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Directing cell migration using micropatterned and dynamically adhesive polymer brushes

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ABSTRACT

Micropatterning techniques, such as photolithography and microcontact printing, provide robust tools for controlling the adhesive interactions between cells and their extracellular environment. However, the ability to modify these interactions in real time and examine dynamic cellular responses remains a significant challenge. Here we describe a novel strategy to create dynamically adhesive, micropatterned substrates, which afford precise control of cell adhesion and migration over both space and time. Specific functionalization of micropatterned poly(ethylene glycol methacrylate) (POEGMA) brushes with synthetic peptides, containing the integrin-binding arginine–glycine–aspartic acid (RGD) motif, was achieved using thiol–yne coupling reactions. RGD activation of POEGMA brushes promoted fibroblast adhesion, spreading and migration into previously non-adhesive areas, and migration speed could be tuned by adjusting the surface ligand density. We propose that this technique is a robust strategy for creating dynamically adhesive biomaterial surfaces and a useful assay for studying cell migration.

1. Introduction

In recent years, the development of micropatterning techniques in which adhesive or non-adhesive proteins and polymers are precisely patterned on cell culture surfaces have provided many important insights into the basic regulatory mechanisms governing cell adhesion and spreading [1,2], as well as their impact on growth [3,4], survival [4] and differentiation [5,6]. Common micropatterning strategies include microcontact printing of self-assembled monolayers [4,7,8], UV photopatterning [9] and direct protein printing [10]. While each of these techniques allows extracellular matrix (ECM) proteins to be deposited in fixed 2-D patterns, only a few reports have described systems for creating dynamic or stimuli-responsive patterns [11–13]. For example, light or electric potentials can be used to selectively remove non-adhesive regions from a surface [11,12], while light-activated deprotection [13,14] or host–guest chemistry [15] provide additional means for controlling cell adhesion to various biomaterials.

"Click" chemistry offers yet another approach for creating dynamically adhesive surfaces. Click chemistry refers to a class of reactions, which are highly efficient and specific under physiological conditions and are attractive for biological applications. The most well-studied click chemistry reaction is the copper-catalysed azide–alkyne cycloaddition (CuAAC), where an organic azide reacts with an alkyne to form a triazole ring [16]. Until recently, a major disadvantage was the use of toxic Cu (I) as a catalyst [17], but advances in copper-free click chemistry using strained alkynes have since made it possible to perform similar reactions in the presence of cells and create dynamically adhesive substrates [18]. Thiol–ene and thiol–yne reactions belong to another class of click reactions that involve the free-radical-mediated addition of thiols to unsaturated carbon–carbon bonds [19,20]. Although both alkene and alkyne groups can react with a single thiol group, radical additions to alkynes produce vinyl sulphides, which can undergo a second addition of a thiyl radical [19].

Thiol–ene/yne reactions are particularly useful for biological applications since thiol groups are present in cysteines, which can easily be introduced into synthetic peptides and are found in some native proteins. However, the biggest advantages of this reaction are its high efficiency and low toxicity, which make it a good candidate for functionalization of biomaterials [21]. Light-based activation of thiol–ene/yne reactions also provides the ability to spatially and temporally control functionalization [22]. In this study, we describe a method to create dynamically adhesive biomaterial surfaces using thiol–ene/yne coupling of cell adhesive

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ligands to POEGMA brushes. By combining this reaction with microcontact printing, we developed a tunable in vitro assay, which allows us to control and study cell adhesion and migration over both space and time.

2. Materials and methods

2.1. Preparation of micropatterned poly(ethylene glycol) methacrylate (POEGMA) brushes

Micropatterned polydimethylsiloxane (Sylgard 184) stamps were inked with the thiol initiator ω-mercaptoundecyl bromoisobutyrate and stamped onto gold-coated coverslips to deposit the initiator as a self-assembled monolayer [6,8]. Non-patterned substrates were soaked in the thiol initiator overnight. Atom transfer radical polymerization of oligo(ethylene glycol) methacrylate (OEGMA; MW_{avg} 360) was carried out as previously described [6,23]. The polymerization reaction was performed at room temperature for 1 h, resulting in an estimated 60 nm thick brush [23]. After polymerization, substrates were activated with N,N′-disuccinimidyl carbonate (DSC, 0.1 M) and dimethylamine pyridine (0.1 M) in N,N-dimethylformamide (DMF), overnight at room temperature. After washing twice with DMF and water, substrates were incubated overnight at room temperature with 1% allylamine or propargylamine in DMF. Both allylamine and propargylamine were handled inside the fume hood, using protective goggles, gloves and a lab coat. All experiments were performed after washing substrates with 70% ethanol for 10 min and twice with phosphate-buffered saline (PBS). All reagents and solvents were obtained from Sigma–Aldrich.

2.2. Thiol–ene and thiol–yne reactions

Thiol–ene/yne reactions were performed in the presence of a photoinitiator, Irgacure 2959 (2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone, PI; Sigma–Aldrich), and UV light (~23 mW cm^{-2}, λ_{max} = 365 nm). Irgacure 2959 was resuspended at 0.5% (w/v) in Dulbecco's modified Eagle's medium (DMEM) without phenol red and allowed to dissolve overnight at 37 °C. Substrates were cut to 1 cm^2 and immersed in 500 μl total volume, with varying concentrations of Irgacure 2959 and **C**GG**RGD**SP (arginine–glycine–aspartic acid, RGD) or **C**GG**RGE**SP (RGE) synthetic peptides (Insight Biotechnology Inc., UK). The GG sequence was used as a spacer, and the SP sequence was added to enhance the integrin–RGD binding [24]. The coupling reaction was initiated by exposing the substrates to UV light from an LED array (Cetoni) for specified amounts of time. After exposure, substrates were immediately washed three times with PBS in the absence of cells or with DMEM in the presence of cells. For the photomask experiments, the reaction was performed in 5 μl total volume of 1 mg ml^{-1} RGD and 0.5% Irgacure 2959. The photomask was placed on top of the UV lamp, then the substrate was inverted onto the 5 μl reaction mixture and exposed to UV for 1 min followed by three PBS washes.

2.3. Cell culture, adhesion and migration

NIH 3T3 fibroblasts and Madin–Darby canine kidney (MDCK) cells were maintained in high-glucose DMEM (Life Technologies) supplemented with 10 or 5% foetal bovine serum (Life Technologies), respectively, and 1% penicillin/streptomycin (Life Technologies). To examine fluorescence, MDCK cells were transfected with 0.7 μg of pEGFP-N1 plasmid DNA (Clontech) and 2.5 μl of Lipofectamine 2000 (Life Technoliges) per well of a six-well plate (70% confluent).

For adhesion assays, substrates were blocked for 1 h with 2% bovine serum albumin in PBS (BSA/PBS), then cells were seeded onto non-patterned substrates in serum-free DMEM at a density of 10,000 cells per cm^2 (1 substrate per well of a 24 well plate) and allowed to adhere for 1 h 30 min at 37 °C in a 5% CO_2 atmosphere. Non-adherent cells were rinsed off, and adherent cells were fixed and stained as described. For migration studies, cells were seeded onto alkyne-modified micropatterned substrates at a density of 50,000 per cm^2, rinsed after 1 h, and cultured overnight. Photoactivated coupling reactions were performed as described above, and migration was monitored by phase contrast imaging with an Incucyte Zoom microscope (Essen Bioscience), image acquisition every 15 min, for 24 h. The Incucyte Zoom image analysis software was used to automatically detect cell edges and generated an overlay mask, which was used to calculate the cell coverage area.

2.4. Enzyme-linked immunosorbent assay (ELISA)

POEGMA substrates were prepared as described and functionalized with the peptide **C**GG**RGD**SP–biotin (RGD–biotin). To ensure that the measurements were within the linear range of the ELISA, the RGD–biotin was diluted 1:100 with non-biotinylated RGD. Substrates were blocked for 1 h with 2% BSA/PBS and incubated with 0.1 μg ml^{-1} horseradish peroxidase (HRP)-conjugated streptavidin (Thermo Fisher) for 1 h at room temperature. After three washes with PBS, substrates were incubated with the one-step 3,3′,5,5′-tetramethylbenzidine ELISA buffer (Thermo Fisher) for 15 min at room temperature to detect HRP activity. The reaction was stopped with 2 M sulphuric acid. Absorbance was measured at 450 nm in a microplate spectrophotometer (BMG Labtech).

2.5. Immunofluorescence staining and imaging

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.2% TritonX-100/PBS for 10 min. Samples were blocked for 1 h with 2% BSA/PBS, then antibodies were diluted in a solution of 2% BSA/PBS and incubated for 1 h at room temperature. Coverslips were mounted on glass slides with Mowiol reagent (Sigma–Aldrich), and images were acquired with a DM5000B epifluorescence microscope (Leica) or LSM 710 confocal microscope (Carl Zeiss Inc.). The mouse anti-paxillin antibody (P13520; 1:500; BD Biosciences), DAPI (1:1000) and Phalloidin Alexafluor 488 (1:1000; Invitrogen) were used.

2.6. Statistical analyses

For quantification of adherent cells, the numbers of DAPI positive nuclei were counted in five random images per sample. Triplicates for each condition were analysed for three individual experiments with ImageJ software. Migration was quantified with the Incucyte Zoom image analysis software or ImageJ by measuring the cell coverage of at least four micropatterned islands per condition in three independent experiments and reported as the percentage change in cell area. All data were reported as the mean ± standard error of the three experiments and analysed by two-tailed unpaired *t*-tests. A probability value of $p < 0.05$ determined significance.

3. Results

3.1. Characterization of thiol–ene/yne reaction efficiency

The overall synthesis and reaction strategy is described in Fig. 1. First, POEGMA brush substrates were generated by surface