds.

Previous studies have shown that MSCs exhibit increased actin anisotropy [26] and focal adhesion length [27] when they undergo osteogenic differentiation. Thus, in addition to overall morphology, we also investigated the effects of micropatterns on actin anisotropy and focal adhesion length since these metrics may precede MSC differentiation. MSCs were cultured on co-continuous PDTEC ribbons (D40) and discontinuous islands (D20) and pits (D80) in bipotential AD/OS induction medium, and after 3 days in culture actin anisotropy and focal adhesion length for single MSCs were evaluated.

Actin anisotropy was quantified by determining the percentage of actin fibers within a region of interest (ROI) using ImageJ (Figure 2d). MSCs cultured on ribbon micropatterns (D40) had a higher fraction of actin anisotropy than those in the discontinuous (D20, D80) conditions and flat control (D100) (Figure 2f). To quantify focal adhesion length, MSCs were stained with actin to visualize the cell body and immunostained with phosphorylated focal adhesion kinases (pFAK). Previous studies have used pFAK as an adequate stain to visualize focal adhesion of MSCs [28]. At least 20 focal adhesions were measured per cell (Figure 2e). Activation of pFAK, as measured by pFAK maturation, is an indicator of downstream cytoskeletal response to local cell-material traction forces [29,30]. Furthermore, focal adhesion involvement is known to be involved in osteogenic differentiation via mechanosensation [31]. In particular, biomaterials that restrict adhesion spacing have been demonstrated to affect cellular adhesion activity by restricting pFAK geometry [28,29]. MSCs on D40 micropatterns had longer and more pronounced focal adhesions than in the discontinuous (D20, D80) conditions and flat control (D100) (Figure 2g). Interestingly, no significant changes in cell spread area and aspect ratio were observed and hence these differences were independent of overall cell morphology.

3.3 Micropatterns induce divergent MSC differentiation outcomes in bipotential medium

To probe the effects of micropatterns on MSC differentiation, we evaluated the presence of intracellular triglycerides (lipids) and alkaline phosphatase (ALP) (Figure 3a). These markers were chosen based on their wide use in assessing MSC differentiation into adipogenic and osteogenic lineages, respectively [1,11]. MSCs were cultured on co-continuous PDTEC ribbons (D40) and discontinuous PDTEC islands (D20) and pits (D80) for two weeks in bipotential AD/OS induction medium and assessed for adipogenic and osteogenic differentiation markers. Using images of samples stained for ALP and lipids, three outputs were acquired: ALP(+), Lipid(+), and MSCs not expressing ALP or lipids, ALP/Lipid (-). MSCs that expressed lipids and ALP were demarcated as Lipid(+) since ALP can also be expressed in some adipogenic cells [34].

Quantification of MSCs on different micropatterns show that co-continuous and discontinuous micropatterns have no effect on the percentage of Lipid(+) cells (Figure 3b). In contrast, co-continuous micropatterns (D40) exhibit an increased percentage of ALP(+) cells in comparison to discontinuous topographies and flat control (Figure 3c). Interestingly, a higher percentage of MSCs on discontinuous micropatterns were AD/Lipid(-) when

compared to the co-continuous D40 condition (Figure 3d). MSCs cultured on non-patterned PDTEC (D100) films resulted in ~35% ALP(+) cells and ~55% ALP/Lipid(–) MSCs, respectively. As expected, no significant differences in differentiation were found between D0 (glass) and D100 non-patterned surfaces (Figure S4), confirming that lineage commitment is a result of the topographic features rather than surface chemistry.

We suspect that increased osteogenic differentiation in the co-continuous ribbon micropatterns is due to early changes in morphology. Specifically, conditions that promote increased focal adhesion length and actin anisotropy (i.e., D40) favor osteogenic differentiation [27,35]. In contrast, MSCs cultured on discontinuous micropatterns (i.e., D20, D80) feature significantly smaller focal adhesions and less aligned actin, which does not favor osteogenesis. It is well established that MSCs cultured on stiff substrates (e.g., tissue culture plastic) preferentially differentiate to cell types present in stiff environments (e.g., osteoblasts), so we suspect that the discontinuous topographies lower the extent of early changes in morphology (focal adhesion length, actin anisotropy) and thus end up not expressing adipogenic or osteogenic markers (Figure 3c, 3d) since there is no additional stimuli (e.g., a soft underlying substrate) to induce differentiation into other phenotypes (e.g., adipocytes). Taken together, these data indicate that long-term differentiation profiles observed for co-continuous (D40) and discontinuous (D20, D80) micropatterns can be explained by early changes in cytoskeletal organization.

3.4 Micropattern gradients induce spatially-dependent differentiation profiles

To study the effects of varying micropatterns on MSC differentiation within one substrate, we developed a system to create a continuous gradient of PDTEC micropatterns onto one glass slide. By slowly lifting a glass slide from a solution in which the concentration of PS in PDTEC solution was gradually increased, it was possible to systematically change the micropatterns from one extreme (i.e., non-patterned PDTEC) to another (i.e., sparse PDTEC islands) (Figure 4a). The result was a glass slide with a PDTEC-rich regime with discontinuous pits (Z1), followed by co-continuous (Z2 through Z5) and discontinuous PDTEC domains (Z6) (Figure 4b). MSCs were cultured on glass slides in bipotential AD/OS induction medium and stained for ALP, lipids, and nuclei to identify cell location.

To assess the effects of different topographies in these gradients, confocal tile scan microscopy was used to image the whole micropattern gradient glass slide which was then divided into six regions for analysis (Figure 4c and Figure S5). For quantitative measurement of osteogenesis and adipogenesis, mean gray values of fast blue and AdipoRed were measured using ImageJ and normalized to DAPI mean intensity. Figure 4d and 4e represent normalized mean gray values for osteogenesis and adipogenesis across different regions. Our analysis shows that the micropattern gradients have no effect in the presence of intracellular lipids (Figure 4d). In contrast, intracellular ALP was significantly higher in regions within the continuous gradient substrate in comparison to flat and discontinuous topographical regions (Figure 4e).

Findings from this study corroborate observations from discrete micropatterns (Figure 3). This suggests that our dip coating process, which can be used to imprint patterns on complex surfaces, is a viable process to modify surfaces of prefabricated devices to modify the cell

response. Furthermore, this gradient pattern can be used to study other cell behaviors including migration, proliferation, and differentiation into other lineages.

3.5 Translational potential and limitations

The demixing process demonstrated here is a non-lithographic, non-printing, high throughput technique that is scalable and has the potential to create textures on surfaces with complex geometries including non-planar prefabricated implants, such as bone plates, vascular grafts, and braided sleeves. Furthermore, this technique lends itself to inline surface modification of biomedical structures. For instance, the surface of a 100 μm polymer filament was textured in one continuous dip coating process (Figure S6). In addition to glass and polymer substrates, we also created patterns on metal surfaces (Au) (data not shown). More importantly, this process can be used to fabricate a range of patterns on a single substrate for high-throughput studies of cell responses to surface topography. For the demixing process to be applicable, the two polymers used should be soluble in a common solvent, be able to phase-separate, and a solvent be available to selectively dissolve the sacrificial polymer while leaving the other polymer intact. PDTEC and PS are suitable for this system and PDTEC can also be modified with several moieties (e.g., poly(ethylene glycol)) to add additional control over how cells respond to underlying micropatterns.

4. Conclusion

Phase separation that occurs by demixing of two immiscible polymers was investigated as a promising technique from the processing standpoint for influencing cell behavior. This method was used to produce a set of distinct micropatterns, co-continuous ribbons and discontinuous islands and pits. Our results show differential effects of co-continuous and discontinuous substrate micropatterns on focal adhesions, actin anisotropy, and lineage differentiation of stem cells. After 14 days in bipotential AD/OS induction medium, MSCs cultured on ribbon micropatterns exhibited increased expression of osteogenic markers, whereas MSCs on discontinuous PDTEC substrates showed a low expression of adipogenic and osteogenic differentiation markers. A micropattern gradient produced by dip coating technique is introduced as a platform for studying the effects of multiple topographies within one substrate. It is important to note that the dip coating technique can be used to deposit micropatterns that induce the expression or absence of differentiation markers onto prefabricated implants with complex shapes, making this system particularly appealing for tissue engineering applications. Additionally, since this system can be used with a variety of polymer chemistries, our platform has the potential to produce biofunctionalized patterns, further extending its utility for a broad range of biomedical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Significance

Gaining a better understanding of how engineered microenvironments influence stem cell differentiation is integral to increasing the use of stem cells and materials in a wide range of tissue engineering applications. In this study, we show the range of topography obtained by polymer demixing is sufficient for investigating how surface topography affects stem cell morphology and differentiation. Our findings show that co-continuous topographies favor early (3-day) cytoskeletal anisotropy and focal adhesion maturation as well as long-term (14-day) expression of osteogenic differentiation markers. Taken together, this study presents a simple approach to pattern topographies that induce divergent responses in stem cell morphology and differentiation.

Figure 1.

Fabrication of discrete micropatterns and characterization. (a) Micropatterns prepared by spin coating PDTEC and PS in THF. Cyclohexane is used to remove sacrificial PS, resulting in micropatterns of PDTEC. (b) Representative optical micrographs of discrete micropatterns show that by varying PDTEC:PS ratios, co-continuous ribbons (D40, D60) and discontinuous islands (D20) and pits (D80) can be robustly achieved (darker regions correspond to presence of PDTEC polymer). (c) The spacing of the patterned features (denoted as “*d*”) increases with increasing PDTEC. Scale bar (b): 100 μm.

Figure 2.

Discrete micropatterns control actin anisotropy and focal adhesion length. (a) Representative images of MSCs on co-continuous ribbons (D40), discontinuous islands (D20), and discontinuous pits (D80) stained for actin (green) and nuclei (blue). Quantification of (b) cell spread area (A) and (c) aspect ratio (AR) of MSCs on co-continuous (D40), discontinuous (D20, D80), and flat (D100) conditions show no significant differences between groups. Schemes to quantify (d) actin anisotropy (phalloidin) and (e) focal adhesion (phosphorylated focal adhesion kinase, pFAK) length for intracellular actin and focal adhesion morphology analysis. Quantification of (f) actin anisotropy and (g) FA length show that MSCs on co-continuous ribbons (D40) have more aligned actin and more pronounced focal adhesions when compared to discontinuous micropatterns and PDTEC films. Error bars represent SEM.; n.s. no significance between groups; *** $p < 0.001$. Scale bars (a, d, e): 25 μm .

Figure 3. Discrete micropatterns control MSC differentiation in bipotential AD/OS induction medium. (a) Representative images of MSCs cultured on micropatterns for two weeks and stained for lipids (fluorescent red), ALP (colorimetric blue), and DAPI (fluorescent blue). Quantification for percentage of (b) Lipid(+), (c) ALP(+) and (d) ALP/Lipid(-) cells shows divergent differentiation trends under different micropatterns. Error bars represent SEM.; n.s. no significance between groups; *** $p < 0.001$. Scale bar: a 100 μm .

Figure 4. Continuous micropattern gradient induces spatial differences in ALP expression. (a) Scheme to prepare continuous gradient substrate. (b) Representative optical images after PS removal showing gradient in topography along the micropatterned substrate that begins with PDTEC-rich region (Z1) to a mixture PDTEC-PS (Z6). (c) Representative images of MSCs on micropattern gradient substrate for the regions indicated shown in Figure 4a. ALP-stained regions appear dark in the figure. Quantification of differentiation markers show (d) no change in lipid-positive cells but a significant increase in (e) ALP-positive cells in regions corresponding to co-continuous PDTEC micropatterns. Error bars represent SEM.; n.s. no significance between groups; *** p < 0.001. Scale bars: b, c 200 μ m.