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Decoupling geometrical and chemical cues directing epidermal stem cell fate on polymer brush-based cell micro-patterns†

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The intricacy of the different parameters involved in cell adhesion to biomaterials and fate decision (e.g. proliferation, differentiation, apoptosis) makes the decoupling of the respective effects of surface properties, extra-cellular matrix protein adsorption and ultimately cell behaviour difficult. This work presents a micro-patterned polymer brush platform to control the adsorption of extra-cellular matrix (ECM) proteins to well defined micron-size areas and consequently control cell adhesion, spreading and shape independently of other chemical and physical surface properties. Protein patterns can be readily generated with brushes presenting a range of hydrophilicity and surface charge density. The surface properties of the selected brushes are fully characterised using a combination of FTIR, XPS, ellipsometry, atomic force microscopy, water contact goniometry, dynamic light scattering and ζ-potential measurements. Interactions of proteins relevant to cell patterning and culture with these brushes are studied by surface plasmon resonance, dynamic light scattering, ellipsometry and immuno-fluorescence microscopy. Finally this platform is used in an assay investigating the relative contributions of matrix geometry and surface chemistry on epidermal stem cell differentiation. It is found that moderate hydrophobicity does not impact stem cell commitment, whereas strongly negative surface potential increases the incidence of differentiation. This correlates with a marked decrease in the formation of focal adhesions (but not cell spreading).

Introduction

Tissue engineering and stem cell-based therapies are progressing fast and will contribute to a revolution in the biomedical field.1,2 Stem cells and their ability to recreate in vitro some of the biological features of tissues and organs also offer important opportunities for the screening of drugs and the testing of toxicity.3–5 However, their wide-spread use still requires overcoming important hurdles such as the isolation of well-defined stem cells and their expansion, delivery and ability to safely regenerate tissues without causing inflammation or tumour formation.6 Biomaterials play an important role at several levels...
in this research effort: they offer attractive solutions to problems associated with the delivery of cells and the regeneration of a tissue, they can be used to confer additional functionality to implants and their importance for the control of stem cell fate and expansion is becoming clearer.

A key feature of biomaterials is their ability to provide a well-defined local environment for cells and tissues, at least in the initial stage of the wound healing or tissue regeneration process. In this context, biomaterials not only confer structure to a cell-scaffold construct, but also provide stimuli that impact on cell behaviour. Cells sense a number of cues in their environment, such as cytokines, growth factors, extra-cellular matrix anchorage and adhesion to other cells, collectively known as the microenvironment. In vivo, this set of signals is perfectly balanced to ensure normal tissue development and homeostasis, but in vitro or upon implantation of a scaffold, this micro-environment is severely disrupted. Research focusing on understanding how cells interact with biomaterials, whether of natural or synthetic origin, is essential to the further development of regenerative medicine as it may allow establishing the minimum set of properties that a biomaterial should display in order to achieve a desired cell or tissue function. Hence it was found that cells and stem cells not only respond to biochemical cues (such as cytokines and growth factors) but also sense physical properties of materials, such as topography, nano-scale topology and mechanical properties. If one adds to this list other physico-chemical properties of materials and surfaces such as charge density, surface energy and wettability, the combination of parameters to vary and potentially control in the design of biomaterials quickly becomes overwhelming.

![Fig. 1](https://academic.oup.com/ib/article-fig/5/6/899/5096904)

**Fig. 1** Design of pattern geometries controlling single cell spreading independently from interactions with surface chemistry. (a) Geometry of patterns used. (b) Cell spreading and localisation of focal adhesions on such patterns. (c) Side-view of cell spreading. These patterns result in the control of ECM geometry (20 μm and 40 μm diameter discs, 40 μm diameter rings, arc shaped patterns) and associated cell adhesion and spreading. Overlap between cell membrane and polymer brush (and hence controlled cell–material interactions) are highlighted by arrows and shaded areas.
To tackle such complexity and diversity, high throughput screening tools have been developed to optimise biomaterials performance for culturing cells and stem cells and to determine structure–property relationships. Anderson and co-workers developed a combinatorial materials screening platform for the expansion of fully potent human embryonic stem cells (hESCs) and induced pluripotent stem cells.\textsuperscript{28} Kiessling and co-workers developed a peptide-based high throughput screen to support the culture of hESCs.\textsuperscript{29} Lutolf and co-workers designed hydrogel micro-arrays for screening the differentiation of human mesenchymal stem cells and the self-renewal of mouse neural stem cells.\textsuperscript{30} Other works used polymer libraries to screen cell adhesion and proliferation.\textsuperscript{31,32} These reports are particularly interesting as they not only make clear connections between cell adhesion and expansion and the adsorption of proteins such as fibronectin\textsuperscript{33} or the density of other cell adhesive molecules such as peptides,\textsuperscript{29} but also other materials and surface properties such as hydrophilicity (as assessed by water contact angle measurements), topography and mechanical compliance.\textsuperscript{28,32} These works highlight the complexity of these systems and the intricacy of different properties such as surface chemistry, physical properties, protein adsorption, cell adhesion, spreading and ultimately expansion or differentiation.

In order to decouple these different parameters, engineered platforms that allow dissociating cell adhesion and spreading from other physico-chemical properties such as surface charge density and hydrophilicity are required. We used patterned polymer brushes to restrict extra-cellular matrix (ECM) protein adsorption to well defined micron-size areas and consequently control cell adhesion, spreading and shape, independently of other chemical or physical surface properties. Polymer brushes are attractive coatings for biomaterials as they allow the control of surface chemistry on a wide variety of substrates.\textsuperscript{34} Some of these brushes display extreme protein resistant performance\textsuperscript{35,36} and can be further bio-functionalised to display selective protein binding and coupling,\textsuperscript{37–40} useful for protein arraying and biosensing. We designed a series of four pattern geometries (Fig. 1) allowing the control of cell spreading (20 μm adhesive islands forcing single cells to round up, 40 μm islands allowing full spreading and asymmetric arc-shaped patterns resulting in cell polarisation, see Fig. 1) and the exposure of the basal cell membrane to different types of surface chemistry (chemistry controlled by the type of polymer brush, see Fig. 2: ECM proteins in the case of 40 μm islands, polymer brush under the central part of the cell membrane in the case of 40 μm rings and polymer brush under non-adhesive edges in the case of arc-shaped patterns, see Fig. 1). This system allowed us to

Fig. 2  Polymer brushes with defined surface charge density and hydrophilicity. (a) Schematic representation of a polymer brush and chemical structure of the brushes used in this study. (b) ζ-Potential of brush-coated particles in different media and after immersion of protein solutions, in PBS. (c) Contact angle goniometry of polymer brushes presently studied.
probe the interplay between coating hydrophilicity, surface charge density, ECM topology as well as cell spreading and shape, independently of each other. We generated a series of patterned brushes with different geometry and brush chemistry, and characterised the chemical and physical properties of these coatings and their ability to resist protein adsorption. Finally, we studied their performance for cell micro-patterning and used the resulting platform in an assay probing the roles of matrix topology, surface charge density and hydrophilicity on the differentiation of epidermal stem cells.

Materials and methods

Materials

Oligo(ethylene glycol methyl ether methacrylate) (OEGMA, Mw 300), 2-(methacryloyloxy)-ethyl-trimethyl-ammonium chloride (METAC), 3-sulfopropylmethacrylate (SPMA), 2-(methacryloyloxy)-ethyl[dimethyl-(3-sulfopropyl)ammonium hydroxide (MEDSAH), (3-aminopropyl)trimethoxysilane (APTMS), CuCl2, CuCl2, CuBr2, 2,2′-dipyridyl (bpy) and phosphate-buffered saline (PBS, 150 mM) were purchased from Aldrich and used as received. Silica particles (320 nm, dry powder) were obtained via a procedure similar to that of POEGMA brushes. The following reagents were used: CuCl2 (77 mg, 0.57 mmol), CuCl (144 mg, 1.45 mmol), bpy (560 mg, 3.6 mmol), MEDSAH (20.0 g, 72 mmol), methanol–water 1/1 (40 mL). Polymisation time: 90 min. Dry thickness: 17 ± 3 nm.

Preparation of brush-coated substrates

ATRP initiator-modified substrates were generated by depositing a monolayer of o-mercaptopundecylbromoisobutyrate (5 mM ethanolic solution) on a gold-coated substrate (glass with 1.5 nm chromium, 100 mm diameter, silicon wafer, 100 mm diameter, 1.5 nm chromium) using a Synergy system from Millipore. Preparation of brush-coated particles

X-ray Photoelectron Spectroscopy (XPS) analysis was performed using a Kratos Axis Ultra DLD electron spectrometer under monochromatic Al Kα radiation (1486.6 eV). Pass energy of 160 eV and a step size of 1 eV were used for survey spectra. For high energy resolution spectra of regions a pass energy of 20 eV and a step size of 0.1 eV were used. Charging neutralising equipment was used to compensate sample charging and the binding scale was referenced to the aliphatic component of C 1s spectra at 285.0 eV. The concentrations obtained (error less than ±10 relative%) are reported as the percentage of that particular atom species (at%) at the surface of the sample (<10 nm analysis depth). The analysis area was 0.3 × 0.7 mm. FTIR spectroscopy was carried out with a Perkin-Elmer Spectrum 100 spectrometer. Spectra were taken at a resolution of 4 cm⁻¹ with a total of 128 scans per run.

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Contact angle goniometry was carried out at room temperature by using deionized water and a FTA1000 contact angle instrument (First Ten Ångstroms). The distance between samples and the micro-syringe was about 1 mm. Images were taken using a digital camera once infusion was complete. The hydrodynamic diameter ($D_h$) and z-potential of particles were determined using a Malvern Zetasizer Nano ZS fitted with a 633 nm red laser. For DLS, the scattered light intensity was measured at a scattering angle of 173°. $D_h$ was determined according to the Stokes-Einstein equation. The z-potential of the particles was estimated from the electrophoretic mobility according to the Helmholtz–Smoluchowski equation. For DLS and z-potential measurements, samples were prepared by dispersing particles in the desired medium such that the samples became slightly turbid, followed by sonication for 5 min. For protein adsorption experiments, particles were dispersed in deionised water (500 µL) via 10 min sonication. An equal volume of 2× concentrated PBS was added to the particle dispersion, mixed well and left to equilibrate for 5 min before centrifugation and resuspension of the particles in PBS (500 µL). An equal volume of a protein solution (collagen I, 20 µg mL$^{-1}$; fibronectin, 20 µg mL$^{-1}$; FBS, 20%; BS, 20%) was added to the particle dispersion and incubation was carried out at room temperature for 30 min. Particles were collected via centrifugation, washed using PBS via two centrifugation–resuspension cycles and finally re-dispersed in PBS for $D_h$ and z-potential measurements. Ellipsometry measurements were performed with a z-SE instrument from J. A. Woollam at 70° incidence angle. For dry samples, a simple gold substrate/cauchy film model was used and fitted between 400 and 900 nm. For wet samples, substrates were placed in an in-house built chamber fitted with quartz windows normal to the laser beam path. The gold model and refractive index of the medium were obtained using data recorded for bare gold in the relevant medium (deionised water or PBS). Measurements were carried out in triplicate. For ellipsometry, contact angle, DLS, and z-potential, measurements were carried out in triplicate on three independent samples. Measurements are given as averages ± standard deviation.

Atomic force microscopy (AFM) was performed for dry samples on a Dimension 3100 microscope (Veeco Instruments) using the tapping mode. Olympus OMCL-AC series silicon probes with a resonant frequency of 300 kHz and a spring constant of 42 N m$^{-1}$ were used. Images were acquired at three different points on a sample at a scan rate of 1.0 Hz for an area of 60 × 60 µm$^2$ and processed using the NanoScope software (Veeco Instruments). Surface roughness (root-mean-square) analysis was carried out in triplicate.

Surface plasmon resonance (SPR) was carried out with a Biacore 3000. SPR chips (Ssens) were coated with the desired polymer brush prior to mounting on a substrate holder. Mounted chips were docked, primed with buffer (PBS) twice and equilibrated at 20 µL min$^{-1}$ for 30 min or until a stable baseline was obtained. For protein adsorption experiments, the programmed sequence was the following: wash and equilibrate for 5 min, expose to protein solution for 5 min, wash with PBS for 30 min. The protein adsorption level is measured 30 min after the start of the washing procedure with PBS. The flow rate was 20 µL min$^{-1}$. Measurements were carried out in triplicate.

**Protein deposition on patterned brushes**

For extra-cellular matrix protein deposition, cut micro-patterned substrates (0.8 × 0.8 cm$^2$ squares) were placed in a 24-well plate, sterilized with 70% ethanol for 5 min, washed twice with PBS and incubated in a solution of the protein of interest (0.5 mL in PBS; collagen I, 20 µg mL$^{-1}$; fibronectin, 10 µg mL$^{-1}$) at room temperature for 45 min. Substrates were washed by first diluting with PBS and aspirating the resulting solution without allowing the substrates to dry. This dilution/aspiration protocol was repeated twice. Finally, substrates were washed twice with PBS and used shortly after for cell seeding or immuno-staining.

**Culture of primary human keratinocytes and cell seeding**

Primary human epidermal keratinocytes (HEKs) were isolated from neonatal foreskin and were cultured with feeder cells (J2 3T3 fibroblasts) as previously described (FAD medium used for culture was prepared as follows: 1 part Ham’s F12, 3 parts DMEM, 10% FBS, 0.5 µg mL$^{-1}$ hydrocortisone, 5 µg mL$^{-1}$ insulin, 10−10 M cholera toxin, 10 ng mL$^{-1}$ EGF).45 The feeders were removed using a solution of versene (no trypsin, Gibco) before detachment of HEKs (passage 2 to 8) using trypsin (0.25% from Gibco, dilution of versene/trypsin 9/1 from stock solutions) and re-seeded onto the micro-patterned substrates (pre-coated with collagen I or fibronectin, in a 24-well plate) at the desired density (100 000, 50 000, 50 000 and 75 000 cells per mL for 20 µm, 40 µm, 40 µmR and arc-shaped patterns respectively, 0.5 mL per well) in FAD medium. Cell adhesion was allowed to proceed for 2 h before thoroughly rinsing with fresh medium (three times 0.5 mL). Care was taken not to let the substrate dry during this washing step. Work with human material was carried out in compliance with the UK Human Tissue Act (2004) and approved by the National Research Ethics Service (08/H0306/30).

**Antibodies and immuno-staining**

The mouse anti-vinculin (hVIN1; 1 : 200) and TRITC–phalloidin (1 : 500) were used to detect actin filament structure. 3-Aminopropyltriethoxysilane (Dow Corning) was used to derivatize the surface before the adsorption of antibodies. Antibodies and immuno-staining was performed at room temperature for 20 min. The mouse anti-vinculin (hVIN1; 1 : 200) and TRITC–phalloidin (1 : 500) were from Sigma Aldrich. Collagen type I (1 : 500) and fibronectin (1 : 500) were from Abcam. The Mouse anti-involucrin (SY7; 1 : 1000) was prepared by Cancer Research UK central services. For immuno-fluorescence staining, cells were fixed in paraformaldehyde (4%, 10 min), permeabilized with Triton X-100 (0.2%, 5 min) and blocked for 1 h (10% PBS plus 0.25% gelatin) at room temperature. Samples were incubated with primary antibodies for 1 h at room temperature, washed, incubated with conjugated secondary antibodies (1 : 1000; Alexafluor 488 and 555; Invitrogen) for 1 h and finally washed, at room temperature. TRITC–phalloidin was included in the blocking solution and DAPI (4,6-diamidino-2-phenylindole) was included in the secondary antibody solution, where indicated. Samples were mounted on glass slides with Mowiol reagent (4-88 Reagent, Calbiochem). For viability and cell patterning performance studies, a live/dead kit from Invitrogen was used according to the protocol provided by the manufacturer.
Fluorescence microscopy images were acquired with a Leica DMI4000B fluorescence microscope (CTR 4000 laser, excitation filter BP480/40, suppression filter B527/30, dichromatic mirror 505, 20 × 0.5 and 63 × 1.23, Oil, lenses). Confocal fluorescence imaging was carried out using a Zeiss Axio Observer (63 × 1.4 NA, Oil, HCX Plan-Apo lens), using an ApoTome (Zeiss) for sectioning.

Data and statistical analysis

Generation and analysis of heatmaps. This analysis was carried out following a reported procedure. Single pattern images (n > 50, from triplicates) were acquired, aligned and cropped (using ImageJ software). The resulting collection of images was stacked and overlayed (Z Project/Sum Slices in ImageJ) into a greyscale image.

Involucrin expression assay. This assay was performed following a previously reported procedure. Image acquisition was carried out using the DAPI channel for focusing. Involucrin expression levels were measured using a region of interest of 15 μm diameter centred on the nucleus of each cell (using the DAPI channel). For each experiment, 100–200 cells were counted and experiments were carried out in biological triplicates. Involucrin expression thresholds were determined using four times the average involucrin intensity 2 h after seeding.

Focal adhesion analysis. Confocal vinculin images were acquired and analysed using ImageJ. For total vinculin quantification, images were thresholded to correct for the background intensity. For focal adhesion distribution analysis, a higher threshold was used (10% of the max intensity) and particle analysis (ImageJ) was performed without any constraints on object size. Measurements were carried out on three biological replicates (n > 30).

Data were analysed by Tukey’s test for posthoc analysis. Significance was determined by **P < 0.05, ***P < 0.01.

Results and discussion

Patterned brushes with controlled surface charge and wettability

In order to generate cell patterns with controlled surface charge, a series of five polymer brushes was selected (Fig. 2): POEGMA is neutral, yet displays medium water contact angle, whereas PMETAC, PSPMA and PMEDSAH are positive, negative and zwitterionic brushes. In addition, a copolymer brush of METAC and SPMA (1/1 random copolymer, COPO) was generated based on its global neutral charge and protein resistance. The ability of changing the brush structure independently of other parameters was first studied.

Brushes were generated with a thickness between 15 and 30 nm. Such thin brushes were chosen on the basis that they offered the best compromise between protein resistance and cell patterning, as in the case of POEGMA brushes. IR spectroscopy (presence of bands at 1439, CH₂ bending POEGMA, 1483, CH₂ bending PMETAC, PMEDSAH, COPO, and 1045 cm⁻¹, sulfonate asymmetric stretching PSPMA, PMEDSAH, COPO) and XPS data (peaks at 167.4 ± 0.1 eV, S2p3/2, and 402.6 ± 0.2 eV, N1s quaternised amine of PMETAC, PMEDSAH and COPO) are consistent with the structure of the resulting brushes (Fig. S1, ESI†). In particular, PMEDSAH and COPO brushes combine features of PMETAC and PSPMA, although the ratio of S/N measured by XPS was found to be 1.1 instead of 1.0 in both cases, an observation consistent with other reports. Brush swelling in PBS was found to fall between 1.3 and 2.4 for PMEDSAH and PMETAC (Fig. S2, ESI†), respectively, whereas the roughness of dry brushes was 1–4 nm (Fig. S3, ESI†).

Surface charge and brush hydrophilicity were investigated via ζ-potential and water contact angle measurements (Fig. 2). As expected, in PBS solutions, the ζ-potential of POEGMA, PMETAC and PSPMA brushes are neutral (−1 ± 1 mV), positive (23 ± 2 mV) and negative (−29 ± 1 mV), respectively, in line with other reports. The ζ-potential of PMEDSAH and COPO brushes, although relatively small (−7 ± 1 mV), remained slightly negative despite their expected globally neutral brush structure. In deionised water, the ζ-potential of these brushes was markedly lower, comparable to that of PSPMA, suggesting a slight imbalance in the charge distribution within these brushes, but arising from relatively weak negative charges easily screened at higher ionic strength. Such observations may be related to the slight S/N imbalance measured in these brushes via XPS (S/N of 1.1, Fig. S1, ESI†). In the case of COPO brushes, this imbalance may be a reflection of the difference of reactivity of SPMA and METAC monomers, but is also observed in unreacted MEDSAH monomer by XPS (results not shown). In terms of hydrophilicity, POEGMA brushes displayed higher water contact angles (45° ± 1) than the rest of the brushes studied (below 10°), in line with the poorer solvation of oligo(ethylene glycol) side chains compared to MEDSAH or charged monomers. Overall our results confirm that the range of brushes studied display a range of surface charge (strongly negative to positive) and hydrophilicity (highly hydrophilic and moderately hydrophobic).

Protein adsorption on neutral and charged brushes

For efficient cell arraying using micro-patterned polymer brushes, selective extra-cellular matrix (ECM) deposition to un-protected gold patterns is required. We first investigated protein adsorption of polymer brushes under typical cell patterning and culture conditions using surface plasmon resonance (Fig. 3): we followed protein adsorption from specific diluted ECM protein solutions (fibronectin and collagen I, 10 μg mL⁻¹) and diluted sera relevant to cell culture (10% FBS and BS). We found that POEGMA resisted to protein adsorption (below 3 ng cm⁻²) from the different protein solutions tested. Similarly, PMEDSAH and COPO brushes resisted the adsorption of fibronectin and collagen at concentrations relevant to protein patterning and when incubated in 10% FBS, in agreement with previous reports. This suggests that these brushes would be efficient at promoting selective ECM protein adhesion to unprotected areas and subsequent cell patterning. However, these brushes displayed relatively high fouling (170–190 ng cm⁻²) from 10% BS, perhaps reflecting that some proteins or other constituents present in this medium interact with residual negative charges observed for these brushes in PBS. The ζ-potential of PMEDSAH and COPO brushes remained unchanged.
Protein and cell patterning with polymer brushes

Given the lack of significant protein adsorption to neutral brushes and that of fibronectin and collagen I to PSPMA and PMETAC, respectively (adsorption below 5% of that measured to unprotected gold surfaces), micro-patterning of polymer brushes should allow efficient protein patterning. Polymer brush patterns were generated via micro-contact printing of an ATRP initiator thiol, followed by polymerisation of the relevant monomer mixture. ECM protein patterning was investigated using arc shaped patterns to unprotected gold surfaces. Micro-patterning with the expected shape and dimensions were obtained (control experiments omitting the primary antibody during the staining step did not result in any detectable fluorescence). To quantify the quality of the pattern, high resolution single pattern images were acquired, stacked and overlayed. Average protein localisation and protein deposition profiles obtained from the resulting heatmaps were compared for the different brush–protein combinations (Fig. S5, ESI†). Although the general distribution of proteins were found to be of similar quality, the profiles highlighted that the background protein density on the polymer brush was higher for PMETAC–collagen I, consistent with the higher protein adsorption measured for this brush–protein pair by SPR. Overall, these results suggested that efficient cell patterning could be expected from most brush–protein pairs tested.

To test cell patterning efficiency, human primary keratinocytes were suspended in culture medium and deposited on protein patterned brushes (20 μm ECM adhesive islands). After 2 h of adhesion, non-adherent cells were removed by replacing the medium and remaining cells were stained to probe their patterning and viability (live/dead assay, Fig. 4 and Fig. S6, ESI†). Cell viability was very high on all surfaces, except PMETAC for which cell death was almost 100%. Cationic polymers and surfaces such as PMETAC typically display such cell toxicity. Highly charged cationic polymers used for siRNA or plasmid delivery are known to induce cell death, especially when not conjugated to charged RNA or DNA molecules. Similarly, positively charged surfaces have been reported to promote rapid “trapping” of cells and toxicity, observations that correlate with surface charge density. Hence, the cell toxicity observed in PMETAC brushes is not surprising, despite the lowering of its ε-potential upon exposure to serum (Fig. 2; 10% FBS contained in keratinocyte medium). In addition, despite the availability of un-coated areas presenting ECM proteins, very few keratinocytes were found alive or patterned, suggesting that even partial exposure to PMETAC is sufficient to induce cell death. The high densities of cells found with PMETAC substrates also suggest a very rapid non-specific capture of cells, presumably due to favourable electrostatic interactions with cell membranes.

The ability of neutral and PSPMA brushes to give rise to efficient cell patterns was next examined. Patterning efficiencies (% cells patterned/total cell population on substrate) were high for all brush–protein combinations (Fig. 4), even on PSPMA brushes for which protein adsorption from keratinocyte medium (containing 10% FBS) was observed. This is in line
with ECM protein patterning results and suggests that proteins adsorbing to PSPMA do not contribute to keratinocyte adhesion significantly. Patterning occupancy (% islands occupied) was defined as the ratio of the number of cells patterned to the total number of available ECM protein adhesive islands. It was found to be around 75% for all combinations of brush and ECM. Although slightly lower in average for PSPMA–fibronectin patterns (63%), cell occupancy was not statistically different (p between 0.27 and 0.99) from that of other patterns. This is important to ensure that similar populations of cells are compared.

Impact of brush chemistry and ECM topology on keratinocyte differentiation

In order to probe independently the roles of cell spreading and surface chemistry, a series of four patterns were chosen (Fig. 1): 20 μm diameter islands that had previously been shown to promote keratinocyte differentiation,46 40 μm islands on which these cells did not differentiate,46 40 μm rings presenting a total ECM protein area identical to that of 20 μm islands but an outer diameter of 40 μm, and arc-shaped asymmetric islands presenting an intermediate total ECM protein area. The first two types of patterns allow cells to spread on ECM-coated areas only and the cell membrane is only in contact with brushes at the periphery. In the latter two types of patterns, the cell is forced to spread on a combination of ECM- and brush-coated areas.

POEGMA, PMEDSAH and COPO patterns coated with collagen I allowed to test the role of brush hydrophilicity independently from ECM topology and cell spreading, whereas comparing fibronectin-coated PMEDSAH and PSPMA patterns allowed to probe the role of surface charge.

Keratinocytes were seeded on these patterns and cultured for 24 h before staining for the differentiation marker involucrin (Fig. 5a and b). It was found that single cells differentiated to the same extent on all 20 μm islands, suggesting that the surrounding brush does not influence further cell fate decision. Similarly, cells allowed to fully spread on 40 μm islands did not commit to differentiate on all brushes tested. Finally, keratinocyte differentiation on 40 μm rings was as low as that measured on 40 μm islands, giving further evidence that cell shape and spreading, rather than total ECM-coated area, are key determinants of cell fate. With these latter patterns, the cell membrane was in contact with brushes at the periphery and in the centre of the island (see Fig. 1). Hence with these matrix geometries, surface hydrophilicity and charge density had no measurable impact on keratinocyte differentiation. However, when cells were forced to spread on asymmetric arc-shaped patterns, cell commitment was sensitive to surface charge and cell differentiation was intermediate between the basal level and that of cells differentiating on 20 μm islands (Fig. 5a). Hence our results suggest a cross-talk between matrix topology and surface chemistry,
although matrix topology and the associated cell spreading seem to remain the dominating factors.

**Cross-talk between matrix topology and surface charge on focal adhesion formation**

Given the role of cell adhesion on keratinocyte differentiation, effects of surface charge on cell adhesion were next investigated (Fig. 6), focusing on the case of cells spreading on PSPMA and PMEDSAH patterns. The possible effect of surface chemistry on cell spreading was first examined (Fig. 6a). We found no measurable difference in cell spreading (cell area) on PSPMA and PMEDSAH brushes for all four patterns tested. However, when analysing focal adhesion distribution via vinculin staining, a systematic decrease in overall intensity was observed (Fig. 6b and c). Focal adhesion formation was further analysed by counting the number, size and intensity of adhesion sites in vinculin images (Fig. S7, ESI†). Pattern geometry had an effect on the number of adhesions, with cells on 40 µm islands forming more adhesions than those on other patterns tested. This effect was more pronounced on PSPMA patterns: although the distributions of adhesions were similar for both brushes on 40 µm islands, a more pronounced decrease in object numbers was found for PSPMA-based rings (40 µmR) and arc-shaped patterns (see Fig. S7a, ESI†). In contrast, the pattern geometry and type of brush had no marked effect on vinculin intensity at single adhesion sites (Fig. S7b, ESI†). Our measurements showed a general increase (1.7–2.0 fold) in vinculin intensity as adhesion sites increased from 0.29 µm² (equivalent to a 600 nm diameter adhesion) to above 10 µm² (3.6 µm adhesion). This trend was insensitive to the nature of the pattern on which

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**Fig. 5** Impact of geometrical and chemical cues on epidermal stem cell differentiation. (a) Epidermal stem cell differentiation on patterned brushes with controlled geometry and chemistry. Involucrin expression is used as differentiation marker. Cells were incubated in FAD medium for 24 h before fixation and staining (n.s., non significant; ***p < 0.01). (b) Associated fluorescence images (green, involucrin; blue, DAPI). Scale bar: 100 µm.
cells spread, suggesting that pattern topology and surface chemistry alter focal adhesion formation \emph{via} a change in the number of adhesion sites formed and their maturation. However, adhesions that were able to further mature in size displayed the normal pattern of vinculin recruitment and reinforcement.\textsuperscript{58}

Integrin signalling is an important determinant of keratinocyte fate decision and mediates physical cues. Decreased \(\beta_1\)-integrin adhesion reduces mitogen-activated protein kinase activation and induces keratinocyte differentiation \emph{in vitro}\textsuperscript{56} whilst reducing proliferation \emph{in vivo}.
\textsuperscript{59} This signalling mechanism is involved in the transduction of matrix physical properties such as morphology and ECM protein tethering\textsuperscript{58} and mechanical stretch.\textsuperscript{60} Similarly, cell spreading, shape and cytoskeleton organisation are important regulators of keratinocyte differentiation\textsuperscript{46} and are tightly connected to integrin-mediated adhesion. However, integrin-mediated adhesion and cell shape can signal and dictate keratinocyte differentiation\textsuperscript{56} \emph{via} distinct mechanisms. The present findings suggest the existence of a cross-talk between ECM matrix topology and surface charge, which in turn control cell shape and focal adhesion formation, respectively. The mechanism by which strong negative surface charge, such as that of PSPMA, modulates integrin-mediated adhesion independently of cell spreading is unclear, but these results demonstrate that physicochemical cues can combine to alter cell behaviour. Similar cross-talks have been reported between mechanical and topological cues,\textsuperscript{61} cell–cell and cell–matrix cues\textsuperscript{62} or between ECM anchorage and growth factor signalling.\textsuperscript{63} At the transcriptional level, diverse genetic and epigenetic programs interact with each other to control cell fate decision.\textsuperscript{64} Therefore the emerging picture points towards the cross-talk of multiple physical, chemical, biophysical and genetic interactions to dictate cell behaviour, tissue development and homeostasis.

Conclusion

Cell–cell and cell–matrix adhesions are important mediators of stem cell fate decision and are key constituents of the cell micro-environment. A variety of physical properties (\emph{e.g.} compliance, topography and topology) of the matrix have been shown to modulate these adhesive cues. The present work gives evidence for further cross-talks between geometric and chemical cues: moderate hydrophobicity has no measurable impact on epidermal stem cell differentiation, whereas strong negative surface potential increases the incidence of cell commitment to the differentiation program when combined to specific matrix topology. Given the potential of bio-functionalised polymer brushes for specific ligand–receptor interactions, this platform will prove useful for the systematic study of cross-talks between cell–matrix adhesive cues, cell spreading and shape, and other membrane receptor-mediated signalling pathways. It is likely that cross-talks and synergies between other physical, chemical and biochemical components of the cell micro-environment are widespread. Studies able to probe simultaneously, and potentially in high throughput, multiple parameters

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\centering
\includegraphics[width=\linewidth]{fig6.png}
\caption{Impact of brush chemistry and pattern geometry on cell spreading and focal adhesion formation. (a) Cell spreading is dictated by pattern geometry and not brush chemistry. Cell areas were measured using single cell actin stainings. Red bars are the expected maximum spreading for the respective pattern geometries. (b) Impact of pattern geometry and brush chemistry on focal adhesion (vinculin) intensity. (c) Single cell vinculin images (after 24 h of incubation) obtained on PMEDSAH and PSPMA brushes for different pattern geometries.}
\end{figure}
of the cell micro-environment will have a strong impact on the fields of cell biology and biomaterials design, by speeding up the discovery of novel materials able to improve stem cell expansion, tissue regeneration and the development of drug or RNAi screening platforms.

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