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Supporting Material

Dissecting the Impact of Matrix Anchorage and Elasticity in Cell Adhesion

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Detailed protocols on MACP-PAAm layer preparation and characterisation and FAK phosphorylation analysis by western blot

Substrate Preparation and Characterisation.

Maleic acid copolymer (MACP)-coated polyacrylamide (PAAm) gel samples were prepared based on the method of Wang and Pelham (15).

Coverslips (24 × 24 mm and Ø 32 mm, #1, VWR International, Darmstadt, Germany) were freshly oxidised (35) in a mixture of aqueous solutions of ammonia (Acros Organics, Geel, Belgium) and hydrogen peroxide (Merck, Darmstadt, Germany) and were subsequently surface-modified with 20 mM (3-acryloxypropyl)trimethoxysilane (ABCR, Karlsruhe, Germany) in 95% ethanol/water for 2 h. After rinsing in ethanol and deionised water, the coverslips were dried and stored for future use.

For gel film preparation, stock solutions of 80% acrylamide (PlusOne Acrylamide PAGE, Amersham Biosciences, Piscataway, NJ, USA) and 1% bis-acrylamide solutions (Amersham Biosciences) in degassed deionised water were utilised to adjust a final acrylamide concentration of 8% and a bis-acrylamide concentration in the range of 0.04% to 0.3%. The final mixtures were supplemented with 2.5% (v/v) fluorescent microbeads (Fluoresbrite YG Microspheres 0.50 µm Polysciences, Warrington, PA). 0.05% (w/v) ammonium persulfate (Fluka, Seelze, Germany) and 0.075% (v/v) *N*,*N*,*N′*,*N′*-tetramethylethylenediamine (TEMED, Bio-Rad Laboratories, Hercules, CA) were added to initiate polymerisation and mixed thoroughly. A volume of 39 or 55 µL (depending on the size of coverslip) of the acrylamide mixture was immediately pipetted onto a (heptadecafluoro-1,1,2,2-tetrahydrodecyl)dimethylchlorosilane (ABCR)-modified glass slide (to ensure easy detachment) and this was covered by the acryl-silanised coverslip. After 30 min of polymerisation, the coverslip with the PAAm gel was slowly peeled off by immersion in deionised water.

Washing the gel films for 30 min in deionised water was followed by drying at room temperature under vacuum for 30 min. Following this, monomolecular films of MACP on top of PAAm hydrogels were prepared. Poly(styrene-*alt*-maleic anhydride) (PSMA, MW 20,000, special product of Leuna-Werke AG, Germany) and poly(ethene-*alt*-maleic anhydride) (PEMA, MW 125,000, Sigma Aldrich, Deisenhofen, Germany) were spin-coated (RC5, Suess Microtec, Garching, Germany) using 0.14% POMA and 0.3% PEMA copolymer solutions in tetrahydrofuran (Fluka, Deisenhofen, Germany) or acetone/tetrahydrofuran (1:2, w/w, Acros Organics, Geel, Belgium), respectively. Entanglement of the water-soluble MACP chains

with the cross-linked PAAm hydrogel and chain attachment via residual radicals from hydrogel synthesis allow for a gentle surface modification.

A glass ring (Ø 1 cm) was stuck to the coated coverslips using silicone vacuum grease. Prior to use, the polymer-coated coverslips were equilibrated for 24 h in phosphate buffer saline at pH 7.4 (PBS) (Biochrom, Berlin, Germany) to ensure complete hydrolysis of the anhydride groups of the maleic anhydride copolymers to carboxylic acid groups (36) and removal of non-bound polymer molecules as well as a full re-swelling of the PAAm gels under physiological buffer conditions. The final gel films were 70–100 µm thick, as determined by confocal laser scanning microscopy (SP1, Leica Microsystems, Bensheim, Germany) using a 40× immersion oil objective.

FN (purified from adult human plasma following the protocol of Brew et al. (37)) was anchored on the substrate by adsorption from a 50 µg/ml solution in PBS for 1 h at 37 °C. The surface concentrations and anchorage strength were determined by surface concentration measurements using ^{125}I-labelled FN during protein displacement studies with 500 µg/ml bovine serum albumin in PBS over 24 h. In these experiments, FN spiked with 10% ^{125}I-labelled FN was adsorbed from a 50 µg/ml solution in PBS. The coupled amount was determined by rinsing several times with PBS after appropriate time periods in displacement solution. For the measurements, a gamma counter (UMo LB 123, Berthold Technologies) was used to detect ^{125}I-labelled FN. For control experiments on FN reorganisation by endothelial cells, FN was fluorescently labelled prior to adsorption using 5-(and-6)-carboxytetramethylrhodamine (TAMRA, Invitrogen, Carlsbad, CA) by an adaptation of the procedure for the FluoReporter Tetramethylrhodamine Protein Labeling Kit (Invitrogen).

Gel stiffness was determined by scanning force spectroscopy (Bioscope BS2-Z, Veeco, Santa Barbara, CA) of PAAm films with three different bis-acrylamide concentrations. Young's modulus, E, was obtained using a Hertz cone model (38) with a fitting of the first 10–200 nm of indentation profiles using the freely available software PUNIAS (39). The exact spring constants (approx. 0.01 N/m) of the pyramid-tipped SiNi cantilevers (Microlevers, Park Scientific Instruments, ThermoMicroscopes, Sunnyvale, CA) were determined by the thermal noise method (40). The Poisson ratio of PAAm was assumed to be 0.48, as published elsewhere (41). The measurements of the three different bis-acrylamide concentrations were fitted by a second-order polynomial function as introduced by Engler et al. (8). From the fitted curve, Young's moduli for other bis-acrylamide concentrations were calculated.

Western Blot Analysis.

FAK phosphorylation was analysed after 60 min of cell culture on the MACP-PAAm substrates with three different concentrations of bis-acrylamide. After gentle removal of non-adherent cells by washing with warm PBS, the cells were detached from the substrate by adding 800 µL of trypsin-EDTA solution (containing 0.5 g/L porcine trypsin, Sigma-Aldrich) (4 min at 37 °C). Trypsin treatment was stopped by adding 10% foetal calf serum in PBS and samples were placed on ice during the subsequent preparation steps. After centrifugation, the cell pellets were resuspended in PBS with ProteaseInhibitor-Mix Complete Mini (without EDTA) and PhosphataseInhibitor-Mix PhoSTOP (both Roche, Mannheim, Germany). After centrifuging again, the cells were lysed for 15 min in cold modified RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.25% sodium deoxycholate, 10% glycerol, and protease inhibitors as mentioned above) (11). Total protein concentrations were determined by a BCA assay (Micro BCA Protein Assay Kit, ThermoScientific, Rockford, IL). Appropriate amounts of cell lysates were separated by denaturing SDS-PAGE, electroblotted onto nitrocellulose (Hybond-C extra, Amersham Biosciences, Piscataway, NJ) using Mini-PROTEAN 3 Cell, Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories), blocked with 5% BSA in TBST buffer, immunoblotted with specific primary antibodies, and detected using horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark) and ECL Plus Western Blotting Detection System (GE Healthcare, UK) as a chemiluminescent substrate. Primary antibodies were FAK antibody (FAK 3285, Cell Signalling Technology, Danvers, MA), phospho-FAK pTyr397 and pTyr861 antibody (FAK pY397 and FAK pY861 PAb, Invitrogen), and GAPDH antibody (GAPDH (FL-335) HRP, Santa Cruz Biotechnology, Santa Cruz, CA). Densitometric analysis was carried out using a Lumi-Imager F1Workstation with LumiAnalyst 3.0 software (Roche). The blots were analysed using ImageJ software (46) by normalising total FAK and phospho-FAK intensities to the internal standard GAPDH and calculating the phosphorylation ratio (phospho-FAK divided by total FAK).