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Cell adhesion mechanisms on laterally mobile polymer films

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ABSTRACT

In contrast with the majority of substrates used to study cell adhesion, the natural extracellular matrix (ECM) is dynamic and remodeled over time. Here we use amphiphilic block copolymers to create self-assembled supported films with tunable lateral mobility. These films are intended to serve as partial mimics of the ECM in order to better understand cell adhesion responses, specifically in the context of dynamic substrates. Block copolymers are end-labeled with RGD peptide ligands to allow for integrin-mediated cell adhesion, and the addition of a trace hydrophobic homopolymer is used to control the film lateral mobility. We find that NIH 3T3 fibroblasts cultured on these biomimetic films exhibit non-linear spreading behavior in response to substrate mobility. In the absence of RGD ligands, however, fibroblasts do not spread. Employing quantitative analysis of focal adhesions (FA) and integrin ligation, we discover the presence of FA-dependent and FA-independent mechanisms responsible for the biphasic cell spreading behavior. The use of designed biomimetic platforms therefore yields insight into ECM mechanosensing by revealing that cells can engage distinct mechanisms to promote adhesion onto substrates with different time-dependent properties.

1. Introduction

Regulation of cellular function from a genetic or biochemical perspective has been appreciated and studied for many decades. Yet only recently have the biophysical effects on cellular function gained more attention. Towards understanding such effects, a large effort has been dedicated to the development of artificial materials [1,2] that mimic different characteristics of the native extracellular matrix (ECM) [3]. These artificial materials are designed to present cell-adhesive ligands or proteins, while displaying a range of physical properties such as texture [4], geometry [5], and stiffness [6–9]. In turn such materials allow the examination of processes including cell motility, differentiation and tumor progression [6,10–12]. However, with very few exceptions [13], artificial ECM materials involve the static display of signals and therefore are insufficient to mimic the dynamics of the ECM [14,15]. To explore the role of dynamics of the cell–material interface, previous works have either used degradable hydrogels [16–19] or supported phospholipid bilayers [20–22]. In the former case the artificial material is provisional and intended for replacement with native ECM, whereas in the latter case substrates are generally unable to promote cell adhesion and spreading [20–23]. Towards overcoming these drawbacks, patterning techniques have been used to partition lipid bilayers with periodic barriers [24,25]. Nevertheless, both barriers and patterned substrates induce mechanotransduction in response to the static pattern density and/or geometry [22,26–28].

Therefore there is an unmet challenge in development of artifical materials that mimic native ECM characteristics such as: the dynamic display of ligands, cell-induced remodeling, no predefined spatial patterns, and the support of cell adhesion/spreading. This challenge motivated us to examine the directed self-assembly of amphiphilic block copolymers as a potential platform. Such block copolymers share the amphiphilicity and mobility of lipids, while forming more stable structures due to their larger molecular weight [29–32]. These characteristics of amphiphilic block copolymer systems make them suitable candidates to mimic aspects of the native ECM. Indeed, related polymer systems have been used to study the role of ligand clustering [33] and ligand tether spacing [34] on cell migration and spreading, respectively.

Towards achieving partial mimicry of the dynamic character of the ECM, here we fabricate ultrathin supported block copolymer films with independently tunable lateral mobility and ligand spacing. The lateral mobility is tuned by varying the amount of a "lubricating" homopolymer; a strategy inspired by the role of cholesterol in cell membranes [35,36]. Due to the self-assembly

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nature of the fabrication process, the average ligand spacing is easily controlled by the fraction of RGD-labeled polymer. Most importantly, the self-assembly fabrication process means that these films are susceptible to cell-induced remodeling. The effects of substrate lateral mobility on murine fibroblast responses are quantified by cell spreading and adhesion strength. At constant ligand spacing, we find that fibroblasts respond non-linearly to substrate mobility, indicating that cell spreading is not a simple function of typical static properties such as ligand density and substrate elasticity. Analysis of focal adhesions (FA) and integrin ligation leads us to propose that cell spreading can be realized by FA-dependent and FA-independent mechanisms (in the presence of sufficient ligand density). Our results reveal that the dynamic display of ligands, as in the native ECM, plays an important role in cellular responses. Thus the strategic design of biomaterials has the potential to provide critical insight on mechanosensing within the ECM.

2. Materials and methods

2.1. Materials

The polymers 1,2-polybutadiene-*b*-poly(ethylene oxide) (1,2-PBd-PEO) of M_w = 10 kg/mol (PDI = 1.15) and w_{EO} = 0.40, and poly(isobutylene) (PIB) of M_w = 0.9 kg/mol (PDI = 1.3) were obtained from Polymer Source, Inc., (Canada), and were used as received. Bovine serum albumin (BSA) was purchased from Sigma and used as received. Silicon wafers were purchased from International Wafer Service, Inc., (California, USA) and glass coverslips were purchased from Fisher. Trypsin-EDTA solution, Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin solution, and calf bovine serum were supplied from ATCC. Y-27632 dihydrochloride was purchased from Sigma–Aldrich.

2.2. Fabrication of supported block copolymer films

Silicon wafers or glass coverslips were rinsed with EtOH and RO water, subjected to oxygen plasma treatment, and submerged in the RO water subphase of a Langmuir trough. Chloroform solutions of polymers were applied dropwise at the air/water interface and left quiescent for 15 min before compression. The initial surface pressure after the addition of polymer solution and before compression was between 20 and 22 mN/m. The interfacial films were compressed at a rate of 10 mm/min up to a surface pressure of 39 mN/m [37].

For the fabrication of supported monolayers, we used chloroform solutions of PB-PEO or its mixture with PIB homopolymer. Interfacial films were transferred from the air/water interface to the silicon wafers or glass coverslips at a constant deposition pressure and rate (39 mN/m, 1–2 mm/min) using Langmuir–Blodgett (LB) deposition. Within an hour post-fabrication, the supported monolayers were used to create a supported bilayer by the Langmuir-Schaefer (LS) technique. LS deposition was allowed a contact time of 1 min between the supported monolayer and the interfacial film of PB-PEO. The dry thickness of silicon-supported films (Table S1) was determined by ellipsometry (LSE Stokes Ellipsometer 7109-C370, Gaertner).

To allow for fluorescence recovery after photobleaching (FRAP), chloroform solutions of PB-PEO (≈90 vol%) and PB-PEO-FITC (≈10 vol%) were premixed and applied at the air/water interface. The fluorescent interfacial film was introduced as the topmost layer through LS deposition onto neat or PIB-doped PB-PEO monolayers. It has been previously shown that labeling a fraction of the polymer chains up to ≈25 vol% does not alter their diffusion characteristics [38]. Herein, a minimum concentration of 10 vol% of PB-PEO-FITC was necessary to gain the required contrast for the FRAP experiment. FRAP studies were initiated within 1 h after bilayer formation.

For the cell adhesion studies, chloroform solutions of PB-PEO and PB-PEO-RGDS were premixed in a stoichiometric ratio that resulted in the desired RGD spacing. The calculation of the RGD spacing assumes ideal mixing between the polymer chains and employs the deposition surface density. The interfacial film containing PB-PEO-RGDS was introduced as the topmost layer through LS deposition onto neat and PIB-doped PB-PEO monolayers.

2.3. Fluorescence recovery after photobleaching (FRAP)

FRAP experiments were performed on a confocal microscope (Zeiss510) using 50% of argon laser (488 nm) intensity with a 40*x* oil immersion objective. FRAP experiments were conducted with the films immersed in reverse-osmosis water at $T = 20\ ^\circ C$.

The fluorescence intensity was doubly normalized according Phair et al. [39]. Specifically, we corrected for acquisition bleaching by division of the fluorescence intensity at the region of interest $ROI(t)$ with the corresponding intensity of the whole field of view $Tot(t)$. The prefactor $Tot(0)/ROI(0)$ accounts for heterogeneities of fluorescence intensity at the starting point of the experiment,

$$\mathrm{ROI}(t)_{\mathrm{corr}} = \frac{\mathrm{ROI}(t)}{\mathrm{Tot}(t)} \times \frac{\mathrm{Tot}(0)}{\mathrm{ROI}(0)} \quad (1)$$

The corrected fluorescence intensity $\mathrm{ROI}(t)_{\mathrm{corr}}$ was normalized to span between 0 and 1, the ideal limits for no and full recovery respectively,

$$N(t) = \frac{\mathrm{ROI}(t)_{\mathrm{corr}} - \mathrm{ROI}(0)_{\mathrm{corr}}}{\mathrm{ROI}(\infty)_{\mathrm{corr}} - \mathrm{ROI}(0)_{\mathrm{corr}}}. \quad (2)$$

This normalized intensity $N(t)$ was fitted to the fractional recovery curve (MATLAB, R2011a) defined in Soumpasis et al. [40]

$$N(t) = \exp(-2\tau/t)[I_o(2\tau/t) + I_1(2\tau/t)], \quad (3)$$

to extract the characteristic time τ of polymer diffusion at the topmost layer of the films. Using this value of the characteristic time we calculate the diffusion coefficient through $D = A/\tau$, where A is the area of the bleaching spot. The diffusion coefficients obtained are the mean values from independent circular bleaching spots for the corresponding films (Figs S1 and S2).

2.4. Cell projection area and adhesion strength

Synchronized and enzymatically recovered fibroblasts were centrifuged (125 *g*, 10 min, 2×) and then resuspended in complete DMEM. The RO water phase above freshly prepared polymer films was exchanged with PBS solution (3×, 5 mL). PBS was exchanged with BSA solution (1 mg/mL, pH 7.4) (3×, 5 mL) and left quiescent for film passivation ($T = 20\ ^\circ C$, $t = 30$ min). Afterward, the BSA solution was exchanged with complete DMEM (3×, 3 mL). The cell suspension was added above the bilayer films to an initial surface concentration of 3×10^3 cells/cm^2 and incubated for 24 h ($T = 37\ ^\circ C$ and 5% CO_2). Only the cells that did not participate in cell–cell contacts were used for cell projection area measurements. After image acquisition, the incubation wells were filled with complete DMEM of adjusted temperature ($T = 37\ ^\circ C$) and sealed with para-film to avoid bubbles. The sealed wells were centrifuged at 600*g* for 10 min Ref. [33]. Following centrifugation, we discarded the media and counted the fraction of cells that remained attached.

2.5. Immunofluorescence staining

After a seeding period of 24 h, the cells underwent fixation by transferring the coverslips to wells containing 4% formaldehyde (Carson-Millonig Formulation; Fisher Scientific) in PBS containing Ca^{2+} and kept at ambient temperature for 15–20 min. Following three rinses with PBS, free aldehydes were quenched with 0.3 M glycine in PBS (3×, 15 min) and permeabilized with 0.1% Triton X-100 for 5 min. To block non-specific interaction, 2% bovine serum albumin (BSA) in PBS was added and incubated for 60 min at ambient temperature. Cells were rinsed with 0.1 M EDTA in PBS (3×, 5 min) to remove trace metals. Anti-vinculin-FITC (1:50 dilution, Sigma-Aldrich) was added and left in the dark for 60 min at room temperature. After rinsing with 0.1% Triton-X100 in PBS (3×, 2 min) and 0.1 M EDTA in PBS (3×, 5 min), actin-phalloidin-orange (1unit; Molecular Probes) (2% BSA) in PBS was added for 30 min. The coverslips were mounted on microscope slides with ProLong antifade reagent (Molecular Probes) and left to cure overnight in the dark prior to image acquisition.

2.6. Pharmacological experiments

Fibroblasts and polymer films were prepared as described above. Contractility inhibitor (Y-27632) in PBS was added at a final concentration of 50 μM and allowed a 30 min incubation time at room temperature [41] before measuring the new cell projection area and performing the centrifugation–adhesion assay (600*g*, 10 min). The fraction of remaining cells was measured and compared with the corresponding untreated control cells.

2.7. Statistics

Unless otherwise noted, data are reported as mean values and error bars as the standard error of the mean. If analysis by ANOVA (Kaleidagraph 4.1.2) detected significant differences, Student-Newman–Keuls multiple comparison tests were performed for pair-wise comparisons. Because ANOVA did not detect a statistically significant difference for FA sizes on different films, we employed the Student's *t*-test for pair-wise comparisons. The error bars for the normalized area and adhesion strength data are the propagation error [42].

3. Results

3.1. Fabrication and characterization of laterally mobile films

Our supported polymer films are created by Langmuir–Blodgett/Langmuir–Schaefer (LB/LS) self-assembly (Fig. 1), which allows